# Optimisation of Anti-Platelet Therapy in Acute Coronary Syndromes

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by **Richard Joaquim FitzGerald**

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## Abbreviations

| Abbreviation | Term(s) |
|---|---|
| **% inhib** | % inhibition |
| **11dhTXB2** | 11-dehydro thromboxane B2 |
| **AA** | Arachidonic acid |
| **ACE-I** | Angiotensin converting enzyme inhibitor |
| **ACS** | Acute coronary syndrome |
| **ADP** | Adenosine diphosphate |
| **Akt** | Protein kinase B |
| **AmA** | Amino acid |
| **ARB** | Angiotensin-2 receptor blocker |
| **ARU** | Aspirin reaction units |
| **ASA** | Acetylsalicylic acid |
| **AU** | Aggregation units |
| **AUC** | Area under the curve |
| **BD** | Twice daily |
| **CABG** | Coronary artery bypass graft |
| **CagA** | Cytotoxin gene A |
| **cAMP** | Cyclic adenosine monophosphate |
| **CC** | Case-control |
| **CCB** | Calcium channel blocker |
| **CCS** | Canadian Cardiovascular Society score |
| **CD40L** | CD40 Ligand |
| **CEPI** | Collagen-epinephrine cartridge |
| **cGMP** | Cyclic guanosine monophosphate |
| **CI** | Confidence interval |
| **cIMT** | Carotid intima-media thickness |
| **CK-MB** | Creatine kinase -muscle/brain |
| **Cmax** | Maximum concentration |
| **Cont** | Control subject |
| **COX** | Cyclo-oxygenase |
| **CRP** | C-reactive protein |

| | |
|---|---|
| **CT** | Closure time |
| **CV** | Cardiovascular |
| **CVA** | Cerebrovascular disease |
| **CYP** | Cytochrome |
| **DAMP** | Danger associated molecular pattern |
| **DAPT** | Dual anti-platelet therapy |
| **DES** | Drug eluting stent |
| **DNA** | deoxyribonucleic acid |
| **Dom** | Dominant model of inheritance |
| **DOAC** | Direct oral anti-coagulant |
| **ECG** | Electrocardiogram |
| **ECP** | Eosinophilic cationic protein |
| **EGR-1** | Immediate-early response gene |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EM** | Extensive metaboliser |
| **ENT** | Equilibrative nucleoside transporter |
| **FDA** | Food and Drug Administration |
| **Freq** | Frequency |
| **Funct Status** | Functional status |
| **G-CSF** | Granulocyte colony stimulating factor |
| **GOF** | Gain of function |
| **GWAS** | Genome wide association study |
| **Hb** | Haemoglobin |
| **Hct** | Haematocrit |
| **HDL-C** | High density lipoprotein cholesterol |
| **HP** | *Helicobacter Pylori* |
| **HR** | Hazard ratio |
| **hsCRP** | high sensitivity C-reactive protein |
| **HSP60** | Heat shock protein 60 |
| **HTPR** | High on-treatment platelet reactivity |
| **HV** | Healthy volunteer |
| **Hyperlipid** | Hyperlipidaemia |
| **ICER** | Incremental cost-effectiveness ratio |

| | |
|---|---|
| **IFN** | Interferon |
| **IgG** | Immunoglobulin G |
| **IL** | Interleukin |
| **IM** | Intermediate metaboliser |
| **Interproc** | Interprocedural |
| **IntM** | Indeterminate metaboliser |
| **IP3** | Inositol triphosphate |
| **IPA** | Inhibition of platelet aggregation |
| **IPD** | Individual patient data |
| **IQR** | Interquartile range |
| **LD** | Loading dose |
| **LDL-C** | Low density lipoprotein cholesterol |
| **LLN** | Lower limit of normal |
| **LMWH** | Low molecular weight heparin |
| **LOF** | Loss of function |
| **LOX** | Lipo-oxygenase |
| **LOX-1** | Lectin-type oxidised LDL receptor 1 |
| **LTA** | Light transmittance aggregometry |
| **LTA10** | Light transmittance aggregometry, 10 µmol/L ADP |
| **LTA20** | Light transmittance aggregometry, 20 µmol/L ADP |
| **LTA5** | Light transmittance aggregometry, 5 µmol/L ADP |
| **MACE** | Major adverse cardiovascular events |
| **MAF** | Minor allelic frequency |
| **MCP** | Monocyte chemoattractant protein |
| **M-CSF** | Macrophage colony stimulating factor |
| **MD** | Maintenance dose |
| **MDA** | Malondialdehyde-acetaldehyde |
| **MI** | Myocardial infraction |
| **mL** | millilitre |
| **MMP** | Matrix metalloproteinase |
| **MP** | Multiplate |
| **MPA** | Maximal platelet aggregation |
| **MPO** | Myeloperoxidase |

| | |
|---|---|
| **mRNA** | messenger ribonucleic acid |
| **MT** | Mutant type |
| **mTOR** | Mammalian target of rapamycin |
| **Neg** | Negative |
| **NF** | Nuclear factor |
| **NLRP3** | Nucleotide-binding domain, leucine rich protein |
| **nm** | Nanometre |
| **NO** | Nitrous oxide |
| **NS** | Not stated |
| **NSTEACS** | Non-ST elevation acute coronary syndrome |
| **NSTEMI** | Non ST-elevation myocardial infarction |
| **OCT** | Optical coherence tomography |
| **OD** | Once daily |
| **OR** | Odds ratio |
| **OxHDL** | Oxidised HDL |
| **OxLDL** | Oxidised LDL |
| **PAPP-A** | Pregnancy-associated plasma protein A |
| **PAR** | Protease-activated receptor |
| **PC** | Prospective cohort |
| **PCI** | Percutaneous coronary intervention |
| **PCR** | Polymerase chain reaction |
| **PCSK9** | Proprotein convertase subtilisin/Kexin type 9 |
| **PD** | Pharmacodynamics |
| **PEAR** | Platelet endothelial aggregation receptor |
| **PET** | Positron emission tomography |
| **PFA** | Platelet function analyser |
| **PGE1** | Prostaglandin E1 |
| **PGI2** | Prostaglandin I2 / Prostacyclin |
| **P-gp** | P-glycoprotein |
| **PI3K** | Phosphoinositide 3-kinase |
| **PK** | Pharmacokinetics |
| **PKPD** | Pharmacokinetic and pharmacodynamic |
| **PLA2** | Phospholipase A2 |

| | |
|---|---|
| **PM** | Poor metaboliser |
| **PON1** | Paraoxonase 1 |
| **Pos** | Positive |
| **PPCI** | Primary percutaneous coronary intervention |
| **PPI** | Proton pump inhibitors |
| **PRI** | Platelet reactivity index |
| **PRU** | P2Y12 reaction units |
| **QALY** | Quality adjusted life-year |
| **QDS** | Four times daily |
| **RAGE** | Receptor for advance glycation end products |
| **RANTES** | Regulated on activation, normal T cell expressed and secreted |
| **RC** | Retrospective cohort |
| **RCT** | Randomised controlled trial |
| **Rec Angina** | Recurrent angina |
| **Regr** | Regression analysis |
| **RESIST** | Resistant |
| **Revasc** | Revascularisation |
| **ROS** | Reactive oxygen species |
| **RPA** | Residual platelet aggregation |
| **RR** | Relative risk |
| **SCAD** | Stable coronary artery disease |
| **SD** | Standard deviation |
| **SENS** | Sensitive |
| **SMC** | Smooth muscle cell |
| **SNP** | Single nucleotide polymorphism |
| **ST** | Stent thrombosis |
| **STEMI** | ST-elevation myocardial infarction |
| **T2DM** | Type 2 diabetes mellitus |
| **TBR** | Target-to-background ratio |
| **TEG** | Thromboelastography |
| **TG** | Triglycerides |
| **Th1** | T helper 1 cells |
| **TLR** | Toll like receptors |

| | |
|---|---|
| **TLR** | Target lesion revascularisation |
| **TMB** | Tetramethylbenzidine |
| **TNF** | Tumour necrosis factor |
| **TPO** | Thrombopoetin |
| **TVR** | Target vessel revascularisation |
| **TXA2** | Thromboxane A2 |
| **TXB2** | Thromboxane B2 |
| **UA** | Unstable angina |
| **ULN** | Upper limit of normal |
| **UM** | Ultra-rapid metaboliser |
| **VAR** | Variable |
| **VASP** | Vasodilator-stimulated phosphoprotein |
| **VLDL-C** | Very low density lipoprotein cholesterol |
| **VN** | VerifyNow |
| **vWF** | von Willebrand factor |
| **WT** | Wild type |
| **β2GPI** | Beta-2 Glycoprotein I |

## Abstract

### Richard FitzGerald – Optimisation of anti-platelets in acute coronary syndromes

Acute coronary syndromes (ACS) are a major cause of morbidity and mortality worldwide. Platelets are central to the underlying pathology of ACS, and anti-platelet drugs, such as aspirin, clopidogrel and ticagrelor, form a cornerstone of its treatment. However, response to anti-platelet drugs is not uniform, with a substantial proportion of patients being non-responsive to their effects, leading to an increase in risk of adverse cardiovascular events. Several mechanisms underlie this observed non-response, including clinical risk factors, genetic polymorphisms, drug interactions, medication adherence and inflammation. However, the data investigating these mechanisms are often contradictory with no consensus on how non-response should be detected or treated. This thesis sought to investigate easily detectable and potentially modifiable causes for anti-platelet non-response, which may have potential clinical utility.

Through comprehensive meta-analyses of the published literature, we demonstrated a consistent association between carriage of the *CYP2C19* loss-of-function polymorphisms, higher platelet reactivity and an increased risk of adverse cardiovascular events in clopidogrel treated patients. Importantly, we failed to demonstrate the effect of other genes such as *ABCB1, CYP3A5 and PON1*, which had been previously suggested as important in determining response to clopidogrel.

We failed to detect a clear association between genes in aspirin's pharmacokinetic and pharmacodynamic pathway and aspirin response, defined by two platelet function tests, in a cohort of patients with ACS. However, weak associations were detected between poor aspirin response and two polymorphisms in the *UGT1A6* and *TBXA2R* genes prior to correction for multiple testing, which may deserve further investigation in larger numbers of patients.

Given the importance of lipid oxidation in the context of vascular inflammation and the pathogenesis of atherosclerosis, we investigated the role of the OxLDL-β2GPI complex in clinical outcomes, platelet reactivity and lipid profiles in ACS patients. We demonstrated an association between higher levels of OxLDL-β2GPI and a lower risk of adverse cardiovascular events, which is not consistent with published clinical studies but is supported by *in vitro* and non-human data. We also detected a significant association between aspirin non-response and raised HDL to cholesterol ratios in patients, although there was no association between OxLDL-β2GPI levels and platelet reactivity demonstrated.

Finally, we investigated whether *H. Pylori* which has been associated with adverse cardiovascular outcomes due to increased inflammation may be responsible for the well-documented interaction between clopidogrel and proton pump inhibitors, which are often prescribed in patients with non-specific gastrointestinal symptoms. In a cohort of patients with ACS, we failed to demonstrate any association between *H. Pylori* serology and clinical outcome although an association between clinical outcome and carriage of the *CYP2C19*2* allele was observed, but only in *H. Pylori* negative patients. The underlying cause for this finding is unclear but may represent an interaction between PPI use and changes in the gastric flora induced by a higher gastric pH.

In summary, this thesis has identified the *CYP2C19* gene as a critical determinant of clopidogrel response which could be used as a biomarker for stratification of ADP receptor antagonists. In addition, we have identified putative biomarkers for aspirin response including the *TBXA2R* gene and clinical risk factors such as hyperlipidaemia. These data suggest that stratification and personalisation of anti-platelets is possible by using genetic and clinical biomarkers, and further, well-powered, clinical outcome studies are necessary.

# Chapter 1 - Introduction

## 1.1: Cardiovascular disease and acute coronary syndromes

Cardiovascular disease remains one of the leading causes of death worldwide. Coronary atherosclerosis is a chronic process leading to progressive vascular stenosis and ischaemia. Acutely, however, rupture of atherosclerotic plaques and subsequent thrombosis, termed acute coronary syndromes (ACS), lead to substantial mortality and morbidity. Acute coronary syndromes are a spectrum of disorders defined on the basis of symptoms, biomarkers (such as troponin) and changes on electrocardiograms (ECGs). ST-elevation myocardial infarctions (STEMI) represent one end of the ACS spectrum and are responsible for over 1.8 million deaths annually in Europe alone (Ibanez et al., 2018). It is a common disorder, with an incidence rate between 43 and 100 per 100,000 per year and carries a significant risk of mortality both in hospital (4-12%) and after 1 year (10%). Diagnosis of STEMI rests on the presence of persistent ST-elevation on an ECG with typical symptoms and because of the significant mortality associated with it, an early invasive strategy utilising percutaneous coronary intervention (PCI) is generally preferred. Non-ST elevation acute coronary syndromes (NSTEACS) represent a more diverse range of conditions, with a generally lower short-term mortality compared to STEMIs but a broadly similar longer-term mortality beyond 2 years. However, NSTEACS can present with a range of different symptoms and ECG characteristics, with some presentations being low risk and others being very high risk with haemodynamic instability, arrhythmias and ongoing myocardial ischaemia. Consequently, treatment of NSTEACS varies dependent on the clinical presentation of a patient, with some patients requiring an early invasive strategy and others not (Roffi et al., 2016).

### 1.1.1: Platelets as the fundamental agent in acute coronary syndromes

Platelet activation, leading to aggregation and thrombus formation, is fundamental to the underlying pathology of most acute coronary syndromes (Siller-Matula et al., 2013). It is a complex and multi-step process, which can be influenced by a number of non-platelet specific factors such as inflammation, diabetes and hyperlipidaemia.

The initial rupture of an atherosclerotic plaque leads to collagen and von Willebrand Factor (vWF) being exposed to platelets via GPIb receptors (Siller-Matula et al., 2013, Marcucci et al., 2016). Collagen, in particular, is highly thrombogenic (Koltai et al., 2017) and binds directly to the platelet GPVI receptor. Activation of platelets via GPVI receptor binding leads to platelet activation and degranulation with increases in a number of platelet derived

mediators such as adenosine diphosphate (ADP), fibrinogen, P-selectin and Factor V. Release of these mediators activates phospholipase C, changing the conformation of the platelet GPIIbIIIa complex (Koltai et al., 2017). The conformational change in GPIIbIIIa is fundamental to the generation of platelet rich thrombi via several mechanisms. Firstly, it allows cross-linking with fibrinogen leading to both platelet to platelet adhesion and platelet to wall adhesion. Secondly, it activates both diacylglycerol and inositol triphosphate (IP3) leading to calcium influx and release of calcium from platelet stores. Thirdly, it increases intracellular platelet signalling which further increases platelet activation and release of pro-aggregatory mediators (Marcucci et al., 2016). The excess calcium released from intracellular stores and from increased calcium influx leads to conformation change in the platelet including shape change (increased free surface area) and degranulation into the platelet canicular system. Phospholipase A2 (PLA2) activation is also increased (Koltai et al., 2017) by excess calcium, liberating arachidonic acid (AA) from phospholipids, which is then converted to thromboxane A2 (TXA2) by the enzyme cyclo-oxygenase 1 (COX-1). TXA2 causes further activation of platelets (Koltai et al., 2017), acting in tandem with other agonists to significantly amplify platelet activation (Fitzgerald and Pirmohamed, 2011). Importantly, and relevant to the use of aspirin as an anti-platelet drug, TXA2 may also be generated by non-COX-1 dependent pathways, for example via PLA2, and is also a potent vasoconstrictor. Degranulation of platelet alpha granules releases coagulation factors and inflammatory mediators (Knowles and Warner, 2019) which stimulates thrombus formation, further platelet activation and endothelial dysfunction.

ADP released by platelet activation binds to platelet P2Y12 receptors and P2Y1 receptors, amplifying responses to various platelet agonists such as thrombin (Siller-Matula et al., 2013). Thrombin is a highly effective platelet agonist (Marcucci et al., 2016) binding via two thrombin receptors PAR-1 and PAR-4. Both receptors require very low concentrations of thrombin for activation (although PAR-4 requires a higher concentration than PAR-1), several fold lower than required for activation of the clotting cascade (Marcucci et al., 2016). P2Y12 stimulation also leads to platelet activation via inhibition of adenylate cyclase and reduction in platelet levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Platelet cAMP and cGMP act as potent inhibitors of platelet activation and are modified substantially via endothelial interaction within the capillary bed (Knowles and Warner, 2019). Furthermore, the P2Y12 receptor induces release of alpha granules and consequent expression of P-selectin as well as stabilising platelet rich thrombi via GPIIbIIIa and Ia/IIa receptor activation. (Siller-Matula et al., 2013).

In addition, other mechanisms have been suggested to be involved in platelet aggregation. In particular, junctional adhesion molecules (JAM), signalling lymphocyte activation molecules (SLAM) and CD40 ligand have all been suggested as important in platelet rich thrombus formation and platelet activation (Koltai et al., 2017). Consequently, immune activation, inflammation and infection may also be potent stimulators of platelet activation.

Endothelial interactions are also key regulators of platelet activation (Knowles and Warner, 2019, Marcucci et al., 2016). In particular, inhibition of platelet activation can be driven via a number of endothelial derived factors including prostacyclin (PGI2) and nitrous oxide (NO) (Marcucci et al., 2016). Exposure to NO and prostacyclin increases platelet cAMP and cGMP which 'damp' platelet responses to agonists via a number of mechanisms including prevention of shape change, reducing P-selectin expression, inhibition of GPVI dimerization and reduction in calcium release and platelet degranulation (Knowles and Warner, 2019).

Given the pluripotent effects of TXA2, inhibition of COX-1 by Aspirin (Acetylsalicylic acid, ASA) is a bedrock of treating and preventing cardiovascular disease. Similarly, the P2Y12 receptor has multiple effects and is inhibited by thienopyridine drugs such as clopidogrel and prasugrel as well as by non-thienopyridine drugs such as ticagrelor and cangrelor. The combination of both ASA and a P2Y12 receptor inhibitor (dual antiplatelet therapy, DAPT) have become the primary treatment for reducing the risk of recurrent ischaemic events following an acute coronary syndrome, with additional drugs supporting the peri-event or peri-procedure period. In high-risk ACS or during percutaneous coronary intervention (PCI), drugs to inhibit GPIIb/IIIa can be administered in addition to DAPT to reduce the risk of ischaemic complications or stent thrombosis, although routine use in primary PCI (PPCI) is no longer recommended (Ibanez et al., 2018). Furthermore, new anti-platelet agents, such as thrombin receptor antagonists (Vorapaxar), have been licensed in combination with or DAPT or single anti-platelet therapy (Roffi et al., 2016).

## 1.2: Anti-platelet agents

### 1.2.1: Aspirin

ASA has been a bedrock of anti-platelet therapy in the context of ACS for many years. Aspirin irreversibly inhibits cyclooxygenase-1 (COX-1) via acetylation at the serine-529 position. Aspirin inhibits COX-1 in a dose-dependent manner (Patrignani et al., 1982) and is rapidly

absorbed in the GI tract with a half-life of 20 minutes prior to being converted to its inactive metabolite salicylic acid. Acetylation of COX-1 by aspirin is irreversible and its effect continues for the lifetime of the platelet. Given that platelets are replaced at around 10% of volume per day, global platelet function returns over a period of 2-5 days (Cai et al., 2016). A study by Pamuckcu, 2007, demonstrated that a single, 300mg, dose of aspirin can suppress both serum and urine thromboxane B2 (TXB2), a metabolite of TXA2, by up to 95% for 5 days (Pamukcu, 2007). However, recent evidence suggest that platelets may retain mRNA coding for COX-1 which may allow partial recovery from the irreversible COX-1 inhibition by aspirin (Weyrich et al., 2009).

Aspirin has been demonstrated to have a critical place in the management of cardiovascular disease and its prevention. The Anti-thrombotics Trialists' Collaboration (2002) (Antithrombotic Trialists, 2002) meta-analysis of 197 randomised controlled trials and 135, 640 patients, demonstrated a risk reduction of 25% for serious vascular events or cardiovascular deaths. Interestingly, there appeared to be no benefit of receiving high doses of aspirin (300mg) as opposed to lower doses of aspirin (75-150mg). Whilst this MA was performed in high risk patients, who would be assumed to gain greatest benefit from an anti-platelet agent, similar studies in a lower risk population have also shown the benefit of aspirin for prevention of adverse cardiovascular events (de Gaetano and Collaborative Group of the Primary Prevention, 2001) although the data for aspirin's utility in primary prevention alone is contradictory (Cai et al., 2016, McNeil et al., 2018b, McNeil et al., 2018c, McNeil et al., 2018a).

However, response to aspirin is not always uniform, and some patients may not receive the same benefit from aspirin as others (Michelson, 2004). In a recent study, Chen HY et al (Chen and Chou, 2018b) demonstrated a fourfold increase in risk of further cardiovascular events in a cohort of patients with stable cardiovascular disease, with 20% of patients defined as non-responsive to aspirin. However, in a larger, 900 patient cohort of stable cardiovascular disease, non-response to aspirin was not associated with adverse cardiovascular outcomes in stable disease (Larsen et al., 2017). However, meta-analysis of reported trials do demonstrate a consistent effect of aspirin non-response on the rate of adverse cardiovascular events. In a 20 study, 2930 patient, meta-analysis, Krasopoulos et al (Krasopoulos et al., 2008) demonstrated a fourfold increase in the risk of adverse cardiovascular events and a six-fold increase in risk of death. Similarly, a meta-analysis by Snoep et al (Snoep et al., 2007a) also demonstrated a significant adverse effect on patients determined as aspirin resistant across a range of different measures.

### 1.2.2: Clopidogrel

Clopidogrel is a first-generation thienopyridine ADP receptor antagonist used in the treatment of both stable and unstable cardiovascular disease. Clopidogrel is a pro-drug that requires conversion to its active metabolite prior to binding and inhibition of the platelet P2Y12 receptor. It is an irreversible inhibitor of the P2Y12 receptor and, like aspirin, its effect continues for the life of the platelet.

Large randomised controlled trials have clearly demonstrated that clopidogrel significantly reduces mortality and adverse cardiovascular events. In the CURE trial, clopidogrel reduced mortality, non-fatal myocardial infarction and non-fatal stroke from 11.4% in the placebo group to 9.3% in the clopidogrel group (Relative Risk (RR) 0.80; 95% Confidence Interval (CI) 0.72-0.90, p <0.001) (Yusuf et al., 2001). This finding has been mirrored by other large RCTs such as CHARISMA, CREDO and CLARITY-TIMI 28 (Bhatt et al., 2006, Sabatine et al., 2005, Steinhubl et al., 2002). In addition, a meta-analysis by Berger et al of all blinded, randomised controlled trials comparing clopidogrel to placebo, demonstrated a 14% proportional reduction in risk of cardiovascular events (Odds Ratio (OR) 0.86; 95% CI 0.80-0.93) (Berger et al., 2009). Clopidogrel has been evaluated for safety in over 42,000 patients in clinical trials in addition to over 15 years of clinical experience. The commonest adverse event, given its mode of action, is bleeding. The risk of bleeding with clopidogrel (as with other anti-platelets) is modified by the context of its use: bleeding risk is highest in studies including patients with unstable cardiovascular disease as compared to studies only including stable patients. For example, in the CURE study which included patients with unstable cardiovascular disease, the bleeding rate in the first month was 9.6% in the clopidogrel arm and 6.6 % in the placebo arm. The risk of bleeding diminished over the course of follow up, with the risk of bleeding in the clopidogrel arm 1.9% and in the placebo arm 1.0% in the 9 to 12 month period (Yusuf et al., 2001). In contrast to the CURE study, the CHARISMA study, which included patients with stable cardiovascular disease, the incidence of bleeding was much lower with 1.7% of patients suffering a bleed in the clopidogrel arm compared to 1.3% in the placebo arm (Bhatt et al., 2006).

Of recent, non-response to clopidogrel has become an important clinical issue particularly in relation to PCI and the advent of newer, more potent P2Y12 antagonists such as prasugrel and ticagrelor. In a meta-analysis of 25 studies investigating clopidogrel non-response and PCI, Snoep and colleagues reported a 21% prevalence of clopidogrel non-response corresponding to an eight-fold increase in the risk of an adverse cardiovascular event (OR

8.0; 95% CI 3.4-19.0) post procedure (Snoep et al., 2007b). Furthermore, individual studies have highlighted clopidogrel non-response as the single most important factor in predicting both stent thrombosis and cardiovascular outcome following PCI (Lev et al., 2007a). However, as is the case with aspirin non-response, it is not immediately clear how non-response should be looked for nor is it clear how it should be treated if found.

As clopidogrel is a pro-drug, it requires activation to its active metabolite, R-130694. Clopidogrel activation is a two-step process involving several cytochrome P450 (CYP) isoforms, with CYP2C19, 1A2 and 2B6 postulated for the first metabolic step and 2C19, 2C9 and 2B6 responsible for the second (Gurbel et al., 2009). CYP2C19 appears to be the primary CYP isoform for both steps in this process although the 3A4 isoform is also involved in clopidogrel's activation. Given the complex activation process, it is likely that most variability in clopidogrel response can be explained to some degree by it, an argument substantially strengthened by the known variability in CYP isoforms and risks of interactions caused by enzyme induction or inhibition. In addition, clopidogrel is also a substrate for the drug efflux transporter, P-glycoprotein (P-gp), and consequently alterations in the activity of P-gp may alter the intestinal absorption of clopidogrel, with a marked increase (Taubert et al., 2006) in clopidogrel accumulation demonstrated in the presence of P-gp inhibitors. Indeed, the newer anti-platelet drugs have been designed to avoid this risk.

### 1.2.3: Prasugrel

Prasugrel, like clopidogrel, is an irreversible thienopyridine P2Y12 receptor antagonist. It is licensed for the treatment of unstable cardiovascular disease for at least 12 months following an acute coronary syndrome or STEMI. As with clopidogrel, it remains bound to the P2Y12 receptor for the lifetime of the platelet.

Prasugrel is a third generation thienopyridine agent, with a faster onset time compared to clopidogrel (Greenhalgh et al., 2015). In the pivotal TRITON-TIMI 38 trial, prasugrel was demonstrated to be superior to clopidogrel in reducing the primary composite endpoint of Major Adverse Cardiovascular Events (MACE) (comprising cardiovascular death, non-fatal myocardial infarction (MI) and non-fatal stroke). TRITON-TIMI 38 included patients with moderate to high risk non ST-elevation ACS (NSTEACS) (N=10,074) and ST-elevation MI (STEMI) (N=3534) undergoing PCI who were randomised to receive either clopidogrel (loading dose 300mg, maintenance dose 75mg) or prasugrel (loading dose 60mg, maintenance dose 10mg) co-administered with aspirin (75 to 162 mg). During the follow up period (15 months), the primary endpoint occurred in 12.1% of patients treated with

clopidogrel compared to 9.9% of patients treated with prasugrel (Hazard ratio (HR) 0.81; 95% CI 0.73-0.90, P < 0.001). The benefit of prasugrel was seen both early (up to day 3 post randomisation) and late (from 3 days post randomisation to completion of follow up) and significant benefit was observed in both NSTEACS patients and STEMI patients (Wiviott et al., 2007). Interestingly, the benefit of prasugrel over clopidogrel is primarily driven by the reduction in non-fatal MIs in the prasugrel group; other components of the primary outcome occur at similar rates in both the prasugrel and clopidogrel groups, and it should be noted that the loading dose of clopidogrel administered in the trial is lower than conventional practice in the UK currently (300mg vs 600mg). Importantly, in the CURENT-OASIS 7 study (Mehta et al., 2010), there appeared to be no significant difference in outcomes between patients receiving a 600mg loading dose of clopidogrel as opposed to those receiving a 300mg loading dose. However, a subgroup analysis of patients in the study treated with PCI suggests that there may be a benefit of using a 600mg loading dose of clopidogrel versus 300mg and would be more consistent with the trial population in TRITON-TIMI 38. Meta-analysis of other trials comparing 600mg and 300mg loading doses of clopidogrel also support the use of the higher loading dose (Lotrionte et al., 2007).

The TRILOGY-ACS study assessed the benefit of prasugrel versus clopidogrel in a cohort of NSTEACS patients who were managed without PCI/revascularisation. A total of 9326 patients were enrolled and randomised to receive clopidogrel (75mg) or prasugrel (10mg or 5 mg (if <60 Kg, $\geq$ 75 years)) and were followed up for a minimum of 6 months and a maximum of 30 months. In the clopidogrel group, the primary endpoint of CV death, non-fatal MI and non-fatal stroke occurred in 16.0% of patients versus 13.9% for the prasugrel group (HR 0.91; 95% CI 0.79-1.05, P=0.21). Whilst TRILOGY-ACS did not demonstrate a clear benefit of prasugrel over clopidogrel in NSTEACS treated without revascularisation, a time-dependent divergence of the survival curves did occur 12 months post randomisation in patients under the age of 75 years, with prasugrel demonstrating significant reductions in the rates of the primary composite outcome, myocardial infarctions and strokes but not cardiovascular deaths (Roe et al., 2012).

Recent meta-analyses comparing prasugrel and clopidogrel have been inconsistent. A network meta-analysis by Shah and colleagues (Shah et al., 2017) demonstrates that prasugrel is superior to clopidogrel across a range of cardiovascular endpoints: MACE (OR 0.87; 95% CI 0.80-0.94), recurrent MI (OR 0.89; 95%CI 0.82-0.98) and stent thrombosis (OR 0.48; 95% CI 0.36-0.64) but was not superior to clopidogrel in preventing all-cause or cardiovascular death. Interestingly, a further recent network meta-analysis from Westman

et al, including both randomised controlled trials and other non-randomised studies, failed to demonstrate any significant benefit of prasugrel when compared to clopidogrel (Westman et al., 2017), a finding in agreement with a meta-analysis by Bavishi et al (Bavishi et al., 2015). In addition, health economic comparative effectiveness evaluation of prasugrel compared to clopidogrel based on a created retrospective matched cohort of prasugrel and clopidogrel treated patients using a US health insurance database demonstrated that clopidogrel and prasugrel were equivalent for time to hospital admission following initial discharge. Furthermore, clopidogrel appeared to be superior to prasugrel for early hospitalisation (within the first month), but not at 1 year (Olson et al., 2014, Olson et al., 2015). Finally, real world data following acute myocardial infarction using a different US hospital group database suggest that prasugrel treated patients have a lower cardiovascular related hospitalisation rate in comparison to clopidogrel patients at 30 days and 90 days from the index hospital admission, with no significant increase in bleeding related re-admissions (Bae et al., 2014).

From a safety perspective, prasugrel has been associated with a higher bleeding risk when compared to clopidogrel, in keeping with prasugrel being a more potent anti-platelet agent. In TRITON-TIMI 38, 2.4% of patients in the prasugrel arm suffered a TIMI major haemorrhage in comparison to 1.8% in the clopidogrel arm (HR 1.32; 95% CI 1.03 – 1.68, P=0.03). In addition, the risk of life-threatening bleeding was higher in the prasugrel arm compared to clopidogrel (HR 1.52; 95%CI 1.08-2.13) (Wiviott et al., 2007). However, in TRILOGY –ACS, no significant difference in GUSTO Severe or Life-Threatening bleeding or TIMI Major bleeding was observed between the prasugrel and clopidogrel groups (GUSTO Severe / Life Threatening 0.4% vs 0.4% respectively; TIMI Major 1.1 vs 0.8%) (Roe et al., 2012). An excess of major bleeding events from prasugrel in comparison to clopidogrel was also reported by Shah et al's recent network meta-analysis (Shah et al., 2017) (OR 1.26; 95% CI 1.03-1.56) and Bavishi et al's meta-analysis (Bavishi et al., 2015) (RR 1.32; 95% CI 1.05-1.67) but not Westman et al's meta-analysis (Westman et al., 2017).

Despite the overall view that prasugrel has superior anti-platelet activity to clopidogrel, concerns about high on-treatment platelet reactivity on prasugrel have recently emerged. HTPR has been reported in patients taking prasugrel, including in patients with verified compliance. In a cohort of AMI patients, Sato et al (Sato et al., 2017) demonstrated a strong association between MACE and presence of prasugrel related HTPR, which was observed in 19 out of 78 patients. Similarly, in the context of PCI in ACS, 25.2% of patients receiving a loading dose of prasugrel were found to have HTPR, and in those with HTPR, 30 day incidence

of MACE was significantly higher compared to those without HTPR (Bonello et al., 2011). The incidence of HTPR in patients on prasugrel appears also to be a function of dose, similar to HTPR observed in clopidogrel treated patients, with higher doses having lower incidence of HTPR compared to lower doses (Ferreiro et al., 2013). Importantly, the assay used to determine platelet reactivity has a significant impact on the proportion of patients determined to have HTPR, with poor agreement across different assays (Ferreiro et al., 2013). Further discussion on PD assays and impact on platelet reactivity is covered later in this chapter.

Like clopidogrel, prasugrel is a pro-drug that requires activation via a two-step process. On oral administration, prasugrel is converted rapidly to an inactive thiolactone metabolite (R-95913) by intestinal esterases. This thiolactone metabolite is subsequently metabolised to an active metabolite (R-138727) by a number of CYP450 enzymes (3A4, 3A5, 2B6, 2C19, 2C9) which, unlike clopidogrel, does not depend on a specific CYP to undertake the majority of its metabolism. Consequently, there is less variability in prasugrel active metabolite exposure with fewer interacting drugs or genetic polymorphisms as compared to clopidogrel (Siller-Matula et al., 2013).

### 1.2.4: Ticagrelor

Ticagrelor, is a novel, first in-class, P2Y12 receptor antagonist. Structurally, ticagrelor is a cyclopentyl-tiazolo-pyrimidine which, unlike the thioenopyridines, binds to the P2Y12 receptor, reversibly, via an allosteric modulation site which prevents ADP binding to the receptor.

In the pivotal PLATO trial, 18,624 patients with either STEMI or NSTEACS were randomised to receive ticagrelor (180mg loading dose followed by 90mg twice daily) or clopidogrel (300 to 600mg loading dose followed by 75mg once daily) for 12 months. The PLATO study demonstrated that ticagrelor was superior to clopidogrel in reducing the primary composite outcome measure of MACE (defined as cardiovascular death, myocardial infarction or stroke). In patients administered ticagrelor, the endpoint occurred in 9.8% compared to 11.7% in those being treated with clopidogrel (Hazard ratio (HR) 0.84; 95% CI 0.77-0.92, $P<0.001$) (Wallentin et al., 2009). The benefit of ticagrelor over clopidogrel was observed for individual endpoints in addition; including cardiovascular death (4.0% vs 5.1% respectively, $P=0.001$), myocardial infarction (5.8% vs 6.9%, $P=0.005$) and all-cause mortality (4.5% vs 5.9%, $P<0.001$) although the rate of stroke did not differ significantly between ticagrelor and clopidogrel treated patients. Interestingly, the benefit of ticagrelor over clopidogrel

appeared to be greater beyond 30 days from randomisation as compared to before 30 days (composite endpoint event rate, days 1 to 30: 4.8% in the ticagrelor arm versus 5.4% in the clopidogrel arm; HR 0.88; 95% CI 0.77-1.00, P=0.045, days 31-360: 5.3% vs 6.6%; HR 0.80; 95% CI 0.70-0.91, P <0.001). Importantly, the benefit of ticagrelor over clopidogrel was observed in both NSTEACS and STEMI patients, irrespective of whether invasive treatment was used or not. In contrast, prasugrel showed clear superiority over clopidogrel only in the context of an invasive strategy in both STEMI and NSTEACS (as demonstrated in TRITON-TIMI 38) and did not demonstrate superiority over clopidogrel in medically managed NSTEACS patients in the TRILOGY-ACS study.

In the earlier DISPERSE-2 study, ticagrelor (90mg and 180mg doses) was compared with clopidogrel (75mg) in a randomised control trial for 12 weeks. A total of 990 patients were randomised equally to receive the three treatment regimens, with a trend observed of lower MI rate in the ticagrelor arms which did not meet statistical significance (Cannon et al., 2007).

However, in the PHILO study, comparing ticagrelor and clopidogrel in Japan and East Asian countries in patients with either STEMI or NSTEACS, ticagrelor failed to show a clear benefit over clopidogrel (Goto et al., 2015). In this study of 801 Japanese and East Asian patients randomised to receive either ticagrelor (180mg loading dose, 90mg BD thereafter) or clopidogrel (300mg loading dose, 75mg od thereafter), the occurrence of the primary composite endpoint (cardiovascular death, myocardial infarction or stroke) was 9.0% in the ticagrelor arm and 6.3% in the clopidogrel arm (HR 1.47; 95% CI 0.88-2.44). Notably, the sample size for this study was small in comparison to conventional phase III studies, with a high rate of PCI performance which may have contributed to the lack of clear data from this study. Similarly, in the EUCLID study, 13855 patients with peripheral arterial disease (PAD), ticagrelor was not demonstrated to be better than clopidogrel for the prevention of a composite endpoint of cardiovascular death, MI or stroke (13.0% for ticagrelor treated patients versus 13.3% in clopidogrel treated patients (HR 1.01; 95% CI 0.88-1.15, P=0.90)). In addition, there was no evidence that ticagrelor was superior to clopidogrel in reducing acute limb threatening events (Jones et al., 2017).

Whilst existing data suggest that ticagrelor is superior to clopidogrel, no large randomised studies have addressed the relative clinical efficacy of ticagrelor compared to prasugrel. The PRAGUE-18 study (Motovska et al., 2016) randomised 1230 STEMI patients being treated with PPCI. No difference between prasugrel and ticagrelor was observed for the composite primary endpoint of death, re-infarction, urgent target vessel revascularisation (TVR), stroke

or serious bleeding at 7 days (4.0% in the prasugrel group versus 4.1% in the ticagrelor group (OR 0.98; 95% CI 0.55-1.73, P=0.939)) or the composite secondary outcome of cardiovascular death, myocardial infarction or stroke (2.7% vs 2.5% (OR 1.06; 95% CI 0.53-2.15; P=0.864)). Similarly, at 12 months, no significant differences between prasugrel and ticagrelor was demonstrated (Motovska et al., 2018). These data are in keeping with the Bayesian network meta-analysis conducted by Shah et al which failed to demonstrate any significant differences between ticagrelor and prasugrel (Shah et al., 2017).

Like prasugrel, ticagrelor appears to be associated with an increased bleeding risk compared to clopidogrel. In the PLATO trial, whilst the overall rate of major bleeding was similar between ticagrelor and clopidogrel, there appeared to be a significant increase on non-coronary artery bypass graft (CABG) related bleeding in the ticagrelor arm compared to the clopidogrel arm (4.5% vs 3.8%, P=0.03) (Wallentin et al., 2009). However, there was no evidence of a significant increase in bleeding risk from ticagrelor in the PHILO study, although the rate of major bleeding was numerically higher in the ticagrelor arm (10.3% vs 6.8%, HR 1.54; 95% CI 0.94-2.53) (Goto et al., 2015) which is also in keeping with bleeding data from the DISPERSE-2 trial (Cannon et al., 2007). Ticagrelor is also associated with adverse events which appear unrelated to its anti-platelet action and may be related to an increased level of adenosine. In the PLATO trial, dyspnoea was reported significantly more often in the ticagrelor arm compared to the clopidogrel arm (13.8% vs 7.8%, P<0.001) although the rate of discontinuation from this adverse effect was very low (0.9% vs 0.1%, P<0.001). In addition, Holter monitor data demonstrated an increased number of ventricular pauses of greater than 3 seconds in patients administered ticagrelor (5.8% vs 3.6%, P=0.01) although these appeared to be asymptomatic with no significant differences in syncope or PPM insertion between the ticagrelor and clopidogrel groups (Wallentin et al., 2009). A significant increase in dyspnoea in ticagrelor treated patients was also observed in the DISPERSE-2 study (statistically significant) and PHILO study (not statistically significant) (Cannon et al., 2007, Goto et al., 2015).

Dyspnoea secondary to ticagrelor appears to be an adenosine mediated effect via stimulation of A1 and A2A receptors in the airways' vagal C-fibres (Unverdorben et al., 2016). Ticagrelor has been demonstrated to increase adenosine concentrations by reducing adenosine uptake via inhibition of the sodium-independent equilibrative nucleoside transporter (ENT) – 1 (Wittfeldt et al., 2013). Ticagrelor's effect on adenosine metabolism may also explain its superiority over clopidogrel in the PLATO study. Whilst previous anti-platelet studies have demonstrated improvements in outcome measures such as non-fatal

MI, the PLATO study is unusual in that there is an almost universal superiority of ticagrelor over clopidogrel across clinical outcome measures and, most notably, cardiovascular death. In addition, the survival curves for ticagrelor and clopidogrel continue to diverge in the longer term, suggesting that ticagrelor has unexpected effects beyond platelet inhibition. Central to explaining these findings has been the hypothesis that increased adenosine concentrations are fundamental to ticagrelor's off-target beneficial effects (Gurbel et al., 2016). In a healthy volunteer, randomised, double blind, placebo-controlled study, Wittfeldt et al (Wittfeldt et al., 2013) demonstrated that a single 180mg ticagrelor dose significantly increased adenosine induced coronary blood flow velocity and the sensation of dyspnoea in the study subjects. In patients with ACS, ticagrelor increases the plasma concentration of both adenosine and cAMP, an increase which is significantly higher than observed with clopidogrel (Li et al., 2017c). The concentrations of adenosine and cAMP showed only weak correlation with platelet inhibition however, suggesting that the increase in adenosine concentrations has only a limited effect on ticagrelor's anti-platelet action. In addition, ticagrelor also increases adenosine mediated myocardial blood flow in ACS patients as compared to clopidogrel (Pelletier-Galarneau et al., 2017), a finding in keeping with the apparent improvement in endothelial dysfunction (as measured by flow mediated and nitro-glycerin mediated dilation) with ticagrelor compared to clopidogrel (Mangiacapra et al., 2016). Taken together, these data suggests that the 'cryptic' effect of ticagrelor is mediated by inhibition of adenosine reuptake with consequent improvement in blood flow, endothelial dysfunction and, consequently, vascular inflammation (Wittfeldt et al., 2013, Gurbel et al., 2016).

Unlike prasugrel and clopidogrel, ticagrelor is a directly acting anti-platelet drug with no requirement for hepatic conversion into an active metabolite (Holmberg et al., 2013). However, ticagrelor is extensively metabolised to two major metabolites, AR-C124910XX and AR-C133913XX via CYP3A4 and CYP3A5. AR-C124910XX also has anti-platelet properties in addition to the parent drug although its effects on clinical outcomes are relatively small (Holmberg et al., 2015). In addition, ticagrelor is a substrate for P-glycoprotein.

Despite ticagrelor's superiority over clopidogrel in clinical trials and its more potent anti-platelet action, HTPR during ticagrelor treatment has been observed. In a meta-analysis from Lemesle et al (Lemesle et al., 2015) of 14 studies in 1822 patients, the overall HTPR rate was 6.1% with a significantly lower rate in patients taking ticagrelor in comparison to prasugrel (1.5% vs 9.8%, RR 0.27; 95% CI 0.14-0.50, P<0.0001). In addition, HTPR rate varied dependent on the timing and type of dose, with studies investigating loading doses reporting higher HTPR rates than studies investigating maintenance doses. In keeping with other studies, the

rate of HTPR is dependent on the type of platelet function assay being used, with VASP reporting higher rates of HTPR than VerifyNow for example. However, these data suggest that HTPR on ticagrelor is less common than with the thienopyridine drugs, such as clopidogrel and prasugrel. Further detail about platelet function testing and the impact of various factors on HTPR are covered later in this introductory chapter.

### 1.2.5: Cangrelor

Cangrelor is a novel intravenous anti-platelet drug which, similar to ticagrelor, binds reversibly and directly to the P2Y12 receptor (Qamar and Bhatt, 2016). As it is administered intravenously, cangrelor's anti-platelet effect is exerted almost immediately, with maximum plasma concentrations occurring around 2 minutes after administration (Sible and Nawarskas, 2017).

Three large phase III studies have demonstrated the efficacy of cangrelor compared to clopidogrel as part of the CHAMPION programme. CHAMPION PLATFORM (Bhatt et al., 2009) randomised P2Y12 naïve patients to either cangrelor or placebo followed by a 600mg dose of clopidogrel, in the context of PCI and unstable cardiac disease. No significant differences in the primary outcome (composite of death, myocardial infarction or ischaemia-driven revascularisation) was observed in the cangrelor group compared to clopidogrel (7.0% vs 8.0%, OR 0.87; 95%CI 0.71-1.07, P=0.17) although a significant reduction in stent thrombosis and a composite of death and Q wave myocardial infarction was observed for cangrelor treated patients. Similarly, CHAMPION PCI (Harrington et al., 2009) did not detect a significant benefit of cangrelor over clopidogrel in a cohort of patients with unstable cardiac disease. Both trials were stopped early given the low likelihood of reaching significance for the primary outcome measure. However, it is likely that the negative results from both the CHAMPION PLATFORM and PCI studies were, at least partly, caused by the definition of peri-procedural MI (creatine kinase MB (CK-MB) of 3x the upper limit of normal (ULN)) used in both studies which resulted in some patients being classified as having a peri-procedural MI when it was more likely that the CK-MB was continuing to rise from the initial ACS (Faxon, 2010). Subsequent re-analysis of the CHAMPION PLATFORM and PCI trial datasets using the universal definition of MI suggest that, even with premature termination, both trials did demonstrate that cangrelor was superior to the comparator in each of the trials (Sible and Nawarskas, 2017). Moreover, a further RCT, CHAMPION PHOENIX (Bhatt et al., 2013) demonstrated that cangrelor is superior to clopidogrel in a cohort of patients with both stable and unstable cardiac disease undergoing PCI. The primary endpoint of death from any

cause, myocardial infarction, ischaemia driven revascularisation and stent thrombosis occurred in 4.7% of the cangrelor group in comparison to 5.9% in the clopidogrel group (OR 0.78; 95% CI 0.66-0.93, P=0.005). In CHAMPION PHOENIX there was no evidence that cangrelor was associated with an increased risk of either GUSTO defined or TIMI defined bleeding; in both CHAMPION PLATFORM and PCI there was a significant association between GUSTO defined mild bleeding and cangrelor use, however, no association was observed for GUSTO defined major or life threatening bleeding nor any TIMI defined bleeding. A significant increase in dyspnoea events in the cangrelor group was observed across all three CHAMPION studies, in keeping with the existing data on ticagrelor.

Because of the nature of the conformational change in the P2Y12 receptor when cangrelor binds to it, neither prasugrel's nor clopidogrel's active metabolite can bind to the P2Y12 receptor whilst cangrelor is bound to it. Consequently, clopiodgrel and prasugrel must be administered at the end of the cangrelor infusion and not before it, given the short half-life of both of those drugs' active metabolite. Conversely, ticagrelor binds to an alternative site on the P2Y12 receptor and can therefore be administered during the infusion which will ensure sufficiently deep platelet inhibition to prevent ischaemic outcomes post PCI (Sible and Nawarskas, 2017).

### 1.2.6: Direct Oral Anti-Coagulants (DOACs)

Of recent, DOACs have been investigated in the context of ACS and stable coronary artery disease, with a particular focus on apixaban and rivaroxaban whose mechanism of action is inhibition of factor Xa and consequent reduction in thrombin generation (Khan et al., 2018). However, despite a similar mechanism of action, the effect on cardiovascular outcomes is markedly different between apixaban and rivaroxaban.

In the APPRAISE study (Committee et al., 2009) of 1715 patients with recent ACS, apixaban in combination with aspirin and/or clopidogrel failed to significantly reduce further cardiovascular events but did increase the risk of bleeding in a dose-dependent manner. Similarly, the larger APPRAISE-2 study (Alexander et al., 2011) was terminated prematurely following recruitment of 7392 patients with ACS due to significant increase in bleeding risk in apixaban treated patients without demonstrated benefit in reduction of recurrent cardiovascular events. In further analyses of the APPRAISE-2 trial data the risk of bleeding was not dependent on the use of single or dual antiplatelet therapy (Hess et al., 2015) given that over two thirds of patients enrolled into APPRAISE-2 were receiving both aspirin and

clopidogrel. Given these data, apixaban is not recommended for secondary prevention of ischaemic events following an acute coronary syndrome.

Conversely, rivaroxaban is licensed for use in secondary prevention, in addition to aspirin, in patients with unstable and stable cardiovascular disease. In the ATLAS ACS 2-TIMI 51 study (Mega et al., 2012), rivaroxaban significantly reduced the risk of MACE compared to placebo in 15,526 patients with recent ACS (HR 0.84; 95% CI 0.74 – 0.96, P=0.008) although the risk of major bleeding was significantly increased in the rivaroxaban treated patients (HR 3.96; 95%CI 2.96 – 6.38, P < 0.001). In a further sub-analysis of the ATLAS ACS 2-TIMI 51 data (Mega et al., 2013), no significant difference in the MACE outcome was noted for the two rivaroxaban dose schedules used in the study (2.5mg twice daily or 5mg once daily); however, bleeding events were fewer in the 2.5mg twice daily group compared to the 5mg once daily group. Notably, the addition of rivaroxaban to aspirin therapy in patients with stable cardiovascular disease also reduces the risk of further cardiovascular events. In the COMPASS trial (Eikelboom et al., 2017), 27, 395 patients with stable cardiovascular disease were randomised to receive placebo, rivaroxaban 2.5mg twice daily or rivaroxaban 5mg twice daily with or without aspirin. A significant reduction in MACE was observed in the rivaroxaban and aspirin group compared to the aspirin only group (HR 0.76; 95%CI 0.66 – 0.86, P<0.001) with a corresponding increase in bleeding events (HR 1.70; 95%CI 1.40 – 2.05; P<0.001). Interestingly, rivaroxaban did not appear to reduce MACE when administered without aspirin but was associated with a similar bleeding risk whether administered with or without aspirin (Anand et al., 2018). Given the data from ATLAS ACS 2-TIMI51 and COMPASS, rivaroxaban is now licensed for use in both unstable and stable cardiovascular disease in combination with aspirin or, in the context unstable cardiovascular disease, clopidogrel.

### 1.2.7: Duration of Anti-platelet Therapy

The ESC guidelines on STEMI (Ibanez et al., 2018) suggest that DAPT should be continued for a period of at least 12 months which is also reflected in the ESC guidelines on NSTEACS (Roffi et al., 2016). However, both guidelines recognise that longer and shorter courses may be suitable for some patient groups (e.g. high vascular or high bleeding risks).

Of recent, specific focus has been given to shortening the duration of DAPT. In the SMART-DATE trial (Hahn et al., 2018), 2712 patients with an ACS were randomised to receive either 6 months or at least twelve months of DAPT and were followed up for a total of 18 months following the initial cardiac event. No significant differences were noted between the occurrence of the primary outcome, MACE, and duration of DAPT (4.7% in the 6 month DAPT

group and 4.2% in the at least 12 month group (HR 1.13; 95% CI 0.79 – 1.62, p=0.51)) although a significant increase in myocardial infarctions were observed in the 6 month DAPT group (1.8% vs 0.8% (HR 2.41; 95% CI 1.15 – 5.05, p=0.02)). Interestingly, no significant reduction in bleeding events were noted in the 6-month group as compared to the at least 12 months of DAPT group although it should be noted that randomisation was performed without regard to bleeding risk. In the STOPDAPT-2 trial (Watanabe et al., 2019), 3045 patients undergoing PCI for a both stable and unstable cardiac disease were randomised to receive one month of DAPT followed by either continuation of DAPT for 11 months or clopidogrel monotherapy for up to five years. At 12 months, subjects who had received one month of DAPT followed by clopidogrel monotherapy had a lower occurrence of the primary end point (MACE) compared to subjects receiving 12 months of DAPT (2.4% vs 3.7% (HR 0.64; 95% CI 0.42-0.98, P=0.04 for superiority)) with a significantly lower rate of bleeding (0.4% vs 1.5% (HR 0.26; 95% CI 0.11-0.64, P=0.004 for superiority)). Similarly, in the SMART-CHOICE trial (Hahn et al., 2019), a three-month DAPT treatment period was non-inferior to the conventional 12 months of DAPT for prevention of MACE in 2993 patients receiving PCI. In keeping with these data, a network meta-analysis by Yin and colleagues (Yin et al., 2019) of 17 studies and 46, 864 patients undergoing PCI demonstrated similar rates of efficacy in preventing further cardiac events between short term DAPT and standard term DAPT. Importantly, standard duration DAPT was associated with a significantly higher risk of bleeding (OR 1.39; 95% CI 1.01 – 1.92). Extension of DAPT beyond 12 months was also associated with a higher risk of non-cardiac death and bleeding than short- or standard tern DAPT, demonstrating that optimal length of DAPT is likely to be no more than 12 months and, in most patients, less than 12 months. Several other studies have also investigated the use of risk scores, taking into account bleeding risk and platelet reactivity, as a tool for stratification of anti-platelet duration with positive results (Brener et al., 2018, Sibbing et al., 2017).

## 1.3: Detecting and Measuring Response to Anti-platelets

Response to anti-platelet drugs can be measured by a range of platelet function tests (PFTs). Platelet function is best assessed by measuring platelet response and subsequent aggregation to various agonists such as arachidonic acid (AA) for ASA response and ADP for thienopyridine, ticagrelor and cangrelor response. Other tests, such as serum or urinary (Ur) 11-dehydrothromboxane B2 (11dhTXB2) assess the downstream effects of anti-platelet drug

administration (in this case aspirin). In addition, there are a number of tests that can be performed near-patient (such as VerifyNow (VN) and Multiplate (MP)) whereas others, such as light transmittance aggregometry (LTA), flow cytometry and VASP, require specialised laboratory infrastructure and staff in order to standardise and calibrate those assays. However, each assay tests platelet function in slightly different ways, and consequently there is a high degree of variability and correlation between the assays is often poor.

### 1.3.1: Light Transmittance Aggregometry (LTA)

LTA measures platelet aggregation by assessing the increase in light transmission through platelet rich plasma following exposure to various agonists such as ADP, epinephrine and AA. It has long been considered the 'gold standard' platelet function test and can be used to assess platelet inhibition to aspirin (using AA as an agonist) or P2Y12 receptor inhibitors (using ADP as an agonist). Several studies highlight that LTA is associated with clinical outcome. In a large study (N=1789), in patients with ACS administered clopidogrel, Parodi et al (Parodi et al., 2011) demonstrated an absolute risk increase of 5.9% of adverse cardiovascular events in patients determined as having HTPR compared to those with low residual platelet reactivity as defined by LTA using ADP as an agonist, a finding in keeping with other clinical studies (Migliorini et al., 2013, Tang et al., 2015) and a meta-analysis (Aradi et al., 2010). Similarly, response to aspirin, as measured using LTA with AA as an agonist, is also associated with clinical outcome (Spectre et al., 2011). However, LTA may not be as predictive for outcome as other platelet function tests (Breet et al., 2011) and results from LTA may not agree with results from other platelet function tests (Breet et al., 2010), with varying rates of HTPR reported across a single cohort dependent on the PFT used. LTA is also performed without the other cellular components of blood, whereas other PFTs are whole blood assays which may decrease the sensitivity of LTA to other, non-platelet dependent, factors that may affect platelet reactivity. Similarly, LTA is performed, generally, with a single agonist which is not truly representative of platelet activation in vivo, where interactions between platelets, other cellular blood components and collagen are present and may contribute to overall platelet reactivity (Ohmori et al., 2006). In addition, reproducibility of LTA is often poor given the high operator and interpreter dependence (Michelson, 2004) and different studies utilise different measures of platelet reactivity or different concentration of agonists which makes comparison across studies difficult. Furthermore, whilst published guidelines for LTA exist, there are important methodological differences between each guideline which limit their usefulness (Koltai et al., 2017). However, Choi et al (Choi and Kim, 2018), in a study of 904 patients post PCI, determined that LTA is relatively unaffected by

clinical and laboratory variables that have significant influence on other PFTs. Consequently, whilst LTA remains an important reference standard, its utility in routine clinical practice may be limited.

### 1.3.2: VerifyNow

VerifyNow (VN) (Accriva Diagnostics, USA) is a point of care platelet function test which utilises turbidometric optical detection to measure platelet aggregation in response to AA or ADP (for aspirin and P2Y12 inhibitors respectively). Importantly, the measured aggregation is converted into either Aspirin Reaction Units (ARU) or P2Y12 Reaction Units (PRU) which are consistent across different operators and settings, allowing the development of reference ranges and cut-off values for HTPR.

Platelet reactivity measured by VN is strongly associated with clinical outcomes in both aspirin and clopidogrel patients. In aspirin treated patients, high ARU values (>550) are associated with adverse cardiovascular outcomes, with a threefold increase in the risk of death or further cardiovascular events reported in a large, 468 patient study with stable coronary artery disease (Chen et al., 2004). However, other, larger studies have not shown clear associations between HTPR with aspirin and adverse cardiovascular outcomes (Stone et al., 2013), although a large meta-analysis of 15 studies and 11542 patients demonstrate a two fold increase in risk of cardiovascular events in patients on aspirin with HTPR detected using VerifyNow (RR 2.23; 95%CI 1.55-3.21) (Wisman et al., 2014).

Similarly, HTPR identified by the VN P2Y12 assay is associated with adverse cardiovascular outcomes (Breet et al., 2010). In the large, 8665 patient, ADAPT-DES study (Stone et al., 2013), HTPR, defined as PRU > 208, in patients on DAPT post PCI for either stable or unstable cardiac disease, was associated with a significant increase in both myocardial infarction and stent thrombosis but not all-cause mortality. However, in another large, observational study, Park et al (Park et al., 2013a) demonstrated a greater than threefold increase in mortality in patients with ACS who were determined to have HTPR on the basis of the VN P2Y12 assay (HR 3.46, 95% CI 1.18-10.18, P=0.02), a finding in keeping with other studies in ACS (Saia et al., 2013). However, no association between mortality and HTPR was observed in patients with stable coronary artery disease, suggesting that HTPR may not be an important prognostic factor in otherwise stable patients. Similar results have been found in other studies in stable coronary artery disease (Viviani Anselmi et al., 2013). In addition, a meta-analysis of 25 studies including 21667 patients, demonstrates a nearly threefold

increase in risk of composite ischaemic events in patients with HTPR on clopidogrel as defined by VN (Relative Risk (RR) 2.52; 95%CI 2.05-3.10) (Aradi et al., 2010).

However, VerifyNow may be sensitive to haematocrit (Hct), haemoglobin (Hb) and platelet count (Choi and Kim, 2018). A patient level meta-analysis, including 10 studies and 4793 patients, (Kim et al., 2017b) observed a significant inverse relationship between PRU reported by VerifyNow and haematocrit or haemoglobin, which was not seen with other platelet function tests such as LTA and Multiplate. It is not clear why there is an association between Hct or Hb and PRU values, although it is hypothesised that the optical nature of the assay may be influenced by Hb concentration. However, there is no association between Hct and LTA values, another optical based assay; therefore, other mechanisms, such as interactions between erythrocytes and platelets, may be important.

### 1.3.3: Multiplate

Multiplate (Roche Diagnostics) is an impedance based aggregometry platelet assay. Like VerifyNow, it is a point of care device that tests both Aspirin and P2Y12 inhibitor response and reports in standard units (aggregation units (AU)) which supports standardisation and development of cut-off values for HTPR. Whilst this assay can be used in a point of care setting, it does require some sample preparation with sample dilution followed by addition of agonists (AA for determination of aspirin response and ADP for the determination of P2Y12 inhibitor response) required before performing the test. Multiplate measures the changes in whole blood impedance, with greater aggregation leading to more adhesion to the test electrodes and a consequent increase in electrical impedance (Gremmel et al., 2015).

Multiplate has been used to determine the presence of HTPR to both aspirin and clopidogrel in several studies. Like other methods for assessing AA induced platelet aggregation, Multiplate does not correlate well with other assays such as VerifyNow and LTA using AA as an agonist (Gremmel et al., 2015), although some studies do report good correlation (Paniccia et al., 2009). However, in a meta-analysis of three studies, including 700 patients, Multiplate determined AA induced platelet aggregation was not clearly associated with cardiovascular events (RR 1.93; 95% CI 0.81-4.62) (Wisman et al., 2014). In addition, a recent, study by Larsen (Larsen et al., 2017) demonstrated no association between HTPR with Aspirin, as determined by Multiplate, and adverse clinical outcomes, in a cohort of 900 stable coronary artery disease patients. Similarly, in a cohort of STEMI patients, undergoing PCI, HTPR with aspirin, determined by Multiplate, was also not associated with adverse clinical outcome (Mrdovic et al., 2016). It is not clear why the Multiplate AA test does not clearly

associate with clinical outcome, but it is likely to represent additional actions of aspirin beyond the COX-1 pathway, a finding in keeping with the known dose-dependent effects of aspirin (Mrdovic et al., 2016). In addition, aspirin dose timing and the presence of additional anti-platelet agents (e.g. thienopyridines or ticagrelor) are likely confounders which may either over-report the prevalence of aspirin resistance (pseudoresistance) or improve ADP-mediated platelet inhibition which are not accounted for by an AA specific assay (Larsen et al., 2017).

The Multiplate ADP assay has also been extensively studied in the context of the P2Y12 inhibitors. Multiplate correlates with LTA using ADP as an agonist and the VN ADP test (Kozinski et al., 2016) and correlated well with cell markers of platelet activation (Gremmel et al., 2015). However, Multiplate does not correlate well with clopidogrel pharmacokinetics (Danese et al., 2016), but appears to show very good correlation with Ticagrelor and AR-C124910XX concentrations (Kozinski et al., 2016). The Multiplate ADP assay is associated with adverse clinical outcomes, with a MA of six studies and 2716 participants demonstrating a six-fold increase in the risk of cardiovascular events in patients determined to have HTPR using the Multiplate ADP test (RR 6.08; 95% CI 1.85-20.00) (Wisman et al., 2014).

However, it should be noted that Multiplate can be affected by a number of clinical and laboratory variables. In a study of 904 patients post PCI patients receiving DAPT, Choi et al identified a strong positive correlation between the Multiplate ADP assay and platelet count. In addition, smoking was associated with HTPR identified by Multiplate but Hct and Hb were not associated with Multiplate results as compared to VerifyNow (Choi and Kim, 2018).

### 1.3.4: Platelet Function Analyser-100/200

The Platelet function Analyser (PFA)-100/200 tests platelet function in conditions of high shear using whole blood. This mechanism is sensitive to the binding of von Willebrand Factor (vWF) to glycoprotein 1b and is consistent with the mechanism of thrombosis formation in small vessels. Whole blood is forced through a small aperture which is coated with either collagen and epinephrine (CEPI), used to assess response to aspirin, or ADP, used to assess the response to P2Y12 inhibitors. Whilst the CEPI test for aspirin response does not use AA as an agonist, it is regarded to be relatively COX-dependent and may also be sensitive to non-COX dependent actions of aspirin. Values are reported as Closure Time (CT) and measured in seconds, with a variable cut-off used to define HTPR to either aspirin or P2Y12 inhibitors.

PFA-100 has been extensively studied in relation to clinical outcomes and aspirin response. In a meta-analysis of 21 studies and 5222 patients, aspirin related HTPR was associated with a twofold higher risk of adverse cardiovascular events (RR 1.88; 95%CI 1.44-2.47) (Wisman et al., 2014). In addition, the ADP test for P2Y12 inhibitors is also associated with clinical response, with a meta-analysis of 4 studies and 1158 patients demonstrating a nearly threefold risk of adverse cardiovascular events in P2Y12 resistant patients (RR 2.74, 95%CI 1.17-6.41) (Wisman et al., 2014). However, the original PFA-100 ADP test was often criticised as being poorly sensitive to the effects of clopidogrel (Li et al., 2016b) which resulted in the development of a new PFA-200 P2Y test to improve the sensitivity to ADP inhibitors. In a study by Li et al (Li et al., 2016b), the PFA-200 P2Y test demonstrated good correlation with LTA and VerifyNow in a cohort of 93 post-PCI patients taking clopidogrel. In addition, the PFA-200 demonstrated significant association between loss-of-function genotypes and clopidogrel related HTPR, a finding replicated by other studies (Kim et al., 2015). Whilst these data are encouraging, there are insufficient data to determine whether the new PFA-200 P2Y assay will have utility in clinical practice.

Whilst sensitivity to non-COX dependent modifiers of platelet function may be useful in the detection of certain types of aspirin resistance, it may reduce the ability of the PFA-100 to fully discriminate aspirin sensitive patients from aspirin resistant patients. In a healthy volunteer study, platelet function was measured before and after the administration of aspirin using six different laboratory methods. The PFA-100 results showed significant correlation between pre-treatment and post-treatment values whilst other, more COX specific, assays demonstrated very poor correlation between the two (Kovacs et al., 2014). Importantly, the PFA-100 assay is sensitive to other variables such as vWF, haematocrit and platelet count (Fitzgerald and Pirmohamed, 2011, Kovacs et al., 2014) in keeping with its development as a platelet function test for haematological disease rather than for anti-platelet therapy. In addition, there is little consensus in the published literature on the PFA-100 closure time cut-offs that should be used to determine HTPR with aspirin, with multiple different values being used. Evidence synthesis for the PFA-100 is therefore challenging and it cannot be clearly stated that the PFA-100 is truly specific for aspirin resistance or not; a fact highlighted by the recent Health Technology Assessment on the prognostic utility of tests for aspirin resistance (Dretzke et al., 2015).

### 1.3.5: Thromboxane Metabolites

The primary thromboxane metabolite used for determining aspirin response is urinary 11-dehydrothromboxane B2 (Ur 11dhTXB2). It is a stable metabolite of TXA2 and therefore is a direct measure of COX-1 inhibition. TXB2 levels have been associated with adverse cardiovascular outcomes in a number of studies (Dharmasaroja and Sae-Lim, 2014, Temperilli et al., 2015) with a large, multicentre study demonstrating a threefold higher risk of cardiovascular death and twofold higher risk of myocardial infarction in cardiovascular disease patients with an elevated Ur 11dhTXB2 (Eikelboom et al., 2002). However, meta-analysis of the relationship between TXA2 metabolites and cardiovascular outcome does not demonstrate a clear and consistent association (Wisman et al., 2014). It is notable that TXB2 is positively influenced by the severity of cardiovascular disease (Faraday et al., 2006) as a consequence of COX-2 expression in atherosclerotic plaques (Patrignani, 2003). In addition, aspirin has a dose-dependent effect on TXB2 levels in healthy volunteers and cardiovascular disease patients (Harrison et al., 2018) which is likely to be explained by the inhibition of COX-2 at higher doses rather than platelet COX-1, in keeping with the finding that low dose aspirin is as effective as high dose aspirin in preventing cardiovascular events, irrespective of TXB2 levels (Antithrombotic Trialists, 2002). Consequently, TXB2 levels may not correlate with aspirin response and may better represent overall COX-1 and COX-2 function in the context of cardiovascular disease. In addition, TXB2 levels may be significantly decreased by other drugs, such as P2Y12 inhibitors (Bagoly et al., 2016), in keeping with the known effects of P2Y12 receptor activation on AA metabolism. Furthermore, Ur 11dhTXB2 may not always be correlated with serum TXB2 (Harrison et al., 2018) in keeping with the generation of TXB2 from other tissues aside from platelets (Koltai et al., 2017).

### 1.3.6: Vasodilator-stimulated phosphoprotein phosphorylation

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation is a flow cytometry technique, using whole blood incubated with Prostaglandin E1 (PGE1) alone or PGE1 and ADP, to determine P2Y12 inhibitor related platelet reactivity as measured by immunofluorescence to phosphorylated VASP. VASP is a laboratory-based technique that, despite CE marked diagnostic kits being available, still requires skilled operators in order to ensure consistency and reliability of assay results. Consequently, whilst VASP has been used in P2Y12 inhibitor studies extensively, there are relatively limited clinical outcome data for the technique. However, in a meta-analysis of six studies including 1813 patients, HTPR to a P2Y12 inhibitor reported by VASP conferred a nearly fivefold increase in risk of adverse

cardiovascular events (RR 4.82; 95%CI 1.27-18.24) (Wisman et al., 2014). VASP also correlates well with the pharmacokinetics of ticagrelor and its metabolite, AR-C124910XX, in a study of MI patients (Kozinski et al., 2016) as well as demonstrating good correlation with clopidogrel's active metabolite (Danese et al., 2016). VASP appears to correlate well with other platelet function tests (Kozinski et al., 2014), and in particular, the gold-standard LTA using ADP as an agonist.

However, given the technical nature of the assay, VASP remains mostly a laboratory reference assay as opposed to a point-of-care test with the potential for use in patient stratification.

### 1.3.7: Other Platelet Function Assays

**Thromboelastography** – Thromboelastography (TEG) is a group of assays that assess viscoelastic changes during the process of blood clotting (Koltai et al., 2017), with the TEG Platelet Mapping System (Haemonetics, Braintree, MA, USA) being the most appropriate technique for assessing platelet function in the context of anti-platelet drugs. Platelet function is reported as Inhibition of Platelet Aggregation (IPA), a standardised unit, which allows determination of appropriate cut-offs of HTPR and is performed as a point-of-care assay. In the context of P2Y12 inhibitors, HTPR defined by TEG is associated with a significant increase in risk of cardiovascular events in a meta-analysis of two studies and 547 patients (RR 7.11; 95%CI 2.32-21.83) (Wisman et al., 2014). Newer clinical studies are also in keeping with these data, with a 178 post-PCI patient study demonstrating a clear association between TEG identified HTPR to clopidogrel and adverse clinical outcomes (Tang et al., 2015). In addition, this study also demonstrated a clear association between carriage of the CYP2C19 loss-of-function alleles and TEG defined HTPR (Tang et al., 2015). TEG shows variable correlation with other platelet function tests including VerifyNow, with some studies demonstrating correlation (Yao et al., 2016) and others not (Lv et al., 2016), in keeping with a comparative study of five platelet assays (VASP, VerifyNow, Multiplate, LTA and TEG) where TEG displayed high inter-assay variability (Karon et al., 2014). In addition, a specific phenomenon described as 'thrombin breakthrough' occurs with TEG which affects the fibrin readout from the assay. This was noted in four tracings of 91 pairs in this study and is associated with the high variability observed with this test (Karon et al., 2014). TEG can also be used to measure aspirin response, although clinical data are more limited than with P2Y12 inhibitors. However, one study has been included in a meta-analysis of aspirin resistance that failed to demonstrate a clear association between aspirin related HTPR and adverse clinical

outcomes (Wisman et al., 2014) and it remains unclear the clinical utility of TEG in the context of aspirin response given the paucity of trial data (Dretzke et al., 2015).

**PlateletWorks**: PlateletWorks (Helena Laboratories) is a point of care device using specific PlateletWorks tubes, one with EDTA as an anticoagulant (baseline) and one with citrate and 20 mmol of ADP or AA. A cell counter is then used to differentiate between aggregated and non-aggregated platelets, and the difference between the two is used as the measurement of platelet reactivity. PlateletWorks is a simple test to perform but requires fresh whole blood and must be completed within minutes for reliable results. The necessity for rapid analysis has limited the usage of the test in the context of clinical outcome trials (Koltai et al., 2017). In a large cohort of 1069 stable coronary artery disease patients undergoing PCI, HTPR to clopidogrel identified by the PlateletWorks assay was associated with adverse cardiovascular outcomes of all-cause mortality, MI, stent thrombosis and stroke (OR 2.22; 95%CI 1.25-3.93, P=0.005) (Breet et al., 2010). In addition, low on treatment platelet reactivity to clopidogrel, as identified by PlateletWorks, is associated with a higher risk of bleeding (defined according to BARC or ARMYDA-BLEEDS criteria) (Holm et al., 2014). PlateletWorks can also be used to determine aspirin response, however, data from two studies suggest that PlateletWorks may be less sensitive for aspirin response in comparison to other platelet function tests such as LTA and VerifyNow (Lennon et al., 2004, Dichiara et al., 2007).

**Impact-R:** Impact-R (DiaMed) determines platelet aggregation under shear and, like PFA, is representative of thrombosis formation in small calibre arteries (Koltai et al., 2017). However, some sample handling and pipetting has to be performed during the use of the assay, which may limit its role as a point of care device. It measures platelet aggregation by subjecting whole blood (incubated with ADP for P2Y12 inhibitor response or AA for aspirin response) to shear stress in a rotating polystyrene cone which rapidly becomes coated in fibrinogen and vWF, acting as the matrix for platelet adhesion. The well is then stained and image analysis is undertaken by the Impact-R system which calculates the percentage of aggregated platelets as the measure of platelet reactivity. In a study by Spectre et al (Spectre et al., 2011) in patients with ACS undergoing PCI, Impact-R was found to correlate well with LTA AA induced platelet aggregation for aspirin response and clinical outcome for six months. However, in a larger study, assessing P2Y12 inhibitor response, IMPACT-R was not associated with clinical outcome in a cohort of patients with stable coronary artery disease undergoing PCI, despite other platelet function tests such as LTA using ADP as an agonist, VerifyNow and

Plateletworks demonstrating a clear association with adverse outcome in the same cohort (Breet et al., 2010).

### 1.3.8: Correlation between platelet function tests

As detailed above, the overall correlation and agreement amongst individual platelet function tests are generally poor. In a study of five platelet function tests (LTA, VASP, VerifyNow, Multiplate and TEG) in both healthy volunteers and patients taking aspirin and clopidogrel regularly, the agreement between tests was only moderate at best and whilst four assays (LTA, VASP, Multiplate and VerifyNow) were found to have good reliability in measuring clopidogrel, only Multiplate was determined to have moderate reliability for aspirin response (Karon et al., 2014). Similarly, Gremmel et al (Gremmel et al., 2015) compared LTA, VerifyNow, Multiplate and flow cytometry in a study of 316 PCI patients. Measures of P2Y12 inhibitor response using ADP based assays correlated significantly with platelet P-selectin expression and activated GPIIb/IIIa with good inter-assay correlation. However, AA induced platelet activation correlated poorly with P-selectin expression and only LTA correlated with activated GPIIb/IIIa.

It is important to note that individual platelet function tests may be confounded by particular patient or biochemical factors that affect one assay but not another. Examples include the sensitivity to haematocrit with VerifyNow (Kim et al., 2014, Kim et al., 2017b) and platelet count with Multiplate (Choi and Kim, 2018). In addition, race, gender and diet my also significantly affect platelet function as identified by a 64 subject healthy volunteer study by Miller et al (Miller et al., 2014), with assays involving whole blood being affected more than those utilising platelet rich plasma, in keeping with the influence of other cellular and humoral factors on platelet reactivity.

### 1.3.9: Reproducibility over time

Despite previous data that suggest that some assays, for example LTA, are reproducible over time, Miller et al's data demonstrate that platelet reactivity in normal healthy volunteers exhibits significant intra-individual variation over time (Miller et al., 2014). Similarly, in aspirin treated patients with stable coronary artery disease, Muir et al (Muir et al., 2009) demonstrated poor reproducibility over a 21 day period with LTA, PFA-100 and serum/urine TXB2.

## 1.4: Potential modifiers of anti-platelet response

As identified in the previous section, several clinical and biological factors may impact the response to anti-platelet therapy. These include a patient's age, gender, medical history (e.g. diabetes, previous cardiovascular disease) and drug adherence. In addition, given the known metabolic fates of the anti-platelet agents, particularly P2Y12 inhibitors, genetic factors and drug interactions may significantly affect the response to anti-platelets.

### 1.4.1: Compliance

Poor compliance and early discontinuation of medications following an ACS is relatively common and has a significant effect on cardiovascular outcomes. In a recent meta-analysis of 10 studies and 106,002 patients with stable coronary artery disease, good adherence to prognostic medications reduced the risk of all-cause mortality (RR 0.56; 95% CI 0.45-0.69), cardiovascular mortality (RR 0.66; 95% CI 0.51-0.87) and cardiovascular hospitalisation and myocardial infarction (RR 0.61; 95% CI 0.45-0.82) (Du et al., 2017). Similarly, in a high risk cohort of diabetic patients following a high limb amputation, poor adherence to drugs used for secondary prevention was common (57% patients, defined as drug intake </= 80%) and associated with a tenfold higher risk of adverse cardiovascular events (Shalaeva et al., 2017). In the prospective TRANSLATE-ACS study, self-reported adherence to anti-platelets was relatively low, with around 30% of the 7425 patients reporting moderate to low adherence. In the identified non-adherent patients, a non-significant association was observed with adverse outcome (HR 1.35; 95% 0.98-1.87) which is likely to be an under-representation of the true effect given that this was a study with self-reported compliance (Mathews et al., 2015). Importantly, this study also demonstrates that poor adherence occurs early post-MI and it is likely that adherence continues to decrease in the longer term. In addition, in a 10 year follow up study in the Netherlands (Yasmina et al., 2017), distinct groups of patients can be defined who were either fully persistent, restarters or largely non-persistent. The proportion of patients on aspirin or clopidogrel who maintained full persistence reduced substantially over the ten-year period, with the numbers either fully non-persistent or intermittent users (restarts) increasing significantly. Interestingly, the presence of clinical risk factors such as diabetes, hypertension and hypercholesterolaemia significantly decreased the risk of anti-platelet drug non-compliance. Cessation of DAPT for reasons other than non-compliance is also relatively common, with a higher risk in women than in men. Both medically advised cessation (for example for bleeding) and non-compliance is more common

in women than men with an increased risk of adverse cardiovascular outcomes (Yu et al., 2016).

One other important component of anti-platelet efficacy is physician prescribing. Several studies have highlighted that adherence to guidelines in treatment of ACS can be poor, with consequent failure to prescribe appropriate medications (for example appropriate DAPT) and worse clinical outcomes on a population level. A recent study, utilising the Danish national registries and including 28449 patients with a first presentation of MI, identified that only between 68-73% of patients were prescribed DAPT which rose to 88-91% when only patients treated with PCI were included (Green et al., 2016). Furthermore, in this study, ticagrelor but not prasugrel was associated with an increased risk of treatment breaks compared to clopidogrel which may be explained by the known adverse effects of dyspnoea, and in keeping with data from the PLATO study (Wallentin et al., 2009). Whilst ticagrelor's adverse effect profile may increase the risk of poor adherence, a modelling simulation of platelet reactivity using drug dosing histories of 5014 patients receiving cardiovascular medicines, demonstrated that missing one of the two daily ticagrelor doses would still maintain a higher platelet inhibition than clopidogrel (Vrijens et al., 2014). Similarly, analysis of large US based healthcare registries suggests that adherence to prasugrel is reasonably high, although duration of therapy is shorter than advised in many patients. Risk factors for early cessation of prasugrel include heart failure and previous ischaemic heart disease, whereas previous use of cardiovascular drugs, such as statins, are protective (Nordstrom et al., 2013).

However, whilst non-compliance is a likely cause for some patients with HTPR, even with verified compliance, response to anti-platelets remains highly variable. However, adjusting the design of long term clinical outcome studies for the risk of poor compliance is challenging; whilst many techniques exist to assess compliance in studies, quantative and semi-quantative techniques are difficult and costly to implement. Whilst qualitative measures, such as questionnaires, are validated they are essentially self-reported tools that may over-estimate the degree of compliance (Navaratnam et al., 2017).

### 1.4.2: Genetic Factors

**Clopidogrel:** Clopidogrel, as previously mentioned, is a prodrug which requires a two-step activation process to its active metabolite, R-130694, largely dependent on hepatic CYP 450 enzymes. Importantly most CYP isoenzymes display a large number of genetic single nucleotide polymorphisms (SNPs), some of which have been associated with both

pharmacodynamics non-response to clopidogrel as well as an increased cardiovascular risk in patients taking clopidogrel for secondary prevention.

In particular, clopidogrel's major activating enzyme, CYP2C19, is highly polymorphic with up to 25 variant alleles / SNPs (Suh et al., 2006). Most important are the loss-of-function (LOF) alleles (*2, *3, *4, *5) and gain-of-function (GOF) allele (*17) in *CYP2C19*. In a study of 162 healthy volunteers, Mega et al demonstrated a relative reduction of 25% in absolute change in maximal platelet aggregation (MPA) following clopidogrel dosing in subjects with at least one LOF allele, which is consistent with a number of other studies investigating the relationship between *CYP2C19* SNPs and platelet aggregation (Hulot et al., 2006, Kim et al., 2008, Mega et al., 2009). In the context of ACS, Mega demonstrated a strong association between carriage of the LOF allele and cardiovascular outcome. In a cohort of 1477 subjects from the TRITON-TIMI 38 study treated with clopidogrel, subjects with at least one LOF *CYP2C19* allele had a 53% relative increase in the composite outcome of death from cardiovascular causes, non-fatal MI and non-fatal stroke (HR 1.53; 95% CI 1.07-2.19, P= 0.01) (Mega et al., 2009). This finding was replicated by Simon et al in their cohort of 2208 prospectively recruited patients with an acute MI who were taking clopidogrel, with carriers of any two LOF alleles having a higher rate of adverse cardiovascular events (HR 1.98; 95% CI 1.10-3.58) (Simon et al., 2009). Furthermore, a meta-analysis of nine studies, involving 9685 patients, demonstrated a 57% increase in risk of cardiovascular death, MI or stroke in patients with one or two LOF *CYP2C19* alleles (HR 1.57; 95% CI 1.13-2.16, P=0.006) with the greatest effect seen in those carrying two LOF alleles (HR 1.76; 95%CI 1.24-2.50, P=0.002) (Mega et al., 2010b). Moreover, a genome wide association study (GWAS) by Shuldiner et al clearly demonstrated that *CYP2C19*2* was the primary SNP associated with the pharmacodynamic response to clopidogrel in a population of health Amish (Shuldiner et al., 2009).

Despite the clear evidence that *CYP2C19*2* is the primary SNP responsible for clopidogrel non-response, the low rate of variability explained by *CYP2C19*2* allele in Shuldiner's GWAS could be a consequence of other genetic variants or clinical factors. Certainly, it is conceivable that SNPs in other CYP450 enzymes involved in clopidogrel activation could have a role in modulating response given the known SNPs in the CYP3A4, 3A5 or 2B6 enzymes. However, to date, the evidence has been conflicting. Most studies do not demonstrate a clear association between SNPs in CYP3A4, 3A5 or 2B6 despite their postulated role in clopidogrel activation (Mega et al., 2009, Simon et al., 2009). However, a study in Korean patients by Suh et al (Suh et al., 2006) showed a significant association between the presence

of the *CYP3A5*3* polymorphism and both pharmacodynamic and clinical response to clopidogrel. However, this finding is not replicated by larger clinical studies, such as Simon et al, or other pharmacodynamic studies (Simon et al., 2009, Lee et al., 2009). It should be noted that CYP3A4 is the primary isoenzyme in Caucasians, which may explain why no association is seen in studies conducted in primarily Caucasian populations. Furthermore, whilst the *CYP3A5*3* polymorphism does reduce CYP3A5 protein expression, it does not abolish it entirely and therefore any effect on pharmacodynamic and clinical outcomes is likely to be small (Taubert et al., 2006, Frere et al., 2008).

Clopidogrel is also a substrate for the drug efflux transporter, P-glycoprotein (P-gp) and consequently alterations in the activity of P-gp may alter the intestinal absorption of clopidogrel. Taubert et al (Taubert et al., 2006) demonstrated a marked increase in clopidogrel accumulation in the presence of P-gp inhibitors. Furthermore, SNPs in P-gp's gene, *ABCB1*, may also alter clopidogrel response. Several groups have investigated the relationship between the C3435T SNP and clopidogrel response with some studies demonstrating a correlation whilst others do not (Simon et al., 2009, Spiewak et al., 2009). The underlying cause of this finding remains unclear, although the C3435T SNP is part of a three SNP haplotype which probably more accurately represents the effect of genotype on outcome (Leschziner et al., 2007). In addition, it is likely that patients may also be receiving other drugs that are P-gp inhibitors which could, conceivably, alter clopidogrel's pharmacokinetics irrespective of genotype with consequent effects on clinical or pharmacodynamic outcome. Interestingly, the effect of the *ABCB1* C3435T polymorphism may be additive in the presence of the *CYP2C19*2* allele as identified in the pharmacogenetic sub-study of the TRITON-TIMI 38 trial (Mega et al., 2010a).

Clopidogrel's pharmacodynamic target, P2Y12, is also polymorphic with a number of SNPs described. Early studies in clopidogrel pharmacogenetics focussed largely on these *P2Y12* SNPs. The majority of pharmacodynamic studies investigating the three major *P2Y12* SNPs (C34T, T744C, H1/H2) did not detect an association between clopidogrel response and genotype (Angiolillo et al., 2005, Cuisset et al., 2007, Motovska et al., 2009, Bonello et al., 2010b), which is also consistent with the data from large clinical outcome studies. For example, Simon et al failed to demonstrate an association between either the C32T or H1/H2 polymorphism and a composite outcome of cardiovascular death, non-fatal MI and non-fatal stroke (Simon et al., 2009). However, a smaller study by Ziegler et al in a cohort of patients with peripheral arterial disease showed a strong association between those carrying the 34T

allele and neurological events (HR 3.96; 95%CI 1.02-17.48, P=0.048), although no association was detected between genotype and all-cause mortality (Ziegler et al., 2005).

More recently, attention has been placed on the Paraoxonase 1 (PON1) enzyme gene and its Q192R polymorphism. Bouman et al (Bouman et al., 2011) demonstrated a significant effect of the *PON1* QQ192 genotype on both clopidogrel's bioactivation and clinical outcomes. In a case-control study of patients with stent thrombosis, stent thrombosis occurred more frequently in patients with the QQ192 genotype compared to those with the QR or RR192 genotype (OR 3.6; 95%CI 1.6-7.9. P=0.003) with no significant effect observed from *CYP2C19*, *CYP2C9*, *CYP3A4* and *CYP3A5* polymorphisms as previously observed in other studies. Similarly, a large replication study of ACS patients (N=1,982) with a 12 month follow up period confirmed the findings from the initial case-control study and identified a 10 fold higher risk of stent thrombosis in patients with the RR 192 genotype compared to QR and QQ192 genotypes (HR 10.2; 95%CI 4.39-71.43). These outcome data are also supported by quantative metabolomics profile of clopidogrel metabolism, patient level clopidogrel pharmacokinetic data and platelet function data all demonstrating a strong association with *PON1* Q192R genotype. However, despite these data from the Bouman studies, the association with *PON1* Q192R has not been consistently replicated in other studies. In a study by Trenk et al (Trenk et al., 2011), 760 patients receiving clopidogrel post PCI demonstrated no significant association between *PON1* Q192R genotype and platelet reactivity or clinical outcome. Similarly, in a study of 1524 patients undergoing PCI, Sibbing et al (Sibbing et al., 2011) did not detect any significant association between the *PON1* Q192R polymorphism and clinical outcome whereas a significant association was observed between *CYP2C19*2*, a finding in keeping with a smaller study by Hulot et al (Hulot et al., 2011). In addition, further studies have not identified any significant effects from the *PON1* Q192R polymorphism and clopidogrel pharmacokinetics, platelet reactivity or clinical outcome (Frelinger et al., 2013, Palmerini et al., 2014).

The mechanism underlying these divergent data is unclear, although it is important to note the differences in sample sizes and study designs between these studies. Nonetheless, it is clear that the original association in Bouman's paper has not been replicated in similar sized or larger cohorts. Furthermore, no significant association with *PON1* genotype was detected in Shuldiner et al's GWAS. However, PON1 activity and its associated polymorphisms have previously been associated with cardiovascular risk and predisposition to diabetes. It is possible, therefore, that the effect of the *PON1* Q192R polymorphism on clinical outcomes in Bouman's paper may, at least in part, be due to a higher vascular risk rather than being

directly related to clopidogrel. Finally, a meta-analysis of 13 studies by Mega et al (Mega et al., 2016), no association between the *PON1* Q192R polymorphism and clinical outcomes was detected.

**Prasugrel**: Prasugrel, like clopidogrel, is a prodrug that requires metabolism to an active metabolite via a two stage process, although only one step is catalysed by CYP450 enzymes for prasugrel activation. In addition, prasugrel's hepatic metabolism is not conducted with one primary CYP450 enzyme as is the case for clopidogrel, instead prasugrel can be metabolised by a range of CYP450 enzymes (3A4, 3A5, 2B6, 2C19, 2C9), although the CYP3A enzymes are identified as the primary group of metabolising enzymes for prasugrel (Kelly et al., 2012).

In the pharmacogenetic sub-study of the TRITON-TIMI 38 study (Mega et al., 2010a), a clear association between adverse clinical outcomes was observed in clopidogrel treated patients carrying the TT genotype of the *ABCB1* C3435T polymorphism or the *CYP2C19*2* allele. As expected, no clear association was observed between either the *ABCB1* C3435T polymorphism or carriage of the *CYP2C19*2* allele in the prasugrel treated patients, a finding in keeping with other studies (Brandt et al., 2007). However, in a study of 213 patients with acute coronary syndromes administered prasugrel, Cuisset et al (Cuisset et al., 2012) demonstrated a significant increase in platelet reactivity in prasugrel treated patients carrying the *CYP2C19*2* allele which was mirrored by a higher HTPR rate in those carriers. Importantly, carriage of the GOF **17* allele significantly increased platelet inhibition by prasugrel with a consequent increase in bleeding events.

Despite the result of Cuisset et al's study, several other studies do not report any association between the *CYP2C19*2* allele and prasugrel response. In a comprehensive PK-PD study of 90 healthy volunteers (Kelly et al., 2012), *CYP2C19* genotype did not affect the pharmacokinetic profile of prasugrel's active metabolite or the degree of platelet inhibition. However, when volunteers were administered clopidogrel, a significant reduction in the exposure to clopidogrel's active metabolite was detected, with lower levels of platelet inhibition, in carriers of *CYP2C19* LOF alleles. In addition, the PRASFIT-ACS genetic sub-study (Ogawa et al., 2015), which included a total of 773 patients with ACS being treated with prasugrel (N=390) or clopidogrel (N=383), did not detect any significant association between clinical outcome or platelet inhibition and *CYP2C19* genotype in prasugrel treated patients.

Given that prasugrel may be primarily metabolised by CYP3A enzymes, it is conceivable that genetic polymorphisms in those enzymes could affect prasugrel clinical and

pharmacodynamic response. Importantly, prasugrel pharmacokinetics and pharmacodynamics have been demonstrated to be affected by drugs or foods that inhibit CYP3A4 function but, to date, very limited data are available on *CYP3A4* or *3A5* polymorphisms and their relationship to prasugrel response.

Other SNPs have also been investigated in relation to prasugrel response. Given the recent interest in the *PON1* Q192R polymorphism and clopidogrel response, Mega et al (Mega et al., 2016) genotyped 275 healthy subjects and 2922 patients treated with prasugrel in the TRITON-TIMI 38 trial for the *PON1* Q192R polymorphism. No significant association between the *PON1* Q192R polymorphism and clinical, pharmacodynamic or pharmacokinetic outcomes in patients treated with prasugrel was demonstrated, in keeping with the existing data with clopidogrel.

PEAR1, a platelet transmembrane receptor, may have a role as platelet-platelet contact receptor and, consequently, be involved in platelet reactivity. Recently, a number of *PEAR1* SNPs have been identified that may increase platelet reactivity (Xiang et al., 2013) and therefore may reduce anti-platelet response. In healthy volunteers administered a loading dose of prasugrel followed by maintenance dosing for 10 days, *PEAR1* SNPs rs12407843, rs77235035, rs3737224, rs41273215, rs822441 and rs822442 were associated with significantly lower levels of platelet inhibition. However, no data are available in patients or on clinical outcomes and therefore it is unclear whether these SNPs have clinically meaningful effects in patients.

**Ticagrelor:** Unlike clopidogrel and prasugrel, ticagrelor does not require biotransformation into an active metabolite. However, as previously discussed, ticagrelor is metabolised by CY3A4 and 3A5 enzymes into two metabolites, one of which (AR-C124910XX) is active although with less potent anti-platelet effects compared to the parent drug. Conceivably, therefore, polymorphisms in either the *CYP3A4* or *3A5* gene could impact clinical and pharmacodynamic responses to ticagrelor. In addition, several studies have demonstrated that CYP3A4 inhibitors and inducers have significant effects on both ticagrelor pharmacokinetics and pharmacodynamics (Holmberg et al., 2013), which has resulted in the ticagrelor label contra-indicating the use of strong CYP3A4 inhibitors whilst on ticagrelor.

Ticagrelor pharmacokinetics may be affected by variants in the *CYP3A4* and *SLCOB1* genes. In a genetic sub-study of the PLATO trial (Varenhorst et al., 2015), 1,812 ticagrelor treated patients with pharmacokinetic data were entered into a GWAS with replication from a further 1,941 ticagrelor treated patients with PK data from the PLATO study. The GWAS

clearly demonstrated potential loci in the *CYP3A4* (rs62471956, rs56324128), *SLCO1B1* (rs4149056) and *UGT2B7* (rs61361928) genes that alter exposure to AR-C124910XX or ticagrelor. Whilst the potential biological mechanism for *CYP3A4*'s effect on ticagrelor metabolism is well known, the *SLCO1B1* and *UGT2B7* variants could also potentially affect ticagrelor pharmacokinetics. The identified variant in *SLCO1B1* (rs4149056), for example, codes for the organic anion transporter polypeptide (OATP1B1) which is known to increase statin concentrations and could conceivably have a role in ticagrelor metabolism. However, the detected association between ticagrelor pharmacokinetics and this SNP may not be a result of a direct effect on ticagrelor metabolism but could represent an interaction between increased statin concentrations and ticagrelor given that CYP3A4 is responsible for the metabolism of both drugs. The identified *UGT2B7* polymorphism affected AR-C124910XX concentrations but not ticagrelor concentrations, suggesting that the impact of the polymorphism is down stream of the active metabolite. However, it should be noted that effects of the identified SNPs on ticagrelor metabolism and pharmacokinetics were relatively modest and no association between clinical outcome and the SNPs could be detected.

In a study of fourteen healthy Chinese subjects, Liu et al (Liu et al., 2017) demonstrated a significant increase in AR-C124910XX exposure in *CYP3A4*1G* carriers and homozygotes, with a significantly longer half-life of the active metabolite. However, despite the observed increase in active metabolite exposure and half-life, no effects were observed in ticagrelor pharmacokinetics or overall platelet function as measured by LTA. In addition, because of the known linkage between the *CYP3A4*1G* and *CYP3A5*3* polymorphisms, subjects were also genotyped for the *CYP3A5*3* variant with no observed association between carriage of the variant allele and ticagrelor pharmacokinetics or platelet inhibition. Similarly, another study of eighteen healthy subjects did not detect any association between the *CYP3A4*1G*, *CYP3A5*3* or *SLCO1B1*5* polymorphisms and ticagrelor pharmacokinetics and pharmacodynamics (Li et al., 2017a).

Given the potential, beneficial, off-target effects of ticagrelor on adenosine metabolism, Nardin et al (Nardin et al., 2018) investigated the effect of a SNP (rs5751876) in the adenosine receptor 2a (ADORA2a) gene in ticagrelor treated patients (N=244) following an acute coronary syndrome. Carriers of the C allele were demonstrated to have a higher risk of HTPR as identified by the Multiplate ADP assay. Whilst the *ADORA2a* SNP was not identified in the PLATO GWAS, it is important to note that PLATO genetic sub-study only included ticagrelor pharmacokinetic data and did not include platelet function.

Additional studies have been conducted investigating variants in platelet receptors and ticagrelor response. In a study of 196 healthy subjects, Li et al (Li et al., 2014) investigated the effect of variants in the GPIIb/IIIa complex and ex-vivo ticagrelor response, genotyping for the *ITGA2B* rs5911 and *ITGB3* rs4642 and rs4634 variants. In this study, platelet inhibition following ex-vivo addition of ticagrelor to platelet rich plasma prior to LTA was significantly reduced by the *ITGA2B* rs5911 variant with no significant effect from the *ITGB3* polymorphisms. Given that GPIIb/IIIa complex is of critical importance in platelet function, it is conceivable that variants in its genes may alter response to anti-platelets as has been previously observed for aspirin. Interestingly, no effect on platelet inhibition with LTA following ex-vivo addition of ticagrelor was seen from common *P2Y12* receptor SNPs in the same cohort of healthy subjects (Li et al., 2015a) which is in keeping with data from clopidogrel pharmacogenetic studies.

**Aspirin**: Like other anti-platelet drugs, the response to aspirin may be heritable. Faraday et al (Faraday et al., 2007) observed clear heritability of aspirin response in a study of 500, ethnically diverse, US families, suggesting that genetic factors may be important determinants of aspirin related HTPR. Aspirin's pharmacodynamic target, *COX-1*, displays several polymorphisms with reasonably common minor allelic frequencies (MAF) that could be related to variability in aspirin response. Several studies have demonstrated that the *COX-1* C50T polymorphism is associated with reduced pharmacodynamic aspirin response (Lepantalo et al., 2006, Clappers et al., 2008) but data on clinical outcomes are lacking. Equally, there are conflicting data demonstrating that the C50T polymorphism has no effect on pharmacodynamic response to aspirin (Li et al., 2013b, Yi et al., 2013). Similarly, several other *COX-1* polymorphisms have been investigated for association with aspirin response with mixed results. However, these data are confounded by the different patient populations studied and different assays used to assess for aspirin resistance which makes comparison across different studies difficult. In addition, it is likely that *COX-1* haplotypes are more sensitive and specific to aspirin response given the high heritability observed in Faraday et al's study, a finding confirmed by a study Maree et al (Maree et al., 2005) that observed a strong association between *COX-1* haplotypes involving the A -842G, C22T, G128A, C644A and C714A SNPs.

Similarly, conflicting data have been observed for other potential modifiers of aspirin's pharmacodynamic response, such as thromboxane synthesis and the thromboxane A2 receptor. In a study of 192 patients with stable coronary artery disease, Lordkipanidze and colleagues (Lordkipanidze et al., 2011) could not detect any association between the

*CYP5A1*9* (thromboxane synthase) polymorphism and AA induced platelet aggregation or major adverse clinical events. However, the minor allelic frequency in this population was very low with only one heterozygote in the cohort. However, two studies assessing SNPs in the TXA2 receptors (*TBXA2R*) demonstrated an association between genotype and platelet reactivity in response to aspirin. In a cohort of 420 post CABG patients, Wang et al (Wang et al., 2013) demonstrated a strong association between carriage of the TT genotype of the T924C SNP and HTPR whilst being treated with aspirin 100mg. Similarly, Postula (Postula et al., 2011) and colleagues observed a significant association between a *TBXA2R* polymorphism and platelet reactivity in a cohort of 295 diabetic patients. Interestingly, no clear association was detected for the T924C SNP in this study, whilst a significant association was observed for the C795T polymorphism instead. It is unclear whether the two different SNPs may be part of a larger haplotype which may explain this discordance.

Importantly, as aspirin inhibits COX-1, platelet generated arachidonic acid is metabolised predominantly via the lipoxygenase (LOX) pathway in aspirin treated patients (Sharma et al., 2013) to leukotrienes, which may have a pro-inflammatory effects. Several studies have investigated whether polymorphisms in LOX or leukotriene receptors are associated with aspirin response. In a 610 patients with stroke and matched controls, Sharma et al (Sharma et al., 2013) demonstrated a clear association between the A allele carriers of the 5-lipoxygenase activating gene (*ALOX5AP*) SG13S114T/A polymorphism and aspirin resistance as well as poor clinical outcome. This SNP, although it is found in an intronic region of the *ALOX5AP* gene, appears to modulate transcription of LOX with lower levels of mRNA observed in T allele carriers. It is therefore conceivable that poor outcome and aspirin resistance associated with carriage of the A allele is a consequence of increased LOX expression, leukotriene production and inflammation. However, a further comprehensive study of SNPs in the leukotriene pathway in 287 patients with type 2 diabetes failed to demonstrate any association between polymorphisms in *ALOX5*, *ALOX5AP*, *LTA4 hydrolase* and *LTC4 hydrolase* and aspirin response (as defined by the PFA-100 and VerifyNow platelet function tests or leukotriene B4/E4) (Rosiak et al., 2013).

Very few studies have been conducted in relation to genes that may impact aspirin's pharmacokinetics. In a study of 287 diabetic patients on aspirin, Postula and colleagues (Postula et al., 2013) investigated 17 SNPs in six genes important in aspirin's metabolic pathway (*ACSM2*, *ACSM3*, *ACSM5*, *UGT1A6*, *CYP2C9* and *CES2*) for their effect on platelet reactivity, thromboxane B2 level (serum and urine) and salicylic acid. No association was detected between any of the SNPs and platelet reactivity or aspirin metabolites. However, a

study of 20 healthy volunteers administered a single 650mg dose of aspirin, demonstrated a significant effect of the *UGT1A6*2* polymorphism on aspirin metabolite generation, with *1*1 individuals displaying a slower generation of metabolites than *2*2 individuals (Chen et al., 2007), in keeping with a further study in female healthy volunteers (van Oijen et al., 2009).

Like clopidogrel, particular focus has been placed on the relationship between aspirin response and platelet receptors and other platelet surface proteins. Several studies have been conducted assessing putative associations between aspirin response and *P2Y12* receptor polymorphisms with predominantly negative results (Lev et al., 2007b, Isordia-Salas et al., 2012, Ulehlova et al., 2014). Similarly, no consistent association between *P2Y1* polymorphisms and aspirin response have been demonstrated with some studies demonstrating positive associations (Li et al., 2007) and others not (Lev et al., 2007b, Lordkipanidze et al., 2011).

However, the *GPIIIa PIA1/A2* polymorphism appears to demonstrate some degree of association with aspirin response. Several studies, including those with clinical endpoints, demonstrate an association between *GPIIIa* genotype and aspirin response, although the direction of association can differ between different studies (McCaslin et al., 2008, Wang and Tan, 2014). These data are in keeping with a large meta-analysis of 31 studies investigating 50 polymorphisms in 11 genes that showed that aspirin resistance was only associated with the *PIA1/A2* polymorphism (Goodman et al., 2008). However, the association was only detected in healthy subjects and when those data were combined with data from patients, no clear association was observed. Polymorphisms in the *COX-1*, *GPIa*, *P2Y1* and *P2Y12* did not appear to be associated with aspirin response, again, in keeping with the data above. Interestingly, a further meta-analysis (Floyd and Ferro, 2014), looking specifically at the *GPIIIa PIA1/A2* polymorphism, which included a total of 16 studies and 1650 PIA1 homozygotes and 688 PIA2 carriers, demonstrated an association between the PIA2 variant and aspirin sensitivity but only when platelet reactivity was assessed by the PFA-100 device. These two meta-analyses suggest that there is little evidence to suggest a significant interaction between the *GPIIIa PIA1/A2* polymorphism and aspirin related platelet reactivity, and that any interaction observed is dependent on the method for assessing platelet function which, in this case, is not a COX-1 specific assay.

An additional recent meta-analysis (Weng et al., 2013), however, demonstrated a clear association between variants in the *COX-2* and *GPIa* genes that are associated with aspirin

related HTPR. In a meta-analysis of 6 studies, Weng et al demonstrated a nearly two fold higher risk of aspirin resistance in C allele carriers of the *COX-2* G765C polymorphism (OR 1.86; 95%CI 1.44-2.41, P<0.0005) and an over twofold increase in risk for carriers of the T allele in the *GPIa* C807T allele (OR 2.37; 95%CI 1.44-3.89, P<0.0005). Whilst *COX-2* and *GPIa* are not primary pharmacodynamic targets for aspirin, aspirin-insensitive thromboxane synthesis via *COX-2* expression in vascular cells (Pamukcu, 2007) is a recognised cause of aspirin resistance. Similarly, *GPIa* interacts with collagen, enhancing platelet reactivity and aggregation and it is therefore conceivable that polymorphisms in *GPIa* could increase platelet reactivity despite COX-1 inhibition by aspirin (Weng et al., 2013).

Finally, the GeneSTAR study (Mathias et al., 2010) aimed to identify genetic determinants of aspirin response by performing a genome wide association study in 2077 healthy subjects following 14 days of aspirin treatment. At 14 days, all subjects underwent comprehensive platelet function testing including LTA to a variety of agonists, urine 11dhTXB2 and PFA-100. Following the GWAS, a number of SNPs met genome wide significance testing, however, as yet, it is unclear how these SNPs relate to aspirin responsiveness. In addition, some SNPs were only identified in association with a particular platelet function test which, again, emphasises the difficulties in using platelet function testing to assess anti-platelet response. Furthermore, the GWAS did not identify any genes that had previously been associated with aspirin response in meta-analyses (such as *COX-2*, *GPIa*, *GPIIIa*). Whilst it is recognised that there is significant disagreements between individual studies investigating these previously identified genes, it should be noted that this GWAS was performed in subjects who were otherwise healthy and therefore the additional interplay between aspirin's pharmacodynamics and other COX-1 independent factors could not be explored. Given that the phenomenon of aspirin resistance may not be directly related to aspirin's pharmacodynamic effect, and more likely represents the complex interplay between platelets and vascular disease, the results of this GWAS may not be directly applicable to aspirin non-response in patients with cardiovascular disease.

### 1.4.3: Inflammation

**Inflammation and cardiovascular disease**: Atherosclerosis, and the process of its formation, is likely to be, at least partly, driven by inflammation. The effect of inflammation on atherosclerosis is likely to represent several different processes including low-density lipoprotein (LDL) cholesterol oxidation, endothelial cell dysfunction and infiltration of inflammatory cells into plaques (Kragholm et al., 2015). Inflammatory markers and pro-

inflammatory cytokines are elevated in the context of atherosclerosis with higher levels observed in acute coronary syndromes compared to stable disease (Eren et al., 2015). In a study of 81 patients (41 with ACS and 40 with stable angina), Tang et al compared the levels of high-sensitivity CRP (hsCRP) and interleukin (IL)-6, a pro inflammatory cytokine responsible for macrophage stimulation and enhancing expression of IL-1β, in patients with stable and unstable cardiac disease as well as investigating the relationship between IL-6 and the severity of cardiovascular disease on coronary angiography. IL-6 levels were significantly higher in patients who had two or three vessel disease compared to single vessel disease on angiography. In addition, hsCRP and IL-6 levels were both significantly higher in patients with ACS in comparison to stable angina patients. Furthermore, both hsCRP and IL-6 were inversely correlated with high density lipoprotein (HDL) cholesterol, in keeping with the known relationship between atherosclerosis and LDL cholesterol and emphasising the importance of LDL and oxidised LDL in the pathogenesis of atherosclerosis. Similarly, Ertem et al (Ertem et al., 2017) demonstrated significant associations between procalcitonin levels, hsCRP and the Syntax score, used as a measure of severity and complexity of atherosclerotic lesions on coronary angiography, in a cohort of 545 ACS patients. In addition, higher procalcitonin levels, as a marker of inflammation, were significantly correlated with mortality in this study.

A further study of 908 patients from the CHAPS cohort (Odeberg et al., 2016) identified pre-existing inflammation as an important risk factor for further ACS whilst specific inflammatory marker and inflammatory cell profiles differentiated MI from unstable angina patients. Patients with MI had elevated hsCRP and fibrinogen compared to unstable angina with significantly higher neutrophil and monocyte counts and lower eosinophil counts compared to patients with unstable angina. These data further support the known increase in inflammatory cell infiltration in acute coronary syndromes which leads to the expression of pro-inflammatory cytokines. Production of pro-inflammatory cytokines also appears to be a local effect within the region of an unstable plaque and acting as a local inflammasome. Martinez et al (Martinez et al., 2015) demonstrated significant trans-coronary gradients of IL-1β levels between the coronary sinus and coronary artery in patients with acute coronary syndromes but not with stable angina patients. This finding highlights the importance of localised inflammation in unstable plaques which does not occur in chronic, stable, disease. IL-6 levels in this study were similar in both venous samples and coronary sinus samples and were correlated with the IL-1β levels, suggesting that the localised inflammation at the unstable plaque induced a more 'global' pro-inflammatory state by increasing expression of

IL-6. Furthermore, allergic type inflammation, mediated by eosinophil and basophil activation and degranulation, may also have an important role in acute coronary syndromes. In a study of 181 patients presenting with STEMI (Niccoli et al., 2015), eosinophilic degranulation and basophil activation were significantly higher than in stable angina patients, with higher eosinophilic cationic protein (ECP) concentrations being significantly associated with major adverse cardiac events.

Inflammation, and its related biomarkers, is associated with outcome following an AMI. Data from the CLARITY-TIMI 28 trial (O'Donoghue et al., 2016), in 1258 patients with STEMI, clearly demonstrate a correlation between inflammatory markers and risk of further cardiovascular events. Following multivariable adjustment, raised hsCRP increased the risk of a further cardiovascular event twofold (OR 1.96; 95%CI 1.17-3.30, P=0.01) and increases in myeloperoxidase (MPO) and pregnancy-associated plasma protein A (PAPP-A, a matrix metalloproteinase expressed in atherosclerotic plaques) increased the risk threefold. Similarly, data from the CREDO trial (Dosh et al., 2009) of 1468 patients undergoing PCI also demonstrated a clear association between raised inflammatory markers (hsCRP and PAPP-A) and adverse cardiovascular outcomes. Interestingly, for both markers, there appeared to be no benefit of clopidogrel over placebo in those patients with the lowest tertile values, whereas for the second and third tertiles, clopidogrel-treated patients had a lower risk of cardiovascular events compared to placebo treatments. Whilst the event rate in the lowest tertile of inflammatory markers was low, the interaction between clopidogrel's effect and the level of inflammatory markers suggest that there may be an important relationship between anti-platelet response and inflammation. Stent thrombosis, an exquisitely platelet sensitive outcome, is also associated with higher levels of IL-6, further strengthening a link between platelets, anti-platelet treatment and inflammation (Hwang et al., 2011b). In addition, in the LIPID study of 6434 patients following discharge with MI or unstable angina and treated with pravastatin or placebo, high levels of CRP (>4.78 mg/L) were associated with an increased risk of cardiovascular events (HR 1.28; 95%CI 1.07-1.54), despite the stable nature of the patient population and the use of a conventional, rather than high sensitivity, assay for the measurement of CRP levels (Tonkin et al., 2015). Finally, in a cohort of ACS patients using DNA microarray (Takashima et al., 2016), a clear gene expression signature of ACS can be developed which predicts 5-year outcomes. In particular, several potentially important inflammatory pathways were identified, predominantly related to T-cell signalling, including the NF-κB signalling pathway, TLR signalling pathway, TNFR2 signalling pathway, CD40L signalling pathway and the oxidative stress-induced gene expression. These data,

together with the other clinical studies, suggest that inflammation is an important predictor of clinical outcome that may inter-relate with anti-platelet therapy.

**Anti-platelet drugs and vascular inflammation**: Studies suggest that anti-platelet drugs may reduce inflammation and inflammatory markers. In a study of 650 patients undergoing PCI (elective or urgent), samples for hsCRP were obtained prior to and following the loading doses of both aspirin and clopidogrel. Clopidogrel reduced hsCRP levels independent of diagnosis (ACS versus stable disease), other treatments, procedures or risk factors (Hajsadeghi et al., 2016b).

Higher doses of clopidogrel may also reduce inflammatory markers to a greater extent than standard doses. In the ARMYDA-150mg study (Patti et al., 2011), a double dose of clopidogrel (150mg) was associated with a greater reduction in hsCRP compared to standard dose (75mg) and was accompanied by a reduction in platelet reactivity and an improvement in endothelial function. The findings from ARMYDA-150mg are consistent with the DOUBLE study where 54 patients were randomised to receive either 75mg or 150mg of clopidogrel. In the high dose group, hsCRP was reduced by nearly 50% and platelet aggregation was also significantly reduced in comparison to the standard dose (Palmerini et al., 2010).

Similarly, more potent anti-platelet dugs may reduce inflammation more effectively than standard doses of clopidogrel. Whilst both clopidogrel and prasugrel reduced hsCRP in a study of 120 patients attending for PCI (routine or urgent), prasugrel reduced hsCRP to a greater extent than clopidogrel which was independent of the pre-dose hsCRP levels (Hajsadeghi et al., 2016a). In addition, a study of 107 STEMI patients demonstrated that ticagrelor lowered hsCRP more effectively than clopidogrel (Wei et al., 2017b), although there appeared to be no significant differences between ischaemic or bleeding events at 30 days. However, Oh et al (Oh et al., 2016) did not detect any significant differences between CRP levels at 6 months between ticagrelor and clopidogrel in a cohort of 50 patients with carotid atherosclerotic disease. In addition, this study also measured inflammation using Positron Emission Tomography (PET) scanning of the carotid atherosclerotic plaques. In keeping with the CRP results, whilst both ticagrelor and clopidogrel significantly reduced the target-to-background ratio (TBR) post treatment, there was no significant differences between the two anti-platelet agents. Similarly, in an experimental model of inflammation using healthy volunteers treated with intravenous *E. Coli* endotoxin (LPS) after 7 days treatment with either ticagrelor and clopidogrel, ticagrelor and clopidogrel both reduced platelet-monocyte aggregates, TNF-α and IL-6 to similar extents. However, ticagrelor

appeared to have additional effects with significant reductions observed in IL-8, G-CSF and increases in the anti-inflammatory cytokine IL-10 (Thomas et al., 2015). Interestingly, platelet reactivity measured prior to LPS administration but post ticagrelor or clopidogrel dosing strongly correlated with subsequent post-LPS inflammatory response, strengthening the putative link between platelet reactivity and inflammation.

However, the association between anti-platelets and reduction in inflammation has not been universally reported. In the DISPERSE-2 study investigating the effectiveness of ticagrelor and clopidogrel in NSTEACS, no significant changes in CRP, MPO, IL-6 and sCD40L level was observed in 984 patients randomised to ticagrelor or clopidogrel after 4 weeks (Husted et al., 2010). Similarly, in a study of 41 patients with stable coronary artery disease randomised to either clopidogrel or placebo, only sCD40L was significantly reduced after 6 weeks of treatment, with no clear effect on endothelial function, oxidative stress or hsCRP observed (Ramadan et al., 2014). Furthermore, in a cross-over study of 17 type 2 diabetes patients, aspirin administration at either 75mg/day, 300mg/day or 3600mg/day did not significantly reduce hsCRP, endothelial function or oxidative stress (Raghavan et al., 2014).

The mechanism by which clopidogrel and other anti-platelet drugs reduce inflammation is unclear with reductions in platelet-leucocyte interactions being cited as a potential mechanism as well as direct reduction of inflammatory markers by platelets also being suggested (Hajsadeghi et al., 2016b). Additional studies have focussed on the endothelial effects of clopidogrel. Cerda and colleagues (Cerda et al., 2017) demonstrated a reduction in the gene expression of IL-8 and MCP1 following clopidogrel treatment of TNF-α induced human umbilical vein endothelial cells with a corresponding reduction in both *ICAM-1* gene and protein expression also noted. Layne et al (Layne et al., 2016) conducted a vaccine challenge study in 60 healthy volunteers following pre-treatment with aspirin and clopidogrel. Whilst neither aspirin nor clopidogrel significantly reduced the rise in inflammatory markers (hs-CRP, IL-6, TNF-α, IL-1β) post vaccination compared to placebo, the rise in P-Selectin post vaccination was absent in aspirin and clopidogrel treated patients as was the expansion of CD14 high CD16+ monocytes. The increase in CD16+ monocytes also occurs during the pathogenesis of atherosclerotic plaques and its attenuation by anti-platelet drugs may explain their putative anti-inflammatory action. In addition, in a cross-over study of 12 healthy volunteers administered either prasugrel or placebo in a for 7 days, prasugrel was demonstrated to abolish the pro-inflammatory effect of platelets on CD4+ T-cells as measured by IFN-γ release and Th1 and Th17 phenotypic markers (Johnston et al., 2015). Furthermore, in a porcine coronary stent restenosis model, ticagrelor was

demonstrated to be better than either prasugrel or clopidogrel in preventing restenosis and inflammatory cell infiltrates (Kim et al., 2017a). Taken together, these data suggest that clopidogrel and other anti-platelet drugs are likely to have a pleiotropic effect on platelets, white blood cells and vascular endothelium which contribute to the reduction in vascular inflammation.

**HTPR and vascular inflammation**: Given the putative beneficial effects of anti-platelets on inflammation, it is conceivable that HTPR and inflammation may also be related. In a study of 352 patients undergoing PCI, higher levels of sCD40L (but not CRP, IL-6 and P-selectin) was associated with clopidogrel related HTPR as assessed by LTA (Ge et al., 2012). However, in another study of 157 patients with symptomatic coronary artery disease, undergoing PCI, (Muller et al., 2010) and treated with aspirin and clopidogrel, aspirin and clopidogrel response as measured by Multiplate was correlated with levels of CRP, IL-6 and RANTES, a platelet derived chemokine which is instrumental in macrophage and monocyte recruitment (Muller et al., 2010). Furthermore, in a replication cohort of 903 patients with stable coronary artery disease, higher levels of CRP were associated with clopidogrel related HPR as assessed by LTA. In a multivariable analysis, higher CRP was associated with the composite outcome of death and non-fatal MI (HR 1.05; 95%CI 1.00-1.09, P=0.01). Further studies in PCI also demonstrate that other inflammatory markers or pro-inflammatory cytokines may be associated with anti-platelet drug response, but with inconsistent results. Despite the association between sCD40L and platelet reactivity observed in Ge et al's study, a further study in 387 patients undergoing PCI (Osmancik et al., 2012) failed to demonstrate an association between sCD40L level and platelet reactivity as measured by VerifyNow, in keeping with the results of a further study in stable coronary artery disease (Kaufmann et al., 2013). However, Osmancik's study did observe a significant association between IL-10 and clopidogrel related HTPR (OR 1.32; 95%CI 1.07-1.72, P<0.05), in keeping with previous data demonstrating associations between IL-10 and myocardial infarction as well as poor outcome following an ACS (Osmancik et al., 2012).

Aspirin non-response is also associated with pro-inflammatory markers. In a study of 194 patients with acute coronary syndromes, aspirin related HTPR (as assessed by Multiplate) was associated with higher levels of hsCRP (Stolarek et al., 2015). P-selectin levels have also been associated with aspirin response, as assessed by LTA, in a study of 148 patients with stable coronary artery disease (Kaufmann et al., 2013) but sCD40L was not associated with aspirin response. A network reconstruction using a systems biology approach of platelet metabolism by Thomas and colleagues (Thomas et al., 2014) identified a unique signature

for aspirin resistance. The signature generated by their model identifies novel pathways primarily driven by the diversion of metabolites into prostaglandin synthesis which may explain the relationship observed between poor aspirin response, oxidative stress and lipid metabolism.

In a comprehensive, proteomic analysis of platelet function, Caruso and colleagues (Caruso et al., 2015) demonstrated clear differences between clopidogrel responders and non-responders in thirty ACS patients. Non-responders to clopidogrel (as identified by LTA) had higher levels of IL-4, IFN-γ and MCP-1 compared to responders, with upregulated CD226 (a platelet adhesion molecule) and down regulation of peroxiredoxin-4 (an anti-oxidant). Whilst this study demonstrates, like others, that clopidogrel related HTPR is associated with a pro-inflammatory milieu, it does not establish a cause and effect relationship between the two. In concordance with most other studies, this study assesses platelet reactivity and inflammatory phenotype only after the administration of the anti-platelet drug and not before. It is therefore not possible to establish whether any change from baseline inflammation or platelet reactivity has occurred following exposure to the anti-platelet agent, and whether this change relates to post-exposure levels of inflammation and platelet function. However, a recent study by Meyer et al (Meyer et al., 2016), in 40 patients with peripheral arterial disease, suggests that both platelet reactivity and inflammation (as measured by RANTES and CRP) are reduced by clopidogrel treatment. However, whilst both platelet reactivity and inflammatory markers decreased in this study, no formal correlation between the two was performed. It is therefore not possible to determine with certainty from these data that there is a causal relationship between platelet reactivity and the degree of inflammation. Furthermore, a study of 51 patients presenting with acute stroke (Sternberg et al., 2016) demonstrates a clear association between inflammatory mediators and anti-platelet response to both aspirin and clopidogrel. In addition, this study also demonstrates that association between platelet reactivity and inflammatory markers is specific to the method being used to assess platelet reactivity. A total of 5 inflammatory markers were assessed in this study (P-selectin, sCD40L, MMP-9, ICAM-1 and IL-6) and platelet function was assessed using TEG, VerifyNow and impedance aggregometry. Clopidogrel administration significantly reduced the levels of P-selectin, sCD40L and MMP-9, which was comparable to reductions seen in response to aspirin. In patients already on aspirin, clopidogrel also significantly reduced the levels of P-selectin, sCD40L and MMP-9 independently of the effect observed with aspirin. VerifyNow and Impedance Aggregometry

correlated well with the anti-inflammatory effect observed with clopidogrel administration but TEG did not. However, TEG and aspirin induced reductions in P-selectin were correlated.

Whilst anti-platelet drug response appears to be at least partially associated with inflammation, with higher levels of inflammation being associated with HPR, it remains unclear which inflammatory marker best correlates with anti-platelet response and in what setting. It appears that some markers, such as sCD40L, are related to anti-platelet response only in specific contexts, such as unstable coronary artery disease. In addition, the method of assessing platelet reactivity appears to be important with some platelet function tests being correlated with inflammatory mediators and others not. Fundamentally, it also remains unclear whether the association between inflammation and HPR reflects a direct effect of inflammation on the pharmacokinetics or pharmacodynamics of the anti-platelet drug or whether it better represents an increased platelet reactivity caused by inflammation that is insufficiently reduced by the anti-platelet drug.

**Overall conclusions**: Inflammation is closely related to the pathogenesis of atherosclerosis and development of unstable plaques that lead to acute coronary syndromes. Prognosis following an ACS is also closely linked to inflammation, with higher levels of inflammatory mediators or markers conferring a poorer long term prognosis. Anti-platelet drugs may modulate the inflammatory response through a variety of putative mechanisms and are likely to represent a pleiotropic response across platelets, white blood cells and vascular endothelium. Importantly, these mechanisms may relate to the variability in response to anti-platelet drugs and may also interact with other cardiovascular risk factors such as diabetes, hyperlipidaemia and lipid peroxidation. It is therefore possible that the inflammatory milieu and its relationship with other cardiovascular risk factors is one of the major determinants of anti-platelet drug response.

### 1.4.4: Diabetes Mellitus

Diabetes is a major risk factor for the development of cardiovascular disease. It is associated with a significant risk of further cardiovascular risk in the context of DAPT and PCI, with a greater than two fold increase in risk of major adverse cardiovascular events following drug-eluting stent (DES) implantation (HR 2.30; 95%CI 1.01-5.27, P=0.048) (Gargiulo et al., 2016). In addition, diabetes may also reduce the effectiveness of anti-platelet agents in reducing the risk of cardiovascular events. In a comparative study of 32 patients with Type 2 diabetes (T2DM) and 32 patients without diabetes, patients with diabetes had a significantly higher degree of platelet reactivity following administration of clopidogrel compared to non-

diabetics, as assessed using the Multiplate analyser (Schuette et al., 2015). Furthermore, the degree of platelet inhibition was inversely correlated with fasting glucose and glycated haemoglobin levels, suggesting that glycaemic control may be an important factor in determining the degree of platelet inhibition observed in patients. Finally, administration of a loading dose of clopidogrel (300mg) did not appear to overcome HTPR to clopidogrel, raising the possibility that the HTPR observed in T2DM is independent of P2Y12 inhibition. These data have been replicated in other, stable, vascular conditions (Nakagawa et al., 2016). In a larger, two part study, Geisler et al investigated the effect of diabetes on platelet reactivity in clopidogrel treated patients (617 patients with T2DM and 1314 non-diabetics) undergoing PCI, using Multiplate to assess platelet reactivity. Diabetes status strongly predicted HTPR to clopidogrel, with a fourfold increase in the risk of having HTPR after adjustment for other co-variates (OR 4.39; 95%CI 1.95-6.83, P<0.001) (Geisler et al., 2010). In addition, Geisler demonstrated that levels of inflammatory markers such as IL-6 and CRP were significantly elevated in diabetics with poor glycaemic control, and the levels of inflammatory markers were inversely correlated with the degree of clopidogrel induced platelet inhibition. These data suggest that the mechanism by which diabetes leads to HTPR to clopidogrel may be mediated via an inflammatory milieu related to diabetes itself. In addition, diabetes may increase plasma esterase activity, leading to a higher transformation of clopidogrel into inactive metabolites and reducing its effect (Geisler et al., 2010). Finally, other, platelet specific mechanisms, may increase the likelihood of HTPR to clopidogrel, which is discussed further below.

Similar to clopidogrel, aspirin response may also be modified by T2DM. In a study of 21 well controlled T2DM patients and 21 non-diabetic controls (Vernstrom et al., 2018), the response to aspirin was lower in diabetes patients compared to the controls. Interestingly, T2DM patients also had higher numbers of immature platelets secondary to higher platelet turnover, which consequently may also reduce the effect of aspirin. In addition, platelet aggregation to AA prior to administering aspirin was also significantly higher in patients with T2DM compared to controls, suggesting that baseline platelet reactivity in diabetics is higher and, therefore, poorer response to aspirin is a consequence of higher platelet reactivity as opposed to a direct pharmacokinetic or pharmacodynamic effect from diabetes. These data are replicated by a larger study of 2113 subjects (175 diabetes, 1938 without diabetes) where diabetics were noted to have higher baseline platelet reactivity compared to subjects without diabetes (Al-Sofiani et al., 2018). Similarly, the post-aspirin treatment platelet reactivity was significantly higher in diabetics compared to non-diabetics but the overall

response to aspirin (i.e. the comparison between the pre-treatment platelet aggregation value and the post-treatment aggregation value) was similar between diabetics and non-diabetics. These data suggest that it is the increased platelet reactivity, rather than a specific aspirin related phenomenon, that drives the higher platelet reactivity seen in aspirin treated diabetic patients. HTPR to aspirin may also be associated with metabolic syndrome, with one study reporting a nearly fivefold risk of aspirin non-response in patients with metabolic syndrome (OR 4.95; 95%CI 1.44-17.02, P=0.011) (Liu et al., 2016), which did not appear to be associated with the levels of hsCRP. Furthermore, unlike clopidogrel, aspirin response in diabetic patients does not appear to be associated with glycaemic control but appears to be closely associated with hyperlipidaemia and smoking (Labuz-Roszak et al., 2014). Taken together, these data suggest that aspirin non-response in diabetes is likely to be determined by a higher baseline platelet reactivity which does not appear to be associated with inflammatory markers or glycaemic control, but may be associated with hyperlipidaemia. Hyperlipidaemia may also be linked, independently, with platelet response and will be discussed later in this chapter.

Newer anti-platelet agents, such as ticagrelor and prasugrel, may also be affected by the presence of diabetes. A large meta-analysis of 22 studies and 35004 patients determined that there was no significant difference between the clinical effectiveness of ticagrelor in diabetic and non-diabetic patients, the recovery of platelet function was faster in diabetic compared to non-diabetic patients (Tan et al., 2017). A patient level data meta-analysis of 8 studies and 445 patients also demonstrates that diabetes adversely affects ticagrelor's pharmacodynamic response, with higher platelet reactivity in diabetic patients compared to non-diabetic patients (Alexopoulos et al., 2014b). It remains unclear whether the mechanisms underlying the interaction between ticagrelor and diabetes are similar to clopidogrel's. However, a recent study has demonstrated that diabetes may adversely affect ticagrelor metabolism, with significant reductions in the transformation of ticagrelor into its active metabolite, AR-C124910XX (Adamski et al., 2018). These data are limited, and further studies investigating the relationship between ticagrelor metabolism, diabetes and platelet reactivity are necessary.

Several studies have investigated which anti-platelet is the most effective in the context of diabetes. Despite evidence that ticagrelor may be less effective in diabetic patients, it remains more effective than placebo for secondary prevention of cardiovascular events in patients with diabetes who are also taking aspirin in the PEGASUS-TIMI 54 study (Bhatt et al., 2016). In a 1324 patient sub-study from the RENAMI registry, ticagrelor was associated

with a lower risk of death compared to prasugrel in diabetic patients (Conrotto et al., 2018). These findings are mirrored by pharmacodynamic data that suggest that early platelet reactivity is similar between prasugrel and ticagrelor in diabetic patients, but ticagrelor exerts a greater anti-platelet effect at 30 days and possibly beyond (Shang et al., 2018), in keeping with the OPTIMUS-4 study (Franchi et al., 2016). However, the OPTIMUS-4 study also demonstrates that the enhanced effect of ticagrelor over prasugrel may be platelet function test specific, and only observed in ADP specific tests but not in non-ADP specific tests. Prasugrel appears to give better and more consistent platelet inhibition than clopidogrel in diabetic patients. The VERDI study randomised 50 diabetic subjects with HTPR to clopidogrel to either receive standard clopidogrel loading doses or a prasugrel loading dose. Patients randomised to prasugrel all achieved optimal platelet aggregation, whereas only 16% in the clopidogrel group did (Cubero Gomez et al., 2015). However, diabetes may impact prasugrel's metabolism and conversion to its active metabolite (R-138727). In a PK-PD study comparing clopidogrel and prasugrel in patients undergoing PCI for stable angina, a specific interaction between diabetes and reduced conversion of prasugrel to R-138727 was demonstrated, which mirrored the PD effect of prasugrel in those patients. No interaction was detected between clopidogrel metabolism, PD effect and diabetes, although clopidogrel was generally less effective than prasugrel in reducing platelet aggregation (Niijima et al., 2018).

Taken together, these data suggest that diabetes does reduce the effectiveness of anti-platelet drugs. However, the mechanism underlying this observation remains unclear. For prasugrel and ticagrelor, there appears to be a significant interaction between diabetes and metabolism of the two drugs. As previously discussed, both these drugs are sensitive to intestinal CYP3A4 activity, as demonstrated by their interactions with grapefruit juice and other CYP3A4 inhibitors or inducers. Diabetes reduces gastric emptying and increases overall gut transfer time (Niijima et al., 2018), and it is therefore conceivable that this is responsible for the reduction in active metabolite production, similar to the observed interaction between morphine and anti-platelet agents.

In addition to this, diabetes increases platelet aggregation and reactivity via a number of different mechanisms. Firstly, it is likely that P2Y12 signalling is increased significantly in diabetes. In a study of 40 diabetes patients and 29 healthy volunteers, Hu et al (Hu et al., 2017) demonstrated a fourfold increase in P2Y12 expression in diabetic patients compared to healthy controls. The increased P2Y12 expression also correlated to overall levels of platelet aggregation to ADP. Interestingly, the increased P2Y12 expression appears to be

mediated via an increased NF-κB activation, suggesting that the observed increased inflammation in diabetes has a direct link with higher platelet reactivity. Furthermore, higher glucose concentrations increase P2Y12 expression, again mediated via increased NF-κB activation, in keeping with the observed findings on patients where poor glycaemic control tends to worsen platelet reactivity and increase the risk of HTPR. Similarly, hyperglycaemia appears to increase the risk of HTPR to aspirin with an increase observed in the generation of reactive oxygen species (ROS) (Kobzar et al., 2017), which in turn increases cytosolic PLA2 with consequent increases in AA generation. As discussed previously, COX-inhibition by aspirin may increase the production of pro-inflammatory leukotrienes which may further increase platelet reactivity via an increase in inflammatory mediators. In this study, administration of a TXA2 inhibitor did not reduce the effect of glucose on aspirin effectiveness, suggesting that excess TXA2 generation from increased AA is not responsible for the observed increased platelet reactivity.

Emerging data also suggest that increased megakaryocyte proliferation and consequent platelet production may be partly responsible for the increased platelet reactivity observed in diabetic patients. In a recent study Kraakman (Lee and Bergmeier, 2017) demonstrated the role of inflammation and neutrophils in increasing levels of the pro-inflammatory calcium-binding protein, S100A8/A9 which, in turn, bind to the receptor for advance glycation end products (RAGE) on Kupffer cells leading to increased IL-6 production and release of thrombopoetin (TPO) from hepatocytes. Increased TPO release increases platelet production, predominantly reticulated platelets, which are associated with higher levels of platelet reactivity.

### 1.4.5: Hyperlipidaemia

In common with diabetes, hyperlipidaemia may be associated with a pro-inflammatory state which, in turn, may reduce the effectiveness of anti-platelet drugs.

High low density lipoprotein cholesterol (LDL-C) and lower levels of high density lipoprotein cholesterol (HDL-C) are associated with higher levels of platelet reactivity (Chan et al., 2015). In aspirin-treated patients, administration of a lipid challenge significantly increases urinary thromboxane production with a consequent increase in platelet reactivity (Yassine et al., 2010). Similarly, in a cohort of T2DM patients, hyperlipidaemia was significantly associated with HTPR to aspirin, independent of glycaemic control (Labuz-Roszak et al., 2014).

High density lipoprotein cholesterol (HDL-C) levels are important modifiers of cardiovascular risk, with higher levels reducing the risk of further cardiovascular events (Annema et al., 2016). However, lower levels of HDL-C are associated with increased levels of inflammatory markers and higher risk of further cardiovascular events. In a study of 6134 ACS patients, hsCRP levels were inversely correlated with HDL-C levels, and patients with low HDL-C had a higher mortality compared with normal or high levels (Gonzalez-Pacheco et al., 2015). Importantly, the functionality of HDL-C, primarily cholesterol efflux and anti-inflammatory activity, is critical and may be reduced in ACS patients, irrespective of the overall level of HDL-C, leading to higher low-density lipoprotein cholesterol (LDL-C) oxidation and inflammation (Annema et al., 2016). Oxidised LDL-C (OxLDL) is strongly pro-inflammatory, interacting with multiple immune cell targets such as toll-like receptors (TLRs), CD36 and lectin-like OxLDL receptor-1 (LOX-1) (Zidar et al., 2016). Similarly, higher levels of LDL-C carry a higher risk of cardiovascular risk and are modified substantially by Proprotein Convertase Subtilisin Kexin 9 (PCSK9), which increases degradation of the LDL-C receptor, reducing LDL-C uptake with consequent increases in LDL-C (Gencer et al., 2016). PCSK-9 levels are also associated with higher hsCRP levels in ACS patients as well as higher LDL-C levels but may not be associated with higher risk of adverse cardiovascular outcomes following an ACS (Gencer et al., 2016). In addition, higher PCSK9 levels are associated with greater atherosclerotic plaque necrosis, in keeping with its pro-inflammatory effects (Cheng et al., 2016). Furthermore, adipokines may also have a significant effect on inflammation in cardiovascular disease with several studies demonstrating a link between higher levels of pro-atherogenic adipokines (resistin, leptin) and higher cardiovascular risk, as well as higher levels of anti-atherogenic adipokines (adiponectin) conferring a lower risk (Li et al., 2016a). In a nested case-control study of the PROVE-IT TIMI 22 cohort, on-statin resistin levels were associated with higher hsCRP levels and the risk of adverse cardiovascular effects (Khera et al., 2015), whilst leptin levels, although associated with hsCRP levels, was not associated with the risk of recurrent cardiac events. Notably, a strong correlation was seen between HbA1c levels and both resistin and leptin levels, in keeping with the known adipokine imbalance in diabetes patients and relationship between glycaemic control and risk of future cardiovascular events. Moreover, OxLDL may increase the expression of resistin, emphasising the relationship between dyslipidaemia, inflammation and adipokine imbalance.

Given the relationship between hyperlipidaemia, inflammation, adipokine imbalance and consequent HTPR, it is conceivable that modification of hyperlipidaemia with statins may

improve platelet reactivity and reduce HTPR. In the STATIPLAT study, 145 patients with stable angina, on clopidogrel, were randomised to receive atorvastatin, rosuvastatin or no statin. Chronic treatment with statins significantly reduced platelet reactivity compared with clopidogrel alone, although no acute benefit (within 12 hours of the loading dose of statin) was noted (Godino et al., 2017). Similarly, Pesaro and colleagues (Pesaro et al., 2012) demonstrated a significant improvement in platelet reactivity in stable angina patients on aspirin administered either simvastatin 20mg, ezetimibe 10mg/simvastatin 20mg or simvastatin 80mg, which was mirrored by significant reductions in both LDL-C and OxLDL levels. Interestingly, whilst platelet reactivity improved on statin treatment, no significant change in contemporaneously measured inflammatory markers was noted, in conflict with the hypothesis that inflammation is important in platelet reactivity linked to hyperlipidaemia. Furthermore, ezetimibe 10mg/simvastatin 20mg, and not simvastatin 80mg was demonstrated to be the most efficacious treatment for reducing platelet reactivity, which, again, is not in keeping with the known effect of high dose statins in acute coronary syndromes. However, the lack of significant change in inflammatory markers and the seemingly poorer response to simvastatin 80mg compared to low dose simvastatin may be explained the stable nature of the patients (lower levels of inflammation) and the platelet function test used in this study (PFA-100) which is not considered to represent a COX-specific effect of aspirin on platelets.

The effect of statins on platelets may be explained via several mechanisms. In a study of 182 patients with cardiovascular disease or established risk factors for cardiovascular disease, treated with aspirin, Tacconelli and colleagues (Tacconelli et al., 2018) demonstrated lower serum TXB2 in patients treated with statins compared to those who were not on statins. In vitro studies demonstrated that atorvastatin significantly increases the acetylation of COX-1 at serine529 by aspirin, thereby increases its effect. In acute coronary syndromes, atorvastatin may also exert anti-inflammatory actions via inhibition of the immediate-early response gene (EGR1), reducing pro-inflammatory CD4+ T-lymphocytes and increase anti-inflammatory CD4+lymphocytes (Severino et al., 2017).

In summary, hyperlipidaemia may significantly reduce the effectiveness of anti-platelet therapy. The mechanisms behind this interaction remain unclear, although it seems likely that inflammation and adipokine balance are underlying causes. Treatment with statins appear to improve platelet inhibition in response to anti-platelet treatments, potentially via direct platelet-specific or anti-inflammatory effects.

### 1.4.6: Smoking

Whilst smoking is a well-known cardiovascular risk factor, it may also have a significant impact on response to anti-platelet drugs. Several studies have demonstrated the so-called 'smokers paradox' with clopidogrel, where current smokers appear to have a significantly better response to clopidogrel than non-smokers. In a study of 71 patients undergoing neuro-interventional procedures administered clopidogrel, smokers had a fivefold increase in clopidogrel hyper-responsiveness (Nakagawa et al., 2016). Similarly, in the PARADOX study, smokers had significantly better platelet inhibition and higher levels of clopidogrel's active metabolite compared to non-smokers (Gurbel et al., 2013). The putative mechanism for this observation is induction of CYP1A2 and 2B6 in response to cigarette smoking which consequently increases metabolism of clopidogrel to its active metabolite thereby increasing clopidogrel's pharmacodynamic effect (Gurbel et al., 2013). Interestingly, data from the 17,263 patient CURRENT-OASIS 7 trial indicates that the benefit of double dose clopidogrel over standard dose clopidogrel in reducing the primary outcome was only observed in smokers and not non-smokers (Bossard et al., 2017). However, some studies suggest the apparent synergy between smoking and better outcomes with clopidogrel is a product of smokers generally being younger with fewer co-morbidities and consequently having lower risk of suffering cardiovascular events (Kodaira et al., 2016). Interestingly, in the large TRILOGY-ACS study, prasugrel was superior to clopidogrel in reducing major adverse cardiovascular events in smokers only, with non-smokers demonstrating no significant difference between the two treatments (Cornel et al., 2014) which is at odds with the previously observed 'smokers paradox'. Similarly, the COPTER study (Patti et al., 2016) demonstrated only a weak effect of cigarette smoking on platelet reactivity to ticagrelor, prasugrel and clopidogrel, with a modest improvement in platelet reactivity on smoking cessation which was reversed on resumption of smoking after 2 weeks.

However, a meta-analysis of nine trials and 74,489 patients (Gagne et al., 2013), demonstrated that the benefit of clopidogrel in reducing ischaemic events was mostly observed only in smokers, with non-smokers demonstrating little benefit from clopidogrel. This is in keeping with another, large meta-analysis of 19 studies and 117,790 patients, which also clearly demonstrated a significant improvement in clinical outcome and platelet reactivity in smokers administered clopidogrel compared to non-smokers (Zhao et al., 2014).

Interestingly, the benefits of ticagrelor and prasugrel also appeared to be larger in smokers than non-smokers in the Gagne et al meta-analysis (Gagne et al., 2013). Given that CYP1A2

has a lesser role in prasugrel metabolism compared to clopidogrel metabolism, and no known role in ticagrelor metabolism, it has been suggested that smoking may induce CYP3A4, an enzyme common to the metabolism of all three drugs. In support, recent data suggest that ticagrelor's metabolism may be affected by smoking, with increased production of AR-C124910XX, ticagrelor's active metabolite, in smokers compared to non-smokers (Adamski et al., 2018). However, the interaction between smoking and CYP3A4 is not clear, with some studies suggesting that smoking may increase induction of CYP3A4 in the presence of an existing inducer (Gagne et al., 2013). However, both the PARADOX and TRILOGY-ACS studies failed to detect any significant association between outcome, pharmacodynamic effect and smoking in prasugrel treated patients, whilst the COPTER study demonstrated only a weak effect of smoking on all three anti-platelet agents (Gurbel et al., 2013, Cornel et al., 2014, Patti et al., 2016).

Aspirin response may also be modified by smoking. In a study of 96 patients with T2DM, smoking was found to be strongly associated with HTPR to aspirin (Labuz-Roszak et al., 2014). These data are in keeping with other studies that have demonstrated higher levels of TXB2 (Ikonomidis et al., 2005) in aspirin treated patients who are current smokers, and is consistent with the observed higher levels of macrophage colony stimulating factor (M-CSF) in smokers (Smith et al., 2001, McAdam et al., 2005). Higher levels of M-CSF facilitate greater platelet-monocyte adhesion with consequent increases on TXA2 release from platelets. However whilst studies using TXB2 as a measure of aspirin response have generally demonstrated a clear response between aspirin response and smoking, other studies using different platelet function tests have not (Valles et al., 2007).

### 1.4.7: Gender

Several large observational studies have demonstrated significant gender effects in relation to aspirin treatment. In the Hypertension Optimal Treatment (HOT) study (Hansson et al., 1998), no significant reduction in the risk of MI was observed in women whereas aspirin therapy in men reduced risk by over 40%. Similarly, the Women's Health Study (WHS) of 39,876 women did not demonstrate any significant reduction in risk of MI or total cardiovascular events from aspirin administration (Ridker et al., 2005). These data are in keeping with the observation that baseline platelet reactivity is higher in women, as demonstrated by Tang and Yin's study of over 14000 healthy individuals who had LTA with ADP as agonist which demonstrated a mean platelet reactivity of 72.4% in men and 80.0% in women ($P<0.001$) (Tang and Yin, 2016). Furthermore, Becker et al conducted an intensive

pharmacodynamic study (Becker et al., 2006), which demonstrated that women had a poorer response to aspirin as assessed by multiple platelet assays. In particular, women had non-suppressed response to collagen, epinephrine and ADP despite aspirin therapy, whilst in men there was almost complete suppression in response to these agonists. In addition, women had significantly higher cholesterol and fibrinogen levels in comparison to men, which is also likely to increase platelet reactivity. However, age may also be a variable, with pre-menopausal women displaying reduced platelet reactivity, most likely related to platelet oestrogen receptors (Di Giosia et al., 2017). In addition, gender effect may also be platelet function test specific, with VerifyNow demonstrating higher platelet reactivity in women whereas the converse is true with the Multiplate platform (Danielak et al., 2017).

However, several studies with thienopyridine have failed to detect a consistent gender effect. In a post-hoc analysis of the PLATO study (Husted et al., 2014), ticagrelor appeared to be equally beneficial over clopidogrel in both men and women, with no gender specific effects observed in rates of bleeding. In the TRILOGY-ACS study (Clemmensen et al., 2015), no difference was observed between men and women in ischaemic or bleeding endpoints, although it should be noted that women enrolled in the study tended to have higher numbers of risk factors for adverse outcomes, such as increased age, lower weight and higher rates of previous cardiovascular events. However, in the TRANSLATE-ACS study (Hess et al., 2014), poorer ischaemic outcomes were reported in women although, following adjustment for cardiovascular risk factors and age, this association became non-significant. Nonetheless, the TRANSLATE-ACS study was in agreement with TRILOGY-ACS, in that women had a more adverse risk profile than men. Finally, in TRANSLATE-ACS women had a higher rate of bleeding which persisted after adjustment for risk factors. Increased bleeding rates in women has also been reported by other studies (Xanthopoulou et al., 2017). However, a large, collaborative, meta-analysis of seven trials including 24,494 women and 63,346 men (Lau et al., 2017) administered potent P2Y12 inhibitors demonstrated that MACE is significantly reduced in both genders, with a similar effect size in both men and women. Similarly, bleeding rates are increased by potent P2Y12 inhibitors to similar degrees in both men and women with no gender effect observed.

Whilst the meta-analysis demonstrates that potent P2Y12 inhibitors are equally effective in men and women, both the TRANSLATE-ACS and TRILOGY-ACS studies report that women have a higher risk score than men which is in keeping with data from the ATLANTIC study (Venetsanos et al., 2017) comparing pre-hospital administration of ticagrelor to cath-lab administration. In this study, women had significantly higher TIMI risk scores and a threefold

higher risk of all-cause mortality compared to men and twofold higher risk of bleeding. Real-life data from these three studies demonstrate that women are at higher risk from cardiovascular events, despite the equal effectiveness of potent P2Y12 inhibitors in men and women. This risk may be explained by the gender based differences in platelet reactivity, but it is also clear that the risk profile of women (older, lower weight etc.) may impact choice of anti-platelet agent and therefore opportunity to benefit from more potent P2Y12 inhibition. Institution of standardised protocols may reduce this inequality, as demonstrated by Wei et al's (Wei et al., 2017a) recent study demonstrating that introduction of a STEMI standardised protocol significantly reduces the treatment disparity between women and men.

### 1.4.8: Anti-platelet dose

Given the HTPR observed with all anti-platelet agents, it is conceivable that increasing the dose with the aim of increasing pharmacodynamic effect would reduce platelet reactivity and reduce the incidence of HTPR.

The CURRENT-OASIS 7 study (Mehta et al., 2010) compared double dose clopidogrel to standard dose clopidogrel in 17,263 ACS patients undergoing PCI. Patients received either 150mg or 75mg clopidogrel for seven days with follow-up for clinical outcomes at thirty days. Double dose clopidogrel was superior to standard dose clopidogrel with the occurrence of the composite outcome being reduced from 4.5% to 3.9% in the double-dose arm (HR 0.86; 95%CI 0.74-0.99, P=0.039). This finding is in keeping with pharmacodynamic studies that demonstrate a significant reduction in platelet reactivity in patients taking clopidogrel 150mg as compared to clopidogrel 75mg (Angiolillo et al., 2007, von Beckerath et al., 2007). Importantly, however, increasing the clopidogrel maintenance dose to 150mg may only be effective in patients who do not carry the loss-of-function *CYP2C19*2* allele, as demonstrated by Alexopoulos and colleagues 71 post-PCI patient study (Alexopoulos et al., 2011a).

In addition, increasing the dose of prasugrel may also overcome prasugrel related HTPR. In the context of STEMI, increasing the loading dose of prasugrel from 60mg to 100mg significantly reduces the HTPR rate from 31.4% to 10.6% in one study of 82 patients (Alexopoulos et al., 2014a). Similarly, other studies have demonstrated a clear dose-response relationship between prasugrel dose and platelet reactivity with higher doses being associated with lower platelet reactivity and fewer patients with HTPR (Ferreiro et al., 2013).

A recent meta-analysis of 10 clinical trials and 4,213 patients (Aradi et al., 2013) also demonstrates that intensified anti-platelet therapy with higher doses of clopidogrel and prasugrel significantly reduces the risk of cardiovascular death, MI or stent thrombosis. In addition, intensified anti-platelet therapy does not appear to be associated with an increased risk of either major or minor bleeding events. Taken together, these data suggest that higher doses of thienopyridines are more efficacious without obvious increases in the risk of bleeding.

Several studies have addressed whether increasing the dose of aspirin increases anti-platelet effectiveness and reduces HTPR. In a study of 40 patients with stable coronary artery disease (Dominiak et al., 2013), increasing the dose of aspirin from 75mg to 150mg successfully reduced HTPR in 62% of aspirin resistant patients, in keeping with data from other studies (Gengo et al., 2016). Interestingly, response to the increased dose of aspirin was predicted by male gender and lower baseline platelet reactivity. In another study, two intensified dose regimens were investigated (81mg QDS compared to 325mg OD), demonstrating that whilst both regimens were more effective at reducing platelet reactivity compared to a single 81mg dose daily, the 81mg QDS was more effective than the 325mg OD dose. However, some studies do not clearly demonstrate any significant change in the HTPR rate or platelet reactivity at higher doses of aspirin. In a study of 961 STEMI patients (Mrdovic et al., 2016), an increased dose of 300mg aspirin for 30 days in the 190 patients defined as poor responders did not significantly reduce the occurrence of major adverse cardiovascular events compared to aspirin sensitive patients. Similarly, the large TRANSLATE-ACS study did not detect any significant benefit of high dose aspirin compared to low dose aspirin in 10,213 patients with ACS (Xian et al., 2015). This was independent of the P2Y12 inhibitor used in combination with aspirin. However, a marginally increased risk of minor bleeding (but not major bleeding) was observed in patients taking high dose aspirin.

### 1.4.9: Drug Interactions

As discussed previously, significant drug interactions exist for all P2Y12 inhibitors. Both the thienopyridine drugs, clopidogrel and prasugrel, have a two-step activation process, catalysed by the CYP450 enzymes. Ticagrelor, on the other hand, is a directly acting agent with no necessity for activation. It, however, is metabolised to an active metabolite by CYP3A4. In addition, all three drugs are administered orally and are therefore subject to variability induced by gastric emptying, intestinal CYP3A4 and intestinal P-gp expression.

**Clopidogrel:** Clopidogrel has a number of well-studied interactions, which are a consequence of clopidogrel's activation pathway. Primary amongst them is the potential interaction between proton pump inhibitors (PPIs) and clopidogrel at CYP2C19, clopidogrel's primary metabolising CYP450 isoenzyme. Importantly, PPIs are frequently prescribed in patients with cardiovascular disease, often as prophylaxis of GI bleeding from aspirin administration (Pelliccia et al., 2015). In a double blind RCT of 124 patients comparing the effects of omeprazole on platelet response to clopidogrel, Gilard et al demonstrated that co-prescription of omeprazole with clopidogrel resulted in a fourfold increase in clopidogrel non-response as defined by VASP (Gilard et al., 2008). However, recent studies have not clearly replicated the interaction between clopidogrel and PPIs. In a recent cross-over study enrolling 28 healthy volunteers, Przespolewski and colleagues did not demonstrate any effect of six PPIs on clopidogrel response, as measured by impedance aggregometry (Przespolewski et al., 2018). In the TRANSLATE-ACS study, there was no significant effect of PPI use on the rate of MACE occurrence in either prasugrel or clopidogrel treated patients, although the cumulative incidence was higher in the clopidogrel-PPI group (20.2%) than the clopidogrel-no PPI group (14.0%) (Jackson et al., 2016). Similarly, the TRILOGY-ACS study did not detect any significant difference between platelet reactivity dependent on PPI status, although clinical events were generally higher in the clopidogrel-PPI treated patients compared to clopidogrel-no PPI patients (Nicolau et al., 2015).

Previous meta-analyses have demonstrated a clear association between co-administration of clopidogrel and PPIs and the risk of major adverse cardiac events. In Siller-Matula and colleagues' 2010 meta-analysis of 20 studies, a 29% increase in risk of MACE was observed in patients taking clopidogrel and a PPI compared to clopidogrel alone (Siller-Matula et al., 2010). A more recent meta-analysis by Bundhun and colleagues (Bundhun et al., 2017) of 11 studies and 84,729 patients, published between 2012 and 2016, demonstrated a 37% increase in risk of MACE in patients administered both clopidogrel and a PPI compared to those on clopidogrel only. Given these data, the FDA and other regulatory agencies worldwide issued several warning about the interactions between PPIs and clopidogrel, with a consequent drop in clopidogrel and PPI co-prescription in patients with ACS. In one registry study of over 200,000 inpatient admissions with ACS, PPI-clopidogrel co-prescription prevalence as 34.9% in 2008, dropping by half to 16.4% in two years (Farhat et al., 2019).

Of recent, concern has emerged over the potential interaction between clopidogrel and statins given that the primary metabolising enzyme for atorvastatin and simvastatin is CYP3A4 which is also responsible for clopidogrel activation. However, in a study of 374 post-

PCI or ACS patients, rosuvastatin, but not atorvastatin, was observed to significantly increase platelet reactivity and HTPR rate in patients administered clopidogrel (Verdoia et al., 2015), which is not in keeping with the CYP3A4 interaction hypothesis. Similarly, other studies have not identified any significant interactions between statins and clopidogrel, although some studies have demonstrated a benefit from using non CYP3A4 metabolised statins such as pitavastatin as opposed to atorvastatin in terms of clopidogrel's pharmacodynamic response (Pelliccia et al., 2015, Zhang et al., 2015). Importantly, in a sub-group analysis of the TRITON-TIMI 38 study, no increased risk of cardiovascular death, MI or stroke was observed in 4,794 clopidogrel treated patients who were co-prescribed a CYP3A4 metabolised statin (HR 1.02; 95%CI 0.85-1.22). In addition, given recent concern about the potential interaction between CYP3A4 metabolised calcium channel blockers (CCBs) and clopidogrel, a further sub-group analysis of the TRITON-TIMI 38 cohort was conducted, again demonstrating no significant increase in risk of MACE in patients being treated with both a CCB and clopidogrel (Ojeifo et al., 2013).

Finally, several other drugs, such as erythromycin, ketoconazole and St John's Wort have all been reported to adversely affect clopidogrel activation, predominantly through an interaction with the CYP3A group of isoenzymes (Farid et al., 2007, Gurbel et al., 2009).

**Prasugrel:** Whilst still requiring a two-step activation process, prasugrel's metabolic pathway does not primarily rely on one or two specific CYP450 enzymes for its bioactivation and consequently it displays fewer interactions than clopidogrel. However, in healthy volunteers administered a 10mg oral prasugrel dose with grapefruit juice, a strong inhibitor of intestinal and hepatic CYP3A4, the exposure to the inactive prasugrel metabolite (R-95913) was significantly increased whilst the plasma concentration of and the exposure to the active prasugrel metabolite (R-138727) was significantly reduced. However, the difference in platelet reactivity was not significantly different between subjects receiving grapefruit juice or not receiving grapefruit juice, a finding in keeping with known pluripotent metabolic activation pathway for prasugrel (Holmberg et al., 2015). Similar findings have been observed with prasugrel co-administration with ritonavir, another strong CYP3A4 inhibitor (Holmberg et al., 2015). Furthermore, co-administration of clopidogrel with grapefruit juice demonstrated a much larger and significant decrease in platelet inhibition as compared to prasugrel, which evidences the difference in prasugrel and clopidogrel bioactivation despite both being pro-drugs requiring a two-step activation process (Holmberg et al., 2015).

**Ticagrelor:** Unlike either prasugrel or clopidogrel, ticagrelor does not require metabolic activation. However, ticagrelor is a substrate for P-glycoprotein and is metabolised by CYP3A4 to an active metabolite. In a healthy volunteer study, Holmberg et al (Holmberg et al., 2013) assessed the effect of grapefruit juice on the pharmacokinetics and pharmacodynamics of a single oral 90mg dose of ticagrelor in a cross-over design. Co-administration of ticagrelor and grapefruit juice increased ticagrelor peak concentrations and exposure significantly compared to ticagrelor alone. Importantly, the pharmacodynamic effect of ticagrelor was also substantially increased, with greater platelet inhibition and lower platelet recovery. In keeping with the known metabolic pathway of ticagrelor, the plasma concentration of the active metabolite, AR-C124910XX, was also significantly lower when ticagrelor was administered with grapefruit, indicating that the active metabolite has little impact on ticagrelor's pharmacodynamics effect despite its known anti-platelet effect. Similar data have been generated from ticagrelor interaction studies with potent CYP3A4 inhibitors and inducers (Holmberg et al., 2013) and ticagrelor is contra-indicated in combination with strong CYP3A4 inhibitors as described in the UK Summary of Product Characteristics.

Recent concerns have been raised about an interaction between ticagrelor and morphine, a drug widely used in the context of acute myocardial infarction. In a healthy volunteer study, Hobl et al (Hobl et al., 2016a), co-administration of 5mg intravenous morphine with a 180mg oral ticagrelor dose resulted in a significant reduction in ticagrelor absorption and reduced plasma concentrations and exposures of both ticagrelor and its active metabolite. However, no significant effect was observed in platelet reactivity, despite the alterations in ticagrelor pharmacokinetics. In a clinical cohort of 70 ACS patients (both STEMI and NSTEACS), the IMPRESSION study (Kubica et al., 2016) demonstrated a significant reduction in ticagrelor and AR-C124910XX exposure when ticagrelor was co-administered with morphine compared with a placebo injection which was also associated with an increased rate of HTPR despite ticagrelor. These findings are in keeping with Parodi et al's study (Parodi et al., 2015) of 300 STEMI patients receiving PPCI, where the anti-platelet effect of ticagrelor was significantly delayed in patients administered morphine. Importantly, this effect is also demonstrated in patients administered prasugrel (Parodi et al., 2015), although a healthy volunteer study investigating the prasugrel – morphine co-administration failed to detect any significant differences in platelet inhibition (Hobl et al., 2016b). Clopidogrel's pharmacokinetic and pharmacodynamics effects have also been demonstrated to be negatively impacted on by co-administration of morphine (Hobl et al., 2016b).

**Aspirin:** Aspirin's pharmacodynamic target, COX-1, may be reversibly inhibited by other drugs with a consequent reduction aspirin's effect. Non-steroidal anti-inflammatory drugs, such as ibuprofen, inhibit COX-1 in a reversible manner and when administered at the same time or prior to aspirin may significantly attenuate its effect. Catella-Lawson and colleagues administered ibuprofen either two hours before or two hours after administration of aspirin. Inhibition of TXB2 response was two-fold higher in subjects who received aspirin two hours prior to the ibuprofen administration compared to those who received aspirin two hours after aspirin administration (Catella-Lawson et al., 2001).

Importantly, enteric coating of aspirin may also substantially reduce its effectiveness. In a triple crossover study, Bhatt and colleagues (Bhatt et al., 2017) demonstrated a threefold higher incidence of aspirin non-response in patients treated with enteric coated aspirin compared to plain aspirin (52.8% vs 15.8%, P<0.0001) as defined by a raised serum TXB2. The serum TXB2 mirrored the pharmacokinetic profiles of both plain aspirin and enteric coated aspirin, with enteric coated aspirin having a significantly lower $C_{max}$ and AUC compared to plain aspirin.

Other commonly prescribed treatments for cardiovascular disease may also influence aspirin response. Several studies have demonstrated inhibition of both COX and ADP mediated platelet aggregation by two angiotensin-2 receptor blockers (ARB), valsartan and losartan (Serebruany et al., 2006, Yamada et al., 2007). This positive impact on platelet aggregation may also be associated with an improved response to aspirin. In a recent large study of 831 aspirin treated patients, concomitant ARB therapy was associated with a better aspirin response than those without ARB therapy (Chen and Chou, 2018a). However, to date, there are no data to suggest that this positive interaction improves clinical outcome and consequently its clinical significance is unknown.

## 1.5: Can we optimise anti-platelet therapy?

Variability to anti-platelet agents is a well characterised phenomenon with a number of identified causes and risk-factors. Modification of these risk factors and potential causes of anti-platelet resistance is possible but relies on identifying and validating a specific biomarker that can be used routinely in clinical practice.

The observed variability in anti-platelet response is, of course, likely to be a multifactorial with some causes easily identified and treated and others not. For example, drug interactions

are easily identified by reviewing patient medication histories and, by stopping interacting drugs, anti-platelet response should improve.

Genetic markers, such as the *CYP2C19* LOF polymorphisms, are also easily identifiable and, if identified, could be used as a biomarker for stratification. For example, genotype guided dosing of ADP receptor antagonists could be instituted, with clopidogrel being used for patients with no *CYP2C19* LOF polymorphisms and ticagrelor or prasugrel used for all other patients. However, universal use of ticagrelor is now commonplace and supported by international guidelines and therefore a strong case would have to be made for a change in practice.

Most other causes such as diabetes, hyperlipidaemia and inflammation are easy to detect, but their presence may not always be associated with poor response to anti-platelets. Furthermore, the mechanism by which they cause HTPR is poorly understood, which makes it difficult to define how best to treat them, other than by focussing on conventional treatments such as statins and anti-diabetic drugs.

Finally, an alternate strategy would be to utilise platelet function testing routinely. This would have the advantage of detecting HTPR irrespective of its cause. However, as previously discussed, there are multiple assays available to test platelet reactivity which often poorly correlate with other assays or clinical outcome, and it is not clear which assay best represents platelet function for all patients. In addition, the use of highly potent anti-platelet drugs may largely negate the need for platelet function testing unless a personalised medicine approach could be used for anti-platelet drugs.

Importantly, stratification for anti-platelets would primarily be used only for ADP receptor antagonists and not aspirin. Whilst response to aspirin can be tested using a variety of assays, it is not clear how aspirin non-response should be treated. Unlike the ADP receptor antagonists, there is no obvious alternative agent for aspirin and its response is not associated with a common, easily testable, genetic polymorphism as is the case with clopidogrel. Consequently, the discussion on stratification will focus only on the ADP receptor antagonists.

### 1.5.1: The Potential for Stratification

Given that the key aim of anti-platelet therapy, particularly in the context of ACS, is to generate rapid and profound platelet inhibition, one treatment option is to simply use more potent anti-platelet agents such as prasugrel and ticagrelor. Whilst this has largely been

incorporated into national and international guidelines for the treatment of acute coronary syndromes, there are a number of important points that merit consideration in relation to the universal use of high potency anti-platelets.

Firstly, the superiority of both prasugrel and ticagrelor over clopidogrel for ischaemic endpoints has not always been consistent in major randomised clinical trials. Whilst the TRITON-TIMI 38 (Wiviott et al., 2007) trial showed a significant benefit of prasugrel over clopidogrel, it should be noted that the clopidogrel loading dose used in the trial (300mg) is lower than the loading dose often used in clinical practice (600mg) which may favour patients in the prasugrel arm. In addition, prasugrel's benefit was mostly driven by the reduction in non-fatal MIs in the prasugrel group with no significant differences between other endpoints in the clopidogrel and prasugrel groups. Furthermore, prasugrel was not demonstrated to be superior to clopidogrel in the TRILOGY-ACS study (Roe et al., 2012) which included patients with medically treated NSTEACS. These variable results are mirrored by meta-analyses, with some reporting a clear superiority of prasugrel over clopidogrel whereas others do not. Similarly for ticagrelor, the PLATO trial reported a clear superiority for ticagrelor over clopidogrel, but the PHILO study in East Asian patients did not. In more stable conditions, such as PAD, ticagrelor was also not demonstrated to be superior to clopidogrel in large randomised studies (Wallentin et al., 2009, Goto et al., 2015, Jones et al., 2017). Altogether, these data suggest that the observed benefit of the more potent anti-platelets is not universal and that some patient groups gain more benefit from them than other groups. Secondly, both prasugrel and ticagrelor are associated with a higher risk of non-CABG related major bleeding than clopidogrel (Wallentin et al., 2009, Wiviott et al., 2007), which is associated with significant morbidity and mortality following ACS, particularly in female and elderly patients. Thirdly, ticagrelor has been associated with dyspnoea, a likely adenosine mediated adverse effect, which in the PLATO trial required discontinuation of the drug in 1% of patients. Whilst this is a small proportion of the overall trial population, it is likely to have a greater impact in general usage where adherence to medication is often lower than in clinical trial. This is likely to a greater problem for ticagrelor, given that it requires administration twice daily which may lower long-term adherence even further. Finally, ticagrelor and prasugrel are significantly more expensive than treating with clopidogrel.

### 1.5.2: Genetic Stratification

As previously discussed, one of the major modifiers of clopidogrel response is genotype and specifically carriage of *CYP2C19* LOF polymorphisms. Given that these polymorphisms are

relatively common in all populations (with higher prevalence in Asian populations), it is possible that the benefit of the more potent anti-platelets is only observed in carries of the LOF polymorphisms. This is supported by data from several studies. Data from the genetic sub-study of the TRITON-TIMI 38 trial demonstrated that the composite endpoint event rate in clopidogrel treated patients with a normal *CYP2C19* and *ABCB1* C3435T genotype was 6.3% which compares favourably with the reported composite event rate of 9.0% in prasugrel treated patients with the same genotypes (Mega et al., 2010a). Similarly, in the genetic sub-study of the PLATO trial, ticagrelor was not conclusively superior to clopidogrel in reducing the occurrence of the composite ischaemic endpoint in non-carriers of the *CYP2C19* LOF allele (Wallentin et al., 2010). These findings suggest that the primary driver of the observed benefit of prasugrel and ticagrelor, when compared to clopidogrel, is due to genetics and, in particular, carriage of the *CYP2C19* LOF alleles. Consequently, genotyping for *CYP2C19* could be used as a marker for personalisation or stratification of anti-platelet therapy, either by determining the dose or type of anti-platelet that should be used in an individual patient.

Several studies have addressed the use of personalised, genotype guided, therapy. In a retrospective study of 199 patients, subjects either received personalised anti-platelet therapy on the basis of *CYP2C19*2* genotype (non-carriers received clopidogrel, carriers received prasugrel) or standard treatment with ticagrelor, with a primary outcome of platelet inhibition within a therapeutic window as defined by VerifyNow. Significantly higher numbers of patients in the personalised anti-platelet therapy (PAT) group achieved platelet inhibition within the therapeutic range compared to ticagrelor treated patients (Malhotra et al., 2015), with a twenty fold higher chance of achieving the therapeutic window in PAT treated patients (OR 20.27; 95%CI 4.33-94.82, P=0.0001). Similarly, a group of 50 AMI patients, discharged post-PCI on prasugrel, were genotyped for the *CYP2C19*2* or **3* allele with those carrying the LOF alleles (**2* and **3*) remaining on prasugrel and those with the wild-type alleles switching to clopidogrel. VerifyNow PRU values were assessed after 5 weeks of genotype guided treatment with no significant differences observed between the genotype-guided clopidogrel and prasugrel groups (Lee et al., 2016). These data are also in keeping with a further, prospective, study of NSTEACS patients using ticagrelor for CYP2C19 LOF carriers instead of prasugrel (Ahn et al., 2013), which also demonstrated no significant differences in PRU values between genotype guided ticagrelor and clopidogrel treated patients. Finally, a large, 628 patient study randomised post-PCI patients to either a 'routine group' receiving clopidogrel 75mg/day or an 'individual group' where anti-platelet therapy was guided by CYP2C19 genotype (Shen et al., 2016). Patients with no CYP2C19 LOF allele

(extensive metaboliser, EM) were continued on clopidogrel 75mg/day whereas as those who carried either one LOF allele (intermediate metaboliser, IM) or two LOF alleles (poor metaboliser, PM) received clopidogrel 150mg/day or ticagrelor 90mg twice daily respectively. Clinical outcomes, based on a composite endpoint of death, myocardial infarction or target vessel revascularisation, were assessed at 1, 6 and 12 months, with a significant reduction in the composite endpoint observed in the 'individual' group compared to the 'routine group'. Within the 'individual group', no significant differences were demonstrated between the EM, IM or PM groups, suggesting that genotype guided choice of anti-platelet agent abolished the risk conferred by carriage of the *CYP2C19*2* allele.

The cost-effectiveness of utilising *CYP2C19* based genotype guided dosing of anti-platelets has also been assessed. In a Hong Kong population, use of ticagrelor in *CYP2C19* LOF carriers and clopidogrel in all other post PCI ACS patients was cost-effective, including in comparison to universal ticagrelor or universal clopidogrel usage (Wang et al., 2018e). However, in an Australian model, whilst such a strategy was again demonstrated to be cost-effective, universal ticagrelor was found to be more effective than genotyped guided dosing with an incremental cost-effectiveness that was higher but within acceptable limits for funding (Sorich et al., 2013). However, Sorich's analysis was predicated on an only threefold difference in cost between ticagrelor and clopidogrel, compared to Wang's differential cost of 23-fold; a value more in keeping with the difference between the cost of clopidogrel and ticagrelor in the UK. Indeed, a further cost-effectiveness analysis by Jiang (Jiang and You, 2017) demonstrated that genotype guided dosing of anti-platelet is cost-effective compared to universal high-potency anti-platelet or universal clopidogrel.

### 1.5.3: Pharmacodynamic Stratification

An alternative strategy would be to utilise platelet function tests for stratification instead of the *CYP2C19*2* polymorphism. In the GRAVITAS study (Price et al., 2011), a total of 2214 patients were enrolled and had platelet function assessed using the VerifyNow instrument after PCI, with those determined as having HTPR receiving high dose clopidogrel (600mg loading dose, followed by 150mg once daily) whilst those with satisfactory platelet inhibition remained on a standard dose of clopidogrel. After 6 months of treatment, there was no significant difference in the occurrence of the primary ischaemic endpoint between the high and low dose clopidogrel groups (HR 1.01; 95%CI 0.58-1.76, P=0.97). In the TRIGGER-PCI study, (Trenk et al., 2012), patients with stable coronary artery disease and HTPR whilst on clopidogrel were randomised to receive either clopidogrel 75mg or prasugrel 10mg once

daily. Whilst a significant improvement in platelet inhibition was observed in patients receiving prasugrel compared to clopidogrel, the trial failed to demonstrate any significant difference in the occurrence of the composite ischaemic outcomes between the two groups and was stopped early because of futility. Finally, in the ARCTIC study (Collet et al., 2012), over 2000 patients undergoing PCI were randomised to either standard anti-platelet therapy or tailored anti-platelet therapy using the VerifyNow platform. No significant difference was observed for the primary ischaemic outcome between the two groups. However, the GRAVITAS, TRIGGER-PCI and ARCTIC studies largely included patients with stable coronary artery disease and it is therefore unsurprising that the trials failed to demonstrate any significant benefit from personalised anti-platelet therapy in a group of patients with traditionally low ischaemic event rates. More recently, studies have been conducted largely in patients with ACS. Dridi et al (Dridi et al., 2014) included 237 ACS patients with HTPR whilst on clopidogrel 75mg, as defined by the Multiplate analyser, with 114 remaining on clopidogrel standard doses whilst the remaining 123 received intensified anti-platelet therapy (either high-dose clopidogrel, prasugrel or ticagrelor). Intensified anti-platelet therapy significantly reduced the occurrence of the primary composite ischaemic outcome compared to patients remaining on standard dose of clopidogrel, with event rates comparable to patients with normal platelet reactivity on clopidogrel. Similarly, in the RECLOSE-3 study (Valenti et al., 2015), subjects with HTPR on clopidogrel 75mg (as identified by LTA) were switched to prasugrel 10mg with a significant improvement in platelet inhibition and reduction in occurrence of the primary ischaemic endpoint. In addition, Aradi et al (Aradi et al., 2014) demonstrated a significant improvement in both pharmacodynamic and clinical outcomes following intensification of anti-platelet therapy in patients with clopidogrel related HTPR. In a recent cost-effectiveness analysis (Coleman and Limone, 2013), platelet reactivity driven dosing of anti-platelets was found to be cost-effective compared to universal ticagrelor, prasugrel or generic clopidogrel, although the ICER for platelet reactivity guided dosing in this study was substantially higher than the ICER for genotype guided dosing in Wang's cost-effectiveness analysis (Wang et al., 2018e).

### 1.5.4: Treatment of non-responders

It is generally assumed that in poor responders to clopidogrel, or those that have any CYP2C19 LOF polymorphisms, high potency anti-platelets such as prasugrel or ticagrelor should be used instead of clopidogrel. However, given the dose-response relationship observed with clopidogrel, several studies have investigated whether giving higher maintenance doses of clopidogrel increases platelet inhibition and reduces cardiovascular

events. As discussed previously, higher loading doses of clopidogrel are associated with better clinical outcomes post PCI and therefore it is conceivable that a higher maintenance dose would have a similar effect in longer use (Patti et al., 2005). In a randomised, double blind trial, Von Beckerath (von Beckerath et al., 2007) demonstrated a significant reduction in platelet aggregation in subjects randomised to clopidogrel 150mg once daily compared to those taking the usual maintenance dose of 75mg/day. This is mirrored by other studies which demonstrate similar reductions in platelet aggregation with higher (150mg) doses of clopidogrel (Angiolillo et al., 2007). Furthermore, increasing the clopidogrel maintenance dose also improves clinical outcomes. Abuzhara et al (Abuzahra et al., 2008) randomised 119 patients attending for PCI to either a high dose (600mg loading, 150mg maintenance) or low dose (300mg loading, 75mg maintenance) clopidogrel regimen for 30 days. There was a significant improvement in clinical outcomes with 23.8% of the low dose group suffering the composite ischaemic outcome compared to 10.3% in the high dose group (P=0.04). Importantly, given the increased platelet inhibition at higher clopidogrel doses, there was no significant increase in bleeding complications in the high dose group. In addition, the CURRENT-OASIS 7 (Mehta et al., 2010) trial recruited 25806 patients with ACS, scheduled for PCI and randomised participants to either high or low dose aspirin or high or low dose clopidogrel therapy. In the clopidogrel group, there was a 14% reduction in the occurrence of the primary ischaemic outcome in the high dose group compared to the low dose group (HR 0.86; 95%CI 0.74-0.99, P=0.039) and a 46% reduction in the occurrence of stent thrombosis (HR 0.54; 95%CI 0.39-0.74, P=0.0001). However, this was at the expense of a 39% increase in the risk of major bleeding episodes in the high dose clopidogrel group (HR 1.39; 95%CI 1.07-1.81, P=0.01) which is in keeping with the increased bleeding risk observed with the newer, more potent , anti-platelet agents. Furthermore, in Lemesle et al's study of 2954 patients with unstable or stable coronary artery disease(Lemesle et al., 2009), there was no significant increase in the risk of bleeding in high versus low dose groups.

However, it is unclear whether increasing the clopidogrel maintenance dose in patients defined as having clopidogrel related HTPR is effective. As discussed previously, the GRAVITAS study (Price et al., 2011) failed to demonstrate any benefit of high dose clopidogrel in patients with clopidogrel related HTPR although this may have been confounded by the inclusion of only stable coronary artery disease patients with consequently lower event rates. In the RESET-GENE study (Sardella et al., 2012), 180 ACS patients had platelet inhibition assessed post PCI using the Multiplate analyser, with those identified as normal responders continuing on a clopidogrel maintenance dose of 75mg once daily. Patients identified as poor

responders were randomised to receive either clopidogrel 150mg/day or prasugrel 10mg/day with a cross-over to the alternate treatment at day 15. After 15 days on the alternate treatment, a further assessment of platelet reactivity was performed, with subjects then receiving the treatment (prasugrel or high-dose clopidogrel) that resulted in the highest level of platelet inhibition until 12 months post randomisation. High dose clopidogrel was less effective at reducing HTPR than prasugrel, with no patients randomised to prasugrel exhibiting HTPR compared to 28% of patients randomised to high dose clopidogrel (P=0.001). However, prasugrel and high dose clopidogrel HTPR rates were comparable in patients with no *CYP2C19* LOF (**2*) alleles whereas HTPR was significantly more common in the high dose clopidogrel arm compared to the prasugrel arm in *CYP2C19* LOF allele carriers, demonstrating that *CYP2C19* genotype is critically important in determining response to high dose clopidogrel as well as low dose clopidogrel. In the RAPID STEMI study (So et al., 2016), STEMI patients undergoing PCI were genotyped for the *CYP2C19*2* and **17* polymorphisms in addition to the *ABCB1* C3435T polymorphism. Patients with any *CYP2C19*2* allele or the *ABCB1* TT genotype were randomised to receive either prasugrel 10mg or 'augmented' clopidogrel (150mg/day for 6 days, followed by 75mg/day) and HTPR was assessed using the VerifyNow platform at one month. HTPR rates were significantly lower in the prasugrel arm compared to the clopidogrel arm (0% vs 24.1%, P=0.0046). No significant difference in HTPR rate was observed between non-carriers treated with clopidogrel 75mg/day compared to carriers treated with prasugrel 10mg/day, in keeping with the importance of the *CYP2C19* genotype in clopidogrel response. In addition, ticagrelor also appears to be more efficacious than high dose clopidogrel. In a study of 224 patients with acute coronary syndrome, *CYP2C19*2* homozygotes were randomised to receive either ticagrelor (180mg loading dose, 90mg twice daily thereafter) or high dose clopidogrel (600mg loading dose, 150mg daily thereafter). Platelet reactivity was assessed after 30 days using the VerifyNow platform, with significantly higher HTPR rates in the clopidogrel treated patients as compared to ticagrelor treated patients (Xiong et al., 2015).

These data suggest that high-dose clopidogrel is likely to reduce HTPR effectively only in patients without the *CYP2C19*2* allele, although some studies have suggested that it may be effective in *CYP2C19*2* heterozygotes (Shen et al., 2016). However, in practical terms, a high potency anti-platelet, such as prasugrel or ticagrelor, is likely to be a better choice for stratification. It remains unclear whether ticagrelor or prasugrel is the better drug to overcome clopidogrel related HTPR. In the ISAR-ADAPT-PF study, Bernlochner et al compared prasugrel and ticagrelor in a cohort of 70 patients with clopidogrel related HTPR

(Bernlochner et al., 2016). Both prasugrel and ticagrelor significantly improved the degree of platelet inhibition compared to clopidogrel, with no significant difference detectable between the ticagrelor and prasugrel groups. These data are consistent with previous studies which have failed to demonstrate any significant differences between prasugrel and ticagrelor in clinical use (Shah et al., 2017, Motovska et al., 2018).

## 1.6: Aims of the thesis

Anti-platelets form a cornerstone of treatment of acute coronary syndromes. Whilst large, clinical outcome studies clearly demonstrate the benefit of anti-platelets, their benefit is not uniform across all patients. Assessing response to anti-platelet is challenging, with several different assays available which test platelet aggregation in different ways. In addition, correlation between individual assays is often poor with different clinical and biochemical factors affecting their results.

Anti-platelet response is a complex phenotype, with numerous different factors affecting the response to anti-platelet drugs. Some factors may be related to the drug itself, such as dose and compliance, whilst others may be related to the underlying cardiovascular disease and its risk factors. Furthermore, genetic factors are critical to the response to some drugs such as clopidogrel.

Response to clopidogrel is largely determined by genetic polymorphisms in its pharmacokinetic pathway. Post-hoc analyses of trials comparing prasugrel or ticagrelor to clopidogrel demonstrate that these polymorphisms, and in particular *CYP2C19*2* and *ABCB1* C3435T, may be responsible for the observed superiority of the newer drugs over clopidogrel. Consequently, stratification on the basis of genotype may be possible and has been assessed as potentially cost-effective in comparison to universal use of a more potent anti-platelet agent.

Furthermore, other factors may be important in determining response to anti-platelet drugs. In particular, inflammation and the presence of clinical factors such as hyperlipidaemia and diabetes may substantially reduce the effectiveness of anti-platelet drugs. Incorporating these factors into any type of stratification may be important to best define anti-platelet response and the choice of anti-platelet agent.

Aspirin non-response is also a significant concern with no alternative agent available should non-response be detected. Whilst the phenomenon of aspirin non-response has been well studied, it underlying mechanisms remain unclear. However, comprehensive genetic studies, addressing both pharmacokinetic and pharmacodynamic pathways have not been undertaken, and its relationship with inflammation is also poorly understood.

In conclusion, poor response to anti-platelets is a well-recognised phenomenon with a number of identified clinical, biochemical and genetic factors. However, data are often inconsistent and underlying mechanisms are poorly understood.

Adopting a personalised approach is challenging with either genetic or pharmacodynamic testing being used in previous studies. Data from these studies are often inconsistent which has limited the clinical application of both genetic testing and platelet function testing. Furthermore, the relationship between genetics, pharmacodynamics and clinical outcome remains unclear.

To this end, this thesis will focus on better defining some of the potential modifiers of clopidogrel and aspirin response. In chapter 2, the relationship between clopidogrel response, platelet function testing and genetic polymorphisms will be comprehensively explored. Chapter 3 will focus on the inconsistent associations observed between clinical outcome and genetic polymorphisms in patients taking clopidogrel. Given the importance of aspirin non-response, chapter 4 will assess relationship between aspirin response and comprehensive assessment of polymorphisms in aspirin's pharmacokinetic and pharmacodynamic pathway in ACS patients from the Pharmacogenetics of Acute Coronary Syndromes (PhACS) study. Chapter 5 investigates the relationship between lipid oxidation, hyperlipidaemia, clinical outcome and anti-platelet response in patients from the PhACS study. Finally chapter 6 will assess the relationship between PPI use, *H. Pylori* antibodies and clinical outcome.

# Chapter 2 – Influence of genetic polymorphisms on pharmacodynamic response to clopidogrel: a systematic review and meta-analysis

## 2.1: Introduction

Clopidogrel is a thienopyridine anti-platelet drug that blocks the platelet P2Y12 receptor. Several large randomised controlled trials have demonstrated that clopidogrel significantly reduces adverse cardiovascular events compared to placebo, a finding demonstrated in a meta-analysis of 5 trials and 79,613 patients by Berger et al (Berger et al., 2009).

However, newer anti-platelet agents, such as ticagrelor and prasugrel have largely replaced clopidogrel in clinical practice (Ibanez et al., 2018). Prasugrel and ticagrelor have both been demonstrated to be superior to clopidogrel in clinical trials (Wiviott et al., 2007, Wallentin et al., 2009) and have accordingly become first line therapy for the treatment of acute coronary syndromes.

Non-response to clopidogrel has been identified as an important contributing factor to the superiority of the newer drugs, particularly in the context of acute coronary syndromes and PCI. In a large meta-analysis of 25 clinical trials, Snoep et al described a 21% prevalence of clopidogrel non-response with a corresponding eightfold increase in the risk of further cardiovascular events (Snoep et al., 2007b).

Clopidogrel response is a complex phenotype with clopidogrel bioactivation being one of the most important factors in determining the overall effect of clopidogrel. Clopidogrel is a pro-drug that requires a two-step activation via CYP450 enzymes and, in particular, CYP2C19. The *CYP2C19* gene is polymorphic with a number of loss-of function (LOF) polymorphisms described (**2*, **3*, **4*), with some meta-analyses demonstrating an increase in the risk of further cardiovascular events in those that carry those polymorphisms (Mega et al., 2010b). However, the association between *CYP2C19* LOF polymorphisms and poor clinical outcomes are sometimes not consistent between studies (Bauer et al., 2011). In addition, there are a number of other genes that have been identified as potential modifiers of clopidogrel response, including genes involved in clopidogrel's absorption and binding to the P2Y12 receptor. These include variants involved in clopidogrel's absorption (*ABCB1*), metabolism (paraoxonase-1), binding (*P2Y12*) as well as other platelet receptors. However, the

association between those variants and outcomes are frequently conflicting (Trenk et al., 2011, Wallentin et al., 2010).

Whilst *CYP2C19* variants have the potential to be used as markers of clopidogrel non-response, there are potential limitations to their usage. Clopidogrel response is likely to be affected by a number of clinical variables such as diabetes, hyperlipidaemia and inflammation, which genotype is not sensitive too. Therefore, an alternative strategy to identify clopidogrel non-response is to measure the effect of clopidogrel on platelet reactivity directly. Several platelet function tests are available to measure platelet reactivity, with clear associations with high on treatment platelet reactivity on clopidogrel and adverse cardiovascular outcomes demonstrated in recent meta-analyses. In addition, several studies have demonstrated an association with *CYP2C19* LOF polymorphisms and higher platelet reactivity (Tsantes et al., 2013, Liang et al., 2013). However, these associations are not always demonstrated consistently across different clinical situations or platelet function tests. Moreover, different platelet function tests assess platelet function in different ways, with variable test conditions and agonists. Furthermore, some tests can be described as bedside, requiring little or no sample preparation, whereas others require specially trained staff and highly specialist equipment. Consequently, agreement between assays is often poor and it is difficult to identify which platelet function test best represents in vivo platelet function. It is therefore difficult to determine which test should be used to identify poor responders to clopidogrel (Lemesle et al., 2014).

Whilst prasugrel and ticagrelor have been adopted as first line therapy for treatment of acute coronary syndromes, some studies have demonstrated that prasugrel and ticagrelor may not necessarily be superior to clopidogrel in all circumstances. For example, in medically managed patients with acute coronary syndromes, the TRILOGY-ACS study (Roe et al., 2012) failed to demonstrate a clear benefit of prasugrel over clopidogrel, although sub-group analysis did demonstrate a significant reduction in the occurrence of primary ischaemic outcome in patients under 75 years of age who were treated beyond twelve months post randomisation. Similarly, for ticagrelor, the PHILO study demonstrated similar outcomes in both clopidogrel and ticagrelor treated patients (Goto et al., 2015) with STEMI and NSTEACS in East Asia. These data are in keeping with the inconsistent observations from meta-analyses, with some meta-analyses demonstrating that newer anti-platelets are superior to clopidogrel (Shah et al., 2017) whilst others do not (Bavishi et al., 2015, Westman et al., 2017).

Given the effects of *CYP2C19* genetic variants on clopidogrel response, it is conceivable that LOF variants may be responsible, at least in part, for the superiority sometimes observed with ticagrelor and prasugrel treatment. Indeed, in a genetic sub-study of the TRITON-TIMI 38 study (Mega et al., 2010a), patients with a normal *CYP2C19* and *ABCB1* C3435T genotype treated with clopidogrel compared favourably with prasugrel treated patients. This also appears to be the case in the PLATO study (Wallentin et al., 2010): patients with a wild-type *CYP2C19* genotype treated with clopidogrel also had similar outcomes to ticagrelor treated patients.

Taken together, these data suggest that anti-platelet therapy could be personalised on the basis of either genotype or platelet function. This strategy has the advantage of lowering usage of the newer anti-platelet agents and therefore reducing the risk of adverse effects such as major bleeding and dyspnoea. In addition, personalisation of therapy is likely to be less expensive than using universal ticagrelor or prasugrel given the price differential between the newer anti-platelet agents and generic clopidogrel currently.

Recent studies have focussed on methods to stratify anti-platelet therapy and improve clopidogrel response, either by using higher loading or higher maintenance doses of clopidogrel or by using the newer anti-platelet agents such as prasugrel or ticagrelor in patients identified as being poor responders to clopidogrel (Piccolo et al., 2014). However, identification of these poor responders to clopidogrel is challenging. Whilst genetics, and specifically the *CYP2C19* variants, appear to be an ideal candidate for stratification, genetics alone will not identify patients who have HTPR due to other clinical or biochemical factors such as higher body mass index, diabetes, impaired renal function or compliance (Sweeny et al., 2009). Platelet function tests have the advantage that they are sensitive to these factors (Bonello-Palot et al., 2009, Mangiacapra et al., 2014), but the variability between individual tests and the inter-operator variability that affects some tests may offset its clinical utility.

Therefore, whilst there remains a good argument for personalisation or stratification of anti-platelet agents, there remain a number of critical issues surrounding the relationship between genetic variants and platelet function tests. Firstly, is there a consistent association between *CYP2C19* variants and platelet reactivity as tested by different platelet function tests? Secondly, are there any platelet function tests that are better associated with genetic variants that have been demonstrated to affect clinical outcome? Finally, despite the well-recognised association between *CYP2C19* loss-of-function variants and outcomes, are there any other genetic variants that are consistently associated with platelet reactivity?

In order to address those questions, a comprehensive systematic review and meta-analysis of all published studies, in patients taking clopidogrel, investigating the relationship between genetics and platelet function tests was conducted.

## 2.2: Methods

### 2.2.1: Search Strategy

Relevant citations were identified using a comprehensive search using PubMed (1966 to November 2015) and Scopus Web of Science. In order to find all relevant citations, a broad search term was used with the following terms in combination or as text words with no language restriction: clopidogrel, thienopyridines, *P2Y12*, *CYP2C19*, *CYP2C9*, *CYP2B6*, *CYP3A4*, *P2Y1*, cytochrome P450, gene, genotype, SNP, allele, polymorphism, variant and haplotype. In addition, manual searching of reference lists was undertaken for each of the extracted papers. Conference abstracts were also identified by searching for supplemental issues of major cardiovascular or clinical pharmacology journals.

### 2.2.2: Data Extraction

Data were extracted by two independent reviewers. Initially, all citations were reviewed by title and subsequently by abstract. Inclusion criteria were (a) studies that included patients about to commence or already established on clopidogrel and (b) studies which investigated the effect of genetic variants on the response to clopidogrel. Included data were extracted onto standardised data extraction forms and entered on to a computer spreadsheet. For each study, data were collected on a number of different variables including number of participants, age, setting, risk factors for cardiovascular disease, clopidogrel dose (maintenance and loading), genotype distribution and pharmacodynamic outcomes. Methodological quality was also assessed (Hardy-Weinberg assessment, genotyping methodology).

### 2.2.3: Outcomes

The primary objective of the meta-analysis was to investigate the relationship between genetic variants and platelet reactivity in patients on clopidogrel. The outcome measures investigated were determined by the platelet function tests in each paper. Only comparable measures were combined in the meta-analysis, and therefore the meta-analyses for each test were broken down by method and measure.

### 2.2.4: Statistical Analysis

As the studies often reported measurements for the wild type gene along with measurements for both one and two variant types and /or a combined variant type, we decided to combine the variant types when given separately in a dominant inheritance model. Combining of the variant type measures was done using the standard formulae for pooling means and variance. Consequently, the comparison included in the meta-analysis is one of wild type against any variant type (homo- or heterozygote); with the pooled measured values used when provided and, when not available, the calculated pooled measurement. Meta-analyses were prepared when more than two studies contributed data.

The inverse variance method, using a fixed effect model, was initially used to calculate a pooled mean difference between wild type and mutant type study arms. However, the Q and I^2 measures of statistical heterogeneity demonstrated that the between study variability was significantly higher than appropriate for the fixed effects model. Consequently, it was decided to standardise the mean difference by dividing the mean difference by the pooled standard deviation and then fitting a random effects meta-analysis, to calculate a pooled standardised mean difference, in addition to 95% confidence intervals. The result of the meta-analyses are presented using Forest plots to describe both the individual studies and the overall pooled effect. Funnel plots, standard error of mean difference plotted against mean difference, were also constructed to assess publication bias. All analyses were undertaken using Review Manager (REVMAN), version 5.1, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011.

## 2.3: Results

### 2.3.1: Search Results and Study Characteristics

The initial literature search yielded a total of 652 citations; of those 207 were included on the basis of title, abstract and full text review. 165 of the papers reported on platelet reactivity and were therefore included in this meta-analysis (**Figure 2.1**). 63 Studies reported outcome related to *CYP2C19*2* genotypes, 56 studies reported outcome related to combined *CYP2C19* metaboliser status, 19 reported outcomes to *ABCB1* C3435T genotypes, 18 reported outcomes related to *PON1* Q192R genotypes, 17 reported outcomes to

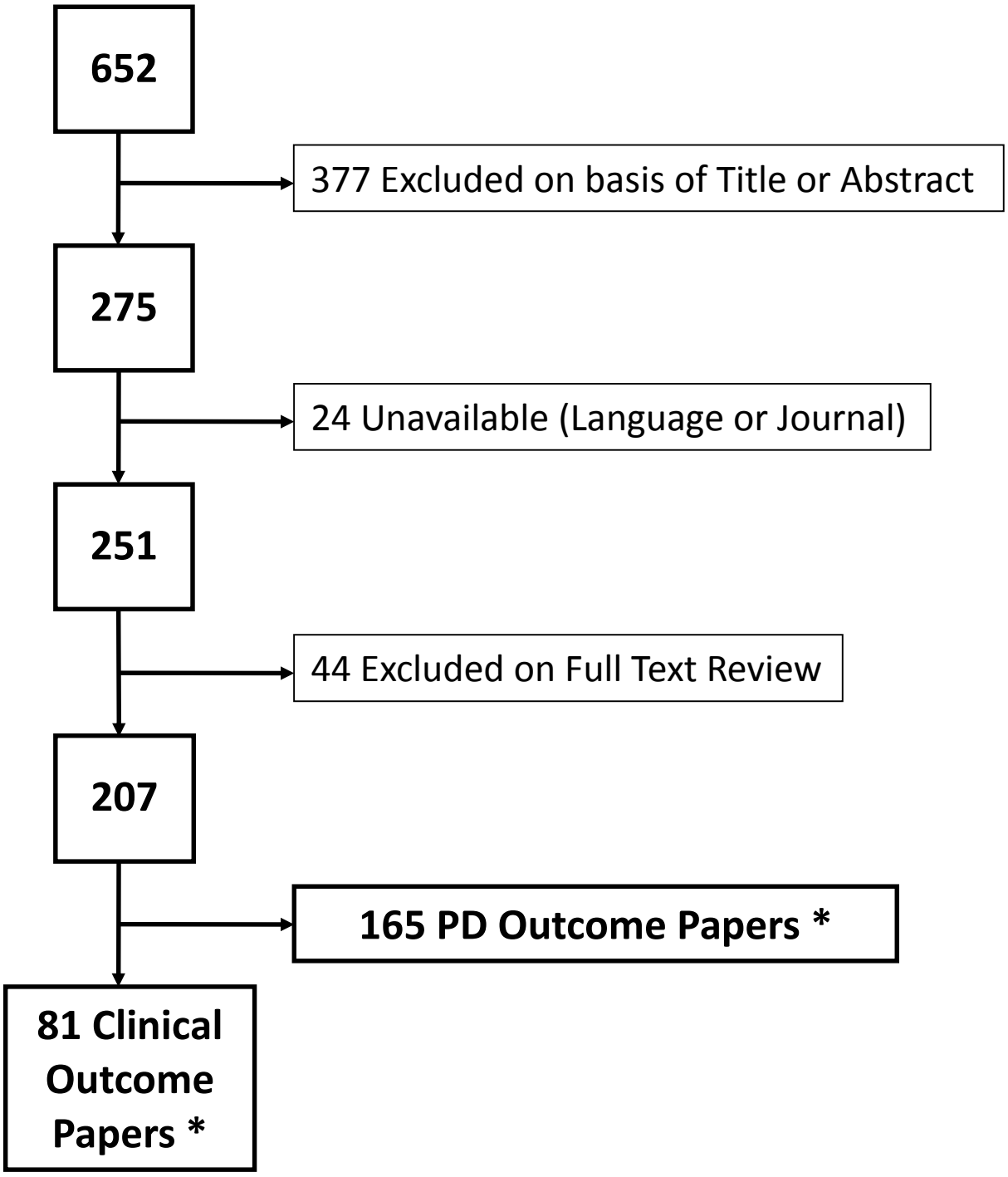


**Figure 2.1 – Literature Search Results** (* 125 papers reported on PD outcomes only, 40 papers reported on both PD and clinical outcomes, 41 papers reported on clinical outcomes only)

*CYP3A5*3*, 17 reported outcomes to *CYP2C19*17* genotypes, 11 reported outcomes to *CYP2C19*3* genotypes and four reported outcomes to *CYP2C9*3* genotypes. A number of polymorphisms were investigated by other studies, but these could not be combined in meta-analyses due to incomparable polymorphisms and/or outcomes (**Table 2.1**).

With regard to outcome measures, 50 studies reported using VerifyNow, 49 reported using Light Transmittance Aggregometry (20 µmol/L ADP as agonist), 34 reported using Vasodilator stimulated phosphoprotein phosphorylation (VASP), 32 reported using LTA (5 µmol/L ADP), 16 reported using LTA (10 µmol/L ADP) and 9 reported using Multiplate. A number of other methods for assessing platelet reactivity were reported by the studies but these could not be combined in the meta-analyses due to small numbers or incomparable polymorphisms (**Table 2.2**).

Therefore, a total of 82 studies, reporting seven polymorphisms (*ABCB1* C3435T, *CYP3A5*3*, *CYP2C9*3*, *CYP2C19*2*, *CYP2C19*3*, *CYP2C19*17*, *PON1* Q192R) and six platelet function tests (LTA 5 µmol/L ADP, LTA 10 µmol/L ADP, LTA 20 µmol/L ADP, Multiplate, VASP and VerifyNow) were included in the final meta-analyses. The characteristics of the included studies are summarised in **Table 2.3**. The studies included a variety of different patient groups, with some studies recruiting healthy volunteers (8 studies) and the others recruiting subjects with either stable or unstable cardiovascular disease. Notably, the clopidogrel loading dose was frequently variable with studies giving up to 1200mg; clopidogrel maintenance doses were typically 75mg once daily although some studies reported using 150mg (ten studies) or 300mg once daily (one study). Furthermore, the studies also reported different outcome measures for individual platelet function tests; for example, LTA was reported using either maximal platelet aggregation (MPA), residual platelet aggregation (RPA) or inhibition of platelet aggregation (IPA). As most studies reported MPA, only studies that reported MPA were included in the final meta-analysis. A summary of all the meta-analyses performed is detailed in **Table 2.4**.

| Gene | SNP | Studies | Study References |
|---|---|---|---|
| *ABCB1* | C3435T | 19 | S1; S2; S3; S5; S6; S7; S9; S10; S11; S12; S13; S14; S15; S22; S23; S24; S25; S26; S27 |
| *ABCB1* | G2677T/A | 3 | S5; S6; S9 |
| *ABCB1* | C1236T | 1 | S6 |
| *ACC3* | -211C/T | 1 | S28 |
| *ARNT* | rs2134688 | 1 | S29 |
| *a2-AR* | rs553668 | 1 | S30 |
| *CES1* | 482G/A | 1 | S31 |
| *CES1* | -816A/C | 2 | S32; S33 |
| *COX2* | rs5277 | 1 | S34 |
| *CYP1A1* | *2C | 1 | S34 |
| *CYP1A2* | *1F | 2 | S2; S13 |
| *CYP1A2* | Met | 2 | S35; S36 |
| *CYP1A2* | *1B | 1 | S34 |
| *CYP2B6* | *4 | 2 | S2; S5 |
| *CYP2B6* | *6 | 2 | S5; S13 |
| *CYP2B6* | Met | 1 | S36 |
| *CYP2C19* | *2 | 63 | S2; S3; S6; S7; S10; S11; S14; S15; S16; S17; S18; S19; S20; S22; S36; S27; S29; S30; S34; S37; S38; S39; S40; S41; S42; S43; S44; S45; S46; S47; S48; S49; S50; S51; S52; S53; S54; S55; S56; S57; S58; S59; S60; S61; S62; S63; S64; S65; S66; S67; S68; S69; S70; S71; S72; S73; S74; S75; S76; S77; S78; S79 |
| *CYP2C19* | Met | 56 | S4; S5; S9; S12; S13; S21; S22; S23; S25; S33; S36; S80; S81; S82; S83; S84; S85; S86; S87; S88; S89; S90; S91; S92; S93; S94; S95; S96; S97; S98; S99; S100; S101; S102; S103; S104; S105; S106; S107; S108; S109; S110; S11; S112; S113; S114; S115; S116; S117; S118; S119; S120; S121; S122; S123; S124 |
| *CYP2C19* | *17 | 17 | S2; S3; S7; S11; S14; S15; S21; S49; S58; S67; S71; S75; S95; S122; S125; S126; S127 |
| *CYP2C19* | *3 | 11 | S2; S10; S11; S14; S15; S21; S49; S58; S67; S71; S75; S95; S122; S125; S126; S127 |
| *CYP2C19* | *4 | 1 | S15 |
| *CYP2C9* | *3 | 4 | S6; S7; S8; S14 |
| *CYP2C9* | *2 | 3 | S6; S8; S14 |
| *CYP2C9* | Met | 2 | S36; S59 |
| *CYP2J2* | Met | 1 | S34 |
| *CYP3A4* | IVS10+G12A (*1G) | 8 | S2; S13; S15; S34; S57; S80; S97; S128 |

| | | | |
|---|---|---|---|
| *CYP3A4* | *1B | 3 | S6; S15; S46 |
| *CYP3A4* | rs2246709 | 1 | S34 |
| *CYP3A4* | *22 | 1 | S29 |
| *CYP3A5* | *3 | 17 | S1; S2; S3; S4; S5; S12; S13; S15; S23; S27; S34: S46; S74; S79; S129; S130; S131 |
| *GPIa* | C807T | 4 | S132; S133; S134; S135 |
| *GPIa* | T837C | 1 | S133 |
| *GPIIIa* | P1A1/A2 | 4 | S79; S132; S136; S137 |
| *GPVI* | C13254T | 1 | S132 |
| *IRS1* | A227497991G | 1 | S15 |
| *IRS1* | G227382808C | 1 | S15 |
| *ITGB3* | T196C | 1 | S15 |
| *P2Y1* | A1622G | 4 | S128; S136;S138; S139 |
| *P2Y12* | T744C | 10 | S11; S34; S57; S63; S70; S74; S136; S140; S141; S142 |
| *P2Y12* | H1/H2 | 8 | S7; S35; S131; S132; S143; S144; S145; S146 |
| *P2Y12* | C32T | 3 | S132; S146; S147 |
| *P2Y12* | G33A | 1 | S27 |
| *P2Y12* | C18T | 1 | S148 |
| *P2Y12* | C34T | 2 | S11; S27 |
| *P2Y12* | G52T | 2 | S11; S27 |
| *P2Y12* | T742C | 2 | S5; S148 |
| *P2Y12* | T2379C | 2 | S5; S148 |
| *P2Y12* | rs6787801 | 1 | S149 |
| *PAR1* | IVSn-A14T | 1 | S132 |
| *PPAR-a* | rs253728 | 1 | S29 |
| *PPAR-a* | rs4823613 | 1 | S29 |
| *PON1* | Q192R | 18 | S1; S5; S9; S12; S13; S15; S16; S17; S18; S19; S20; S21; S22l S25; S37; S72; S93; S101 |
| *PON1* | L55M | 2 | S15; S20 |
| *P-Selectin* | Thr715Pro | 1 | S150 |

**Table 2.1 – Number of studies per gene and SNP** (For all S references, please refer to Appendix 1).

| Test | Studies | Study References |
|---|---|---|
| VN | 50 | S1; S2; S3; S4; S5; S6; S7; S8; S10; S13; S16; S23; S30; S31; S34; S38; S41; S43; S52; S53; S54; S55; S56; S60; S68; S71; S75; S76; S77; S83; S84; S86; S88; S89; S90; S91; S92; S93; S94; S5; S105; S106; S108; S112; S119; S122; S124; S127; S129; S149 |
| LTA 20 ADP | 49 | S1; S4; S6; S10; S11; S12; S16; S17; S18; S22; S23; S26; S28; S29; S32; S36; S39; S42; S43; 45; S46; 49; S53; S58; S59; S65; S80; S82; S84; S85; S86; S87l S99; S100; S101; S106; S107; S113; S115; S116; S118; S121; S128; S134; S136; S138; S141; S146; S149 |
| LTA 5 ADP | 32 | S1; S4; S6; S9; S10; S16; S23; S29; S35; S42; S43; S44; S45; S46; S78; S80; S82; S83; S97; S98; S109; S120; S124; S126; S130; S131l S136; S138; S139; S146; S148; S149 |
| VASP | 34 | S1; S8; S9; S12; S14; S15; S18; S19; S21; S33; S35; S39; S40; S41; S46; S50; S51; S52; S61; S68; S73; S86; S87; S96; S106; S114; S120; S125; S132; S140; S143; S144; S150 |
| LTA 10 ADP | 16 | S8; S16; S27; S46; S47; S48; S57; S62; S70; S79; S125; S133; S140; S143; S149 |
| Flow Cytometry | 11 | S46; S64; S123; S128; S131; S136; S137; S138; S140; S141; S146 |
| Multiplate® | 9 | S8; S37; S39; S52; S67; S69; S72; S83; S110 |
| TEG | 4 | S25; S74; S103; S104; S117: S120; S147 |
| PFA® | 4 | S24; S39; S63; S133 |
| Impedance Aggregometry | 2 | S102; S145 |
| Imp-R | 3 | S8; S52; S77 |
| WBSPC | 1 | S131 |

**Table 2.2 - Number of studies per Platelet Function Test** (For all S references, please refer to Appendix 1).

| Author | Year | Type | PD Test | Measure | Gene | N | Clop LD | Clop MD | Setting | Cohort |
|---|---|---|---|---|---|---|---|---|---|---|
| Alexopoulous D et al [S60] | 2011 | CROSS | VN | PRU | 2C19*2 | 21 | NA | 150 | SCAD | |
| Barker CM et al [S88] | 2010 | PC | VN | PRU | 2C19*2, *3, *4, *17 | 41 | NS | 75-150 | PCI+SCAD | |
| Bin Sayeed MS et al [S75] | 2015 | PC | VN | %inhib | 2C19*2, *17 | 149 | NS | NS | PCI | |
| Bonello L et al [S51] | 2010 | PC | VASP | PRI | 2C19*2 | 411 | VAR | 75 | PCI | |
| Bonello L et al [S19] | 2012 | PC | VASP | PRI | 2C19*2; ABCB1 C3435T; PON1 Q192R | 498 | 600 | 75 | NS | |
| Bonello-Palot N et al [S40] | 2009 | PC | VASP | PRI | 2C19*2 | 73 | 600 | NS | PCI+SCAD | |
| Campo G et al [S3] | 2011 | PC | VN | PRI | 2C19*2, *17; 3A5*3; ABCB1 C3435T | 300 | 600 | 75 | PCI | |
| Chae H et al [S108] | 2013 | PC | VN | PRU | 2C19*2, *3, *17 | 56 | 600 | 75 | PCI | |
| Chan MY et al [S21] | 2012 | PC | VASP | PRI | 2C19*2, *3, *17; PON1 Q192R | 89 | 300 | 75 | SCAD | |
| Chen B et al [S80] | 2008 | PC | LTA5, 20 | | 2C19*2, *3 | 18 | 300 | 75 | HV | |
| Collet JP et al [S53] | 2011 | CROSS | LTA20, VN | RPA, PRU | 2C19*2 | 106 | 300 | 75 | PCI | CLOVIS-2 |
| Fontana P et al [S50] | 2008 | PC | VASP | PRI | 2C19*2 | 81 | 600 | 75 | PCI+ACS | |
| Fontana P et al [S18] | 2011 | PC | LTA20, VASP | MPA, PRI | 2C19*2; PON1 Q192R | 538 | NS | 75 | SCAD | ADRIE |

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|---|---|---|---|---|---|---|---|---|---|---|
| Frelinger AL et al [S1] | 2013 | CROSS | LTA5, 20; VASP; VN | MPA; PRI; PRU | 3A5*3; ABCB1 C3435T; PON1 rs662 | 156 | NA | 75 | HV | |
| Frere C et al [S46] | 2008 | PC | LTA10, FC62 10ADP; VASP | MPA; PRI | 2C19*2; 3A4*1B; 3A5*3 | 603 | 600 | NS | ACS | |
| Gajos G et al [S45] | 2012 | RCT | LTA5, 20 | MPA | 2C19*2 | 63 | NS | 75 | PCI+SCAD | OMEGA-PCI |
| Gladding P et al [S95] | 2008 | RCT | VN | PRU | 2C19*2,*4, *17; ABCB1 C1236T, C3435T, G2677T/A; 2C9*2, *3; P2Y12 H1/H2 | 60 | 600-1200 | 75-150 | PCI | PRINC |
| Gong IY et al [S93] | 2012 | PC | VN | PRU | PON1 Q192R | 21 | NA | 75 | HV | |
| Gremmel T et al [S8] | 2011 | PC | LTA10; VN; VASP; MP; Imp-R | MPA; PRU;PRI; AU; SC% | 2C9*2, *3 | 288 | 300-600 | 75 | PCI | |
| Gremmel T et al [S52] | 2012 | PC | LTA10; VN; VASP; MP; Imp-R | MPA; PRU;PRI; AU; SC% | 2C19*2, *3 | 288 | 300-600 | 75 | PCI | |
| Grosdidier C et al [S96] | 2013 | PC | VASP | PRI | 2C19*2, *17 | 730 | NS | 150 | ACS | |

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|---|---|---|---|---|---|---|---|---|---|---|
| Han Y et al [S122] | 2015 | PC | VN | PRU | 2C19*2, *3, *17 | 339 | NA | 75 | CVA | |
| Harmsze AM et al [S6] | 2010 | PC | LTA5, LTA 20, VN | MPA; PRU | 2C9*2, *3; 2C19*2,*3; 3A4*1B; 3A5*3; ABCB1 C3435T, G2677T/A, C1236T; P2Y1 A1622G | 428 | 300 | 75 | PCI | |
| Hulot JS et al [S47] | 2006 | PC | LTA10, VASP | MPA; PRI | 2C19*2; 3A5*3; 2B6*5; 1A2*1F | 29 | NA | 75 | HV | |
| Hulot JS et al [S20] | 2011 | PC | VN | PRU | 2C19*2; PON1 Q192R, L55M | 371 | VAR | 75 | ACS | AFIJI (CLOVIS-2 not included in extraction) |
| Hwang SJ et al [S42] | 2010 | PC | LTA5,20 | RPA, MPA | 2C19*2 | 134 | NS | 75-150 | PCI | ACCEL-POLYMORPHISM |
| Hwang SJ et al [S43] | 2011 | PC | LTA5,20; VN | MPA, PRU | 2C19*2, *3 | 190 | 300 | 75 | PCI+SCAD | |
| Jeong YH et al [S10] | 2011 | PC | LTA5, 20; VN | MPA; PRU | 2C19*2, *3, *17; ABCB1 C3435T | 266 | 600 | 75 | PCI | ACCEL-AMI |
| Jeong YH et al [S23] | 2010 | PC | LTA5, 20 | RPA, MPA | 2C19*2, *3; 3A5*3; ABCB1 C3435T | 126 | NS | 150 | PCI | ACCEL-DOUBLE |
| Jeong YH et al [S124] | 2012 | PC | LTA5; VN | MPA, PRU | 2C19*2/*3 | 47 | NA | 75 | NS | ACCEL-SWITCH |

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|---|---|---|---|---|---|---|---|---|---|---|
| Kaikita K et al [S86] | 2014 | PC | LTA20; VN; VASP | MPA; PRU, %inhib; PRI | 2C19*2, *3 | 104 | 300 | 75 | PCI+SCAD | CALDERA-PCI |
| Kang MK et al [S82] | 2010 | PC | LTA5,20 | MPA | 2C19*2, *3 | 176 | 300 | 75 | PCI | |
| Kassimis G et al [S7] | 2012 | PC | VN | PRU | 2C19*2, *17; 2C9*3; ABCB1 C3435T; P2Y12 | 146 | 600 | 75 | PCI | |
| Kim HS et al [S112] | 2013 | PC | VN | %inhib | 2C19*2, *3, *17 | 2188 | 300-600 | NS | SCAD+ACS | |
| Kim IS et al [S81] | 2009 | PC | LTA5, 20; VN | MPA; PRU, %inhib | 2C19*2+*3 | 136 | 600 | 75 | PCI+ACS | ACCEL cohort |
| Kim IS et al [S4] | 2012 | PC | LTA5, 20; VN | MPA, RPA; PRU | 2C19*2, *3; 3A5*3; ABCB1 C3435T | 127 | NS | 75 | PCI | ACCEL-2C19 |
| Konishi A et al [S119] | 2015 | PC | VN | PRU | 2C19*2/*3 | 196 | NS | 75 | PCI+SCAD+ACS | |
| Kreutz RP et al [S16] | 2012 | PC | LTA5, 10, 20; VN | MPA; PRU, %inhib | 2C19*2; PON1 Q192R | 151 | 600 | 75 | PCI+SCAD | |
| Kreutz RP et al [S29] | 2013 | PC | LTA5, 10, 20 | MPA | 2C19*2; 3A4*22; PPAR-a rs4253728, rs4823613; ARNT rs2134688 | 211 | 600 | 75 | PCI+SCAD | |
| Latkovskis G et al [S14] | 2014 | PC | VASP | PRI | 2C19*2, *3, *5, *17; 2C9*2, *3; ABCB1 C3435T | 93 | 300-600 | 75 | PCI+SCAD+ACS | |

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|---|---|---|---|---|---|---|---|---|---|
| Lee JB et al [S90] | 2011 | RC | VN | PRU, %inhib | 2C19*2/*3 | 166 | NA | 75 | CVA |
| Lee JM et al [S34] | 2009 | PC | VN | % inhib | 1A1 rs1048943; 1A2 rs2470890; 2J2 rs2280274; P2Y12 rs2046934; 2C19*2, *3; 3A4 rs2242480; 3A5 rs776746 | 387 | 300 | 75 | PCI |
| Li S et al [S76] | 2015 | PC | VN | PRU, %inhib | 2C19*2 | 198 | NS | 75 | PCI+SCAD+ACS |
| Li X et al [S17] | 2013 | PC | LTA20 | MPA, %inhib, %HPR | 2C19*2, PON1 Q192R | 180 | NS | 75 | ACS |
| Liang ZY et al [S11] | 2013 | PC | LTA20 | MPA, %HPR | 2C19*2, *3, *17; 3A4 rs2242480C>T, rs2404955G>A, rs2246709A>G, rs4646437C>T; 3A5 rs3800959T>C, 15524T>C; P2Y12 34C>T, 52G>T, 744T>C; ABCB1 C3435T | 1016 | 600 | 75 | PCI+ACS |
| Liu T et al [S114] | 2014 | PC | VASP | PRI | 2C19*2/*3 | 145 | 300 | 75 | PCI+SCAD |
| Liu XL et al [S44] | 2010 | PC | LTA5 | MPA | 2C19*2 | 722 | 300 | 75 | PCI+SCAD |
| Marcucci R et al [S48] | 2012 | PC | LTA10 | MPA | 2C19*2 | 1187 | 600 | 75 | PCI |

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|---|---|---|---|---|---|---|---|---|---|---|
| Mega JL et al [S41] | 2011 | RCT | VASP; VN | PRI; PRU | 2C19*2 | 333 | NS | 75, 150, 225, 300 | SCAD | ELEVATE-TIMI 56 |
| Miura G et al [S5] | 2014 | PC | VN | PRU, %inhib | 2B6*4, *6; 2C9*3; 2C19*2/*3; 3A5*3; PON1 Q192R; ABCB1 G2677A/T, C3435T; P2Y12 C742T, T2739C | 114 | NA | 75 | SCAD | |
| Nagashima Z et al [S91] | 2013 | PC | VN | PRU, %inhib, %HPR | 2C19*2/*3 | 177 | 300 | 75 | PCI+ACS | |
| Nakata T et al [S106] | 2013 | PC | LTA20; VASP; VN | %HPR; PRI; PRU | 2C19*2, *3 | 155 | 300 | 75 | PCI+SCAD | McLORDD |
| Nishio R et al [S92] | 2013 | PC | VN | PRU | 2C19*2, *3 | 112 | 300 | 75 | PCI | |
| Oestreich JL et al [S127] | 2014 | PC | VN | PRU | 2C19*2/*17 | 98 | NA | 75 | SCAD | |
| Oh et al [S54] | 2012 | PC | VN | PRU, %HPR | 2C19*2 | 2146 | 300-600 | 75 | PCI | SKY |
| Ono T et al [S84] | 2011 | PC | LTA20; VN | MPA; PRU | 2C19*2, *3 | 202 | 300 | 75 | SCAD | |
| Palmerini T et al [S15] | 2014 | PC | VASP | PRI | 2C19*2, *3, *4, *17; 3A4*1G, *1B; 3A5*3; ABCB1 C3435T; IRS1 A227497991G, G227382808C; | 750 | 300-600 | 75 | PCI+ACS | GEPRESS |

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|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | PON1 L55M, Q192R; ITGB3 T196C | | | | | |
| Park JJ et al [S13] | 2013 | PC | VN | PRU | 2C19*2, *3, *17; 1A2*1F; 2B6*6; 3A4 IVS10+12; 3A5*3; PON1 Q192R; ABCB1 C3435T | 1264 | 300-600 | 75 | PCI | CROSS-VERIFY |
| Park JJ et al [S105] | 2013 | PC | VN | PRU | 2C19*2; 3A4 IVS 10 | 1247 | 300-600 | 75 | PCI | CROSS-VERIFY |
| Park KW et al [S2] | 2010 | PC | VN | PRU | 1A2*1F; 2B6*4; 2C19*2, *3, *17; 3A4 rs2242480; 3A5*3; ABCB1 C3435T | 1123 | 300-600 | 75 | PCI | CROSS-VERIFY |
| Park KW et al [S89] | 2011 | RCT | VN | PRU | 2C19*2, *3, *17 | 474 | NS | 75 | PCI | CILON-T |
| Park Y et al [S12] | 2014 | PC | LTA20; VASP | MPA, RPA; PRI | 2C19*2/*3; PON1 Q192R; ABCB1 C3435T; 3A5*3 | 50 | NA | 75 | SCAD | ACCEL-PARAZOL |
| Peace AJ et al [S30] | 2014 | PC | VN | PRU, %inhib | 2C19*2; a2AR rs553668 | 141 | 300 | 75 | PCI+SCAD | |
| Pettersen AAR et al [S68] | 2011 | PC | VASP, VN | PRI, PRU | 2C19*2 | 219 | NA | 75 | SCAD | ASCET |
| Price MJ et al [S94] | 2012 | RCT | VN | Res N | 2C19*2, *17; 2B6*1B, *1C, *9, *6; ABCB1 C3435T; PEAR1; ITGB3; VAV3 | 1170 | 600 | 75, 150 | PCI+SCAD+ACS | GRAVITAS / GIFT |

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|---|---|---|---|---|---|---|---|---|---|---|
| Rideg O et al [S9] | 2011 | RCT | LTA5; VASP | MPA, RPA; PRI | 2C19*2, *3, *17; ABCB1 C3435T, G2677T/A; PON1 Q192R | 189 | 600 | 75-150 | PCI+SCAD | DOSER |
| Roberts JD et al [S55] | 2012 | PC | VN | PRU | 2C19*2 | 187 | 600 | 75 | PCI | RAPID GENE |
| Rossi JS et al [S71] | 2014 | PC | VN | PRU | 2C19*2, *17 | 211 | NA | 75 | SCAD | |
| Sani YN et al [S38] | 2013 | PC | VN | PRU, %HPR | 2C19*2 | 45 | 300 | NA | HV | |
| Shuldiner A et al [S49] | 2009 | PC | LTA 20 | MPA | 2C19*2 | 429 | 300 | 75 | HV | AMISH-PAPI |
| Sibbing D et al [S37] | 2011 | PC | MP | AU*min | 2C19*2, PON1 Q192R | 1524 | 600 | 75 | PCI | |
| Simon T et al [S35] | 2011 | PC | LTA5, 20; VASP | MPA, RPA;PRI | 2C19*2, *3, *17 | 337 | 300-600 | 75-150 | HV | |
| Tang N et al [S120] | 2015 | PC | LTA5; VASP; TEG | MPA; PRI; MA | 2C19*2/*3 | 178 | 300 | 75 | PCI+ACS | |
| Tang XF et al [S147] | 2013 | PC | TEG | % Agg, % HPR | 2C19*2; P2Y12 C34T | 577 | 300 | 75 | PCI | |
| Tousoulis D et al [S56] | 2013 | PC | VN | PRU, %HPR | 2C19*2 | 353 | NA | 75 | SCAD | |

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|---|---|---|---|---|---|---|---|---|---|
| Tsantes AE et al [S39] | 2013 | PC | LTA20; VASP; PFAC; MP | MPA; PRI; %HPR; AU*min | 2C19*2 | 95 | NS | 75 | ACS+SCAD |
| Umemura K et al [S87] | 2008 | PC | LTA20, VASP | MPA; PRI | 2C19*2, *3 | 47 | 300 | NA | HV |
| Xie C et al [S33] | 2014 | PC | VASP | PRI | 2C19*2/*3; CES1A2 -816 A/C | 162 | 300-600 | 75 | PCI+SCAD+ACS |
| Zhang HZ et al [S83] | 2014 | PC | LTA5; VN; MP | MPA; PRU; AU*min | 2C19*2, *3, *17 | 244 | NA | 75 | SCAD |
| Zhang L et al [S22] | 2013 | PC | LTA20 | MPA, %HPR | 2C19*2, *3, *17; ABCB1 C3435T; PON1 Q192R | 520 | 300 | 75 | ACS |
| Zhang S et al [S73] | 2014 | PC | VASP | PRI | 2C19*2, *3 | 95 | 300 | 75 | CVA |
| Zou JJ et al [S85] | 2013 | PC | LTA20 | MPA | 2C19*2, *3 | 617 | 300 | 75 | PCI+SCAD |

**Table 2.3 - Characteristics of the studies included in meta-analysis** (For all S references, please refer to Appendix 1).

| MA | PD TEST | MEASURE | GENE | STUDIES COMBINED | REFERENCES | N | Std Mean Diff | 95% CI | P Value | I^2 |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | LTA5 | MPA | 2C19*2 | 6 | S16; S29; S42; S43; S44; S45 | 1455 | -0.41 | -0.61 to -0.20 | <0.0001 | 59 |
| **2** | LTA5 | MPA | 2C19*2&*3 | 8 | S9; S35; S80; S81; S82; S83; S12; S124 | 1190 | -1.16 | -2.07 to -0.25 | 0.01 | 98 |
| **3** | LTA5 | MPA | ABCB1 C3435T | 4 | S1; S6; S9; S10 | 1031 | 0.05 | -0.09 to 0.19 | 0.52 | 0 |
| **4** | LTA5 | MPA | PON1 Q192R | 3 | S1; S9; S16 | 496 | -0.10 | -0.29 to 0.08 | 0.28 | 0 |
| **5** | LTA10 | MPA | 2C19*2 | 4 | S16; S46; S47; S48 | 1967 | -0.92 | -1.65 to -0.18 | 0.01 | 97 |
| **6** | LTA20 | MPA | 2C19*2&*3 | 7 | S11; S12; S22; S81; S82; S84; S85 | 2697 | -0.51 | -0.61 to -0.41 | <0.00001 | 29 |
| **7** | LTA20 | MPA | 2C19*2 | 9 | S10; S11; S16; S17; S22; S29; S39; S45; S49 | 2832 | -1.02 | -1.76 to -0.28 | <0.00001 | 99 |
| **8** | LTA20 | MPA | 2C19*3 | 4 | S10; S11; S22; S43 | 1840 | -0.53 | -0.83 to -0.23 | 0.0006 | 68 |

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|---|---|---|---|---|---|---|---|---|---|---|
| **9** | LTA20 | MPA | ABCB1 C3435T | 4 | S1; S6; S11; S12 | 1650 | 0.04 | -0.07 to 0.14 | 0.50 | 0 |
| **10** | LTA20 | MPA | PON1 Q192R | 4 | S1; S12; S16; S17 | 537 | -0.14 | -0.34 to 0.07 | 0.20 | 0 |
| **11** | MP | | 2C19*2 | 2 | S37; S39 | 1619 | -0.35 | -0.46 to -0.24 | <0.00001 | 0 |
| **12** | VASP | PRI | 2C19*2&*3 | 8 | S9; S12; S33; S35; S86; S87; S114; S120 | 809 | -0.99 | -1.36 to -0.61 | <0.00001 | 82 |
| **13** | VASP | PRI | 2C19*2 | 13 | S14; S15; S18; S19; S39; S40; S41; S46; S47; S50; S51; S52; S73 | 3833 | -1.35 | -2.11 to -0.60 | 0.0005 | 99 |
| **14** | VASP | PRI | ABCB1 C3435T | 5 | S1; S9; S12; S14; S15 | 1196 | -0.15 | -0.28 to -0.02 | 0.03 | 0 |
| **15** | VASP | PRI | PON1 Q192R | 7 | S1; S9; S12; S15; S18; S19; S21 | 2232 | -0.07 | -0.15 to 0.02 | 0.14 | 0 |
| **16** | VN | %inhib | 2C19*2&*3 | 6 | S5; S23; S81; S84; S90; S91 | 921 | 0.84 | 0.68 to 1.00 | <0.00001 | 23 |

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|---|---|---|---|---|---|---|---|---|---|---|
| **17** | VN | PRU | 2C19*2&*3 | 12 | S4; S5; S13; S81; S84; S89; S90; S91; S92; S119; S122; S124 | 3007 | -1.18 | -1.66 to 0.70 | < 0.00001 | 97 |
| **18** | VN | %inhib | 2C19*2 | 5 | S16; S30; S34; S75; S76 | 990 | 0.56 | 0.31 to 0.80 | <0.00001 | 68 |
| **19** | VN | PRU | 2C19*2 | 17 | S2; S3; S7; S10; S16; S30; S38; S41; S43; S52; S53; S54; S55; S56; S60; S71; S76 | 6038 | -0.67 | -0.95 to -0.40 | <0.00001 | 95 |
| **20** | VN | PRU | 2C19*3 | 3 | S2; S10; S43 | 1436 | -0.34 | -0.48 to -0.20 | < 0.00001 | 0 |
| **21** | VN | PRU | 2C9*3 | 4 | S5; S6; S7; S8 | 909 | 0.14 | -0.15 to 0.43 | 0.35 | 39 |
| **22** | VN | PRU | 3A5*3 | 5 | S1; S2; S3; S4; S5 | 1802 | -0.16 | -0.38 to 0.06 | 0.16 | 0 |

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|---|---|---|---|---|---|---|---|---|---|---|
| **23** | VN | PRU | ABCB1 C3435T | 7 | S1; S3; S5; S6; S7; S10; S13 | 2674 | -0.04 | -0.14 to 0.06 | 0.48 | 16 |
| **24** | VN | PRU | PON1 Q192R | 5 | S1; S5; S13; S16; S20 | 2056 | -0.04 | -0.16 to 0.08 | 0.49 | 0 |
| **25** | VN | PRU | 2C19*17 | 6 | S2; S3; S7; S71; S122; S127 | 1813 | 0.25 | -0.13 to 0.64 | 0.20 | 77 |

**Table 2.4 – Summary of all meta-analyses** (For all S references, please refer to Appendix 1).

### 2.3.2: *CYP2C19*2* and platelet reactivity

A total of 46 studies with 16,808 participants investigated the association between the *CYP2C19*2* polymorphism and platelet reactivity. Meta-analyses were prepared when more than two studies contributed data. A clear association with higher platelet reactivity was demonstrated across all platelet function tests investigated with the carriage of the variant *2 allele.

For LTA 5 µmol/L ADP, a meta-analysis of six studies (Hwang et al., 2010, Hwang et al., 2011a, Gajos et al., 2012, Kreutz et al., 2012, Kreutz et al., 2013, Liu et al., 2010) with 1455 participants demonstrated a strong association between carriage of the variant *2 allele and higher platelet reactivity as measured by MPA (Std Mean Difference -0.41, 95% CI -0.61 to -0.20; P<0.0001) although there was evidence of heterogeneity between the individual studies (I^2: 59%) (**Figure 2.2**).

Four studies (Frere et al., 2008, Hulot et al., 2006, Kreutz et al., 2012, Marcucci et al., 2012) of 1967 subjects demonstrated a significant association between carriage of the variant *2 allele and higher platelet aggregation as measured by LTA 10 µmol/L MPA (Std Mean Difference – 0.92, 95% CI -1.65 to -0.18; P-0.01). However, there was clear evidence of significant heterogeneity between the individual studies (I^2: 97%) (**Figure 2.3**).

Similarly, a clear association was demonstrated between carriage of the *2 allele and platelet aggregation defined by LTA 20 µmol/L ADP MPA in a meta-analysis of 9 studies and 2832 participants (Gajos et al., 2012, Jeong et al., 2011, Kreutz et al., 2012, Kreutz et al., 2013, Li et al., 2013a, Liang et al., 2013, Shuldiner et al., 2009, Tsantes et al., 2013, Zhang et al., 2013) (Std Mean Difference -1.02, 95% CI -1.76 to -0.28; P <0.00001). Significant heterogeneity was evident with an I^2 value of 99% (**Figure 2.4a**). However, when only studies with patients with unstable cardiovascular disease were combined (5 studies, 2023 participants (Jeong et al., 2013, Li et al., 2013a, Liang et al., 2013, Tsantes et al., 2013, Zhang et al., 2013)), the observed heterogeneity was removed with a significant association between higher platelet reactivity and carriage of the *2 allele still demonstrated (Std Mean Difference: -0.44, 95% CI: -0.53 to -0.35; P <0.00001) (**Figure 2.4b**).

Thirteen studies of 3833 subjects demonstrated a significant association between carriage of the *2 allele and higher platelet aggregation as defined by VASP (Std mean difference -1.35, 95% CI -2.11 to -0.60; P=0.0005) although there was significant inter-study

heterogeneity (I^2: 99%) (Bonello et al., 2010a, Bonello et al., 2012, Bonello-Palot et al., 2009, Fontana et al., 2008, Fontana et al., 2011, Frere et al., 2008, Gremmel et al., 2012, Hulot et al., 2006, Latkovskis et al., 2014, Mega et al., 2011, Palmerini et al., 2014, Tsantes et al., 2013, Zhang et al., 2014c) (**Figure 2.5**). Despite analysing separately for stable cardiovascular disease, clopidogrel loading dose, time post loading dose of clopidogrel and assessment of Hardy-Weinberg equilibrium (HWE), the degree of heterogeneity remained significant.

Finally, a meta-analysis of 17 studies and 6038 subjects demonstrated a significant association between higher VerifyNow defined platelet aggregation and the *2 allele (Std mean difference -0.67, 95% CI -0.95 to -0.40; P<0.00001) (Alexopoulos et al., 2011b, Campo et al., 2011, Collet et al., 2011, Gremmel et al., 2012, Hwang et al., 2011a, Jeong et al., 2011, Kassimis et al., 2012, Kreutz et al., 2012, Li et al., 2015b, Mega et al., 2011, Nasyuhana Sani et al., 2013, Peace et al., 2014, Park et al., 2011a, Roberts et al., 2012, Rossi et al., 2014, Oh et al., 2012, Tousoulis et al., 2013) (**Figure 2.6**). However, similar to the previous meta-analyses, there was considerable heterogeneity between the individual studies (I^2: 96%), with no change in the I^2 value following removal of stable patient studies from the meta-analysis. There was considerable variation in clopidogrel loading doses and duration of clopidogrel treatment prior to platelet function testing which probably contributed to the observed heterogeneity. In addition, several studies did not assess for HWE or perform quality control steps for genotyping. As described in the meta-analyses above, despite analysing separately for clopidogrel loading dose, time between clopidogrel loading and platelet function testing and assessment of HWE, the degree of heterogeneity remained significant.

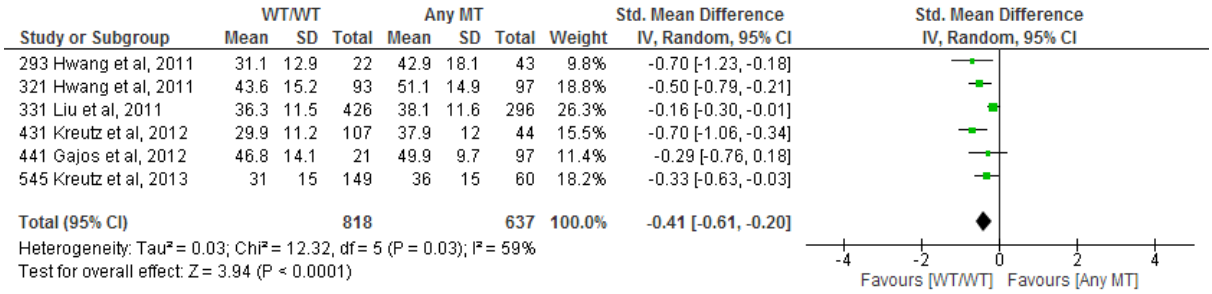


**Figure 2.2 – LTA 5 µmol/L ADP and CYP2C19*2**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

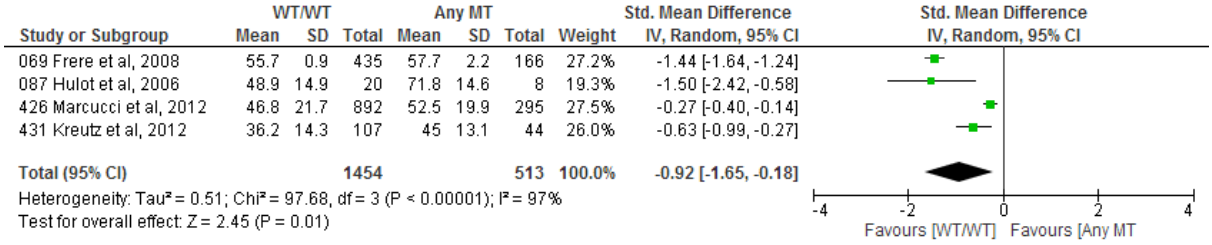


**Figure 2.3 – LTA 10 µmol/L ADP and CYP2C19*2**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

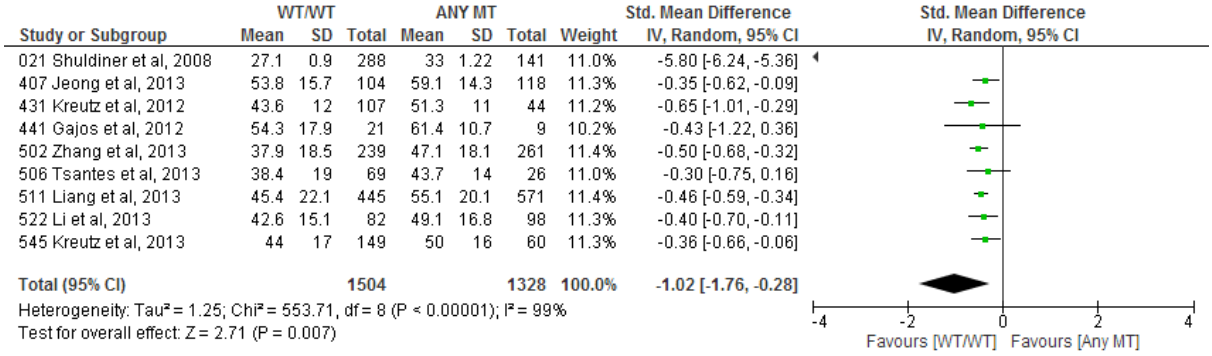


**Figure 2.4a – LTA 20 µmol/L ADP and CYP2C19*2**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

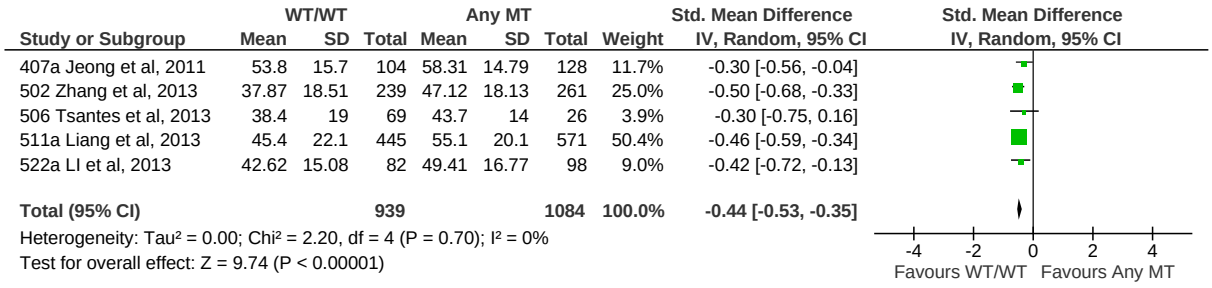


**Figure 2.4b – LTA 20 µmol/L ADP and CYP2C19*2 (Unstable patients only)**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

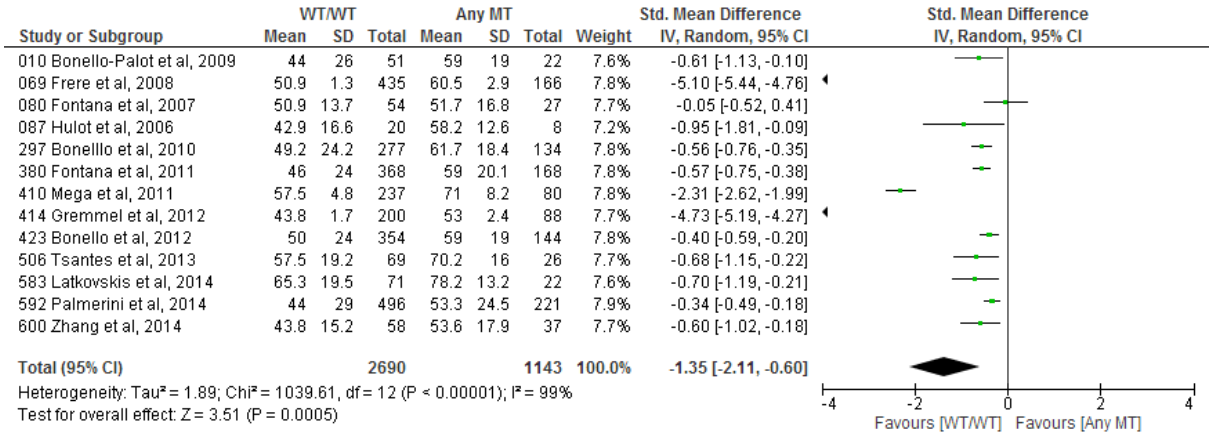


**Figure 2.5 – VASP and CYP2C19*2**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

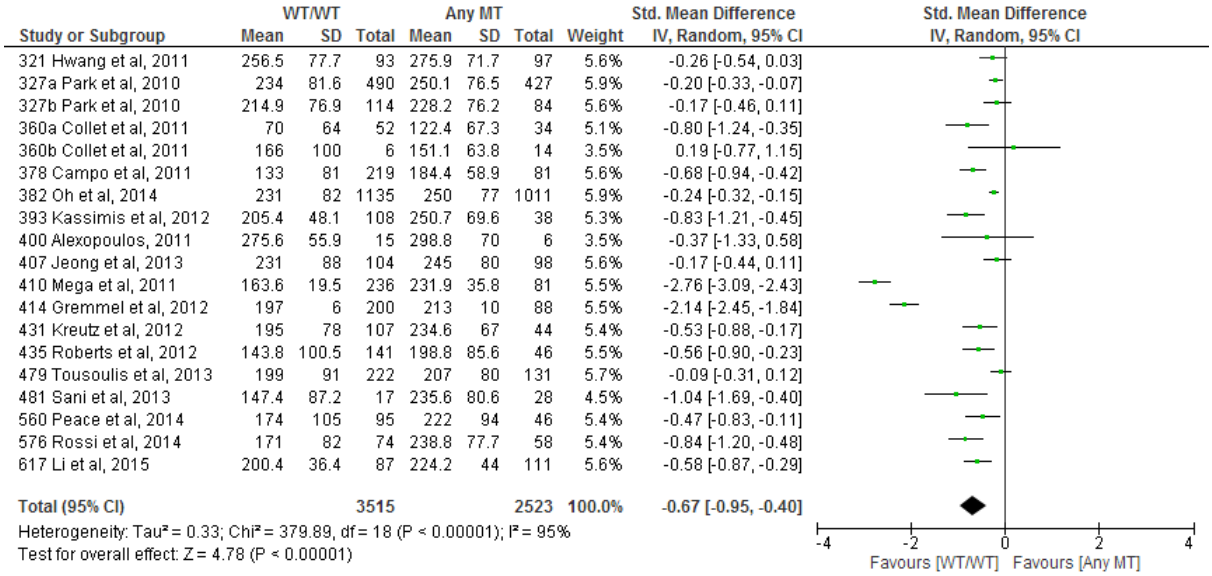


**Figure 2.6 – VerifyNow and CYP2C19*2**
WT homozygotes have significantly lower platelet reactivity than *2 carriers

### 2.3.3: *CYP2C19* metaboliser phenotype and platelet reactivity

A total of 36 studies involving 9524 participants investigated the association between the combined *CYP2C19* loss of function variants (*2 and *3 alleles) and platelet aggregation. A significant association was demonstrated between carriage of the loss of function variants and each of the pharmacodynamic tests investigated in the individual meta-analyses.

A meta-analysis of eight studies and 1190 participants (Chen et al., 2008, Jeong et al., 2012, Kang et al., 2010, Kim et al., 2009, Rideg et al., 2011, Simon et al., 2011a, Tang et al., 2015, Zhang et al., 2014a) demonstrated a significant association between higher LTA 5 µmol/L ADP defined platelet reactivity and carriage of the LOF variant (Std Mean Difference -1.16, 95% CI -2.07 to -0.25; P=0.01) (**Figure 2.7**). Similarly, for LTA 20 µmol/L ADP, in a meta-analysis of 7 studies and 2967 participants, a significant association with higher platelet reactivity was demonstrated with the LOF variants (Std Mean Difference -0.51, 95% CI -0.61 to -0.41; P<0.00001) with a relatively low heterogeneity between the studies (I^2: 29%) (Kim et al., 2009, Kang et al., 2010, Liang et al., 2013, Ono et al., 2011, Park et al., 2014, Zhang et al., 2013, Zou et al., 2013) (**Figure 2.8**). Eight studies with 809 participants were combined to assess the association between VASP defined platelet aggregation and LOF variants, with a significant association with higher platelet reactivity observed (Std Mean Difference -0.99, 95% CI -1.36 to -0.61; P<0.00001) (Kaikita et al., 2014, Liu et al., 2014, Park et al., 2014, Rideg et al., 2011, Simon et al., 2011a, Tang et al., 2015, Umemura et al., 2008, Xie et al., 2014) (**Figure 2.9**). Finally a significant association was also observed between higher VerifyNow defined platelet reactivity and LOF allele carriage in a meta-analysis of twelve studies and 3007 participants (Standard Mean Difference -1.18, 95% CI -1.66 to -0.70; P<0.00001) (Han et al., 2015, Jeong et al., 2012, Kim et al., 2009, Kim et al., 2012, Konishi et al., 2015, Lee et al., 2011, Miura et al., 2014, Nagashima et al., 2013, Nishio et al., 2013, Ono et al., 2011, Park et al., 2011b, Park et al., 2013b) (**Figure 2.10**).

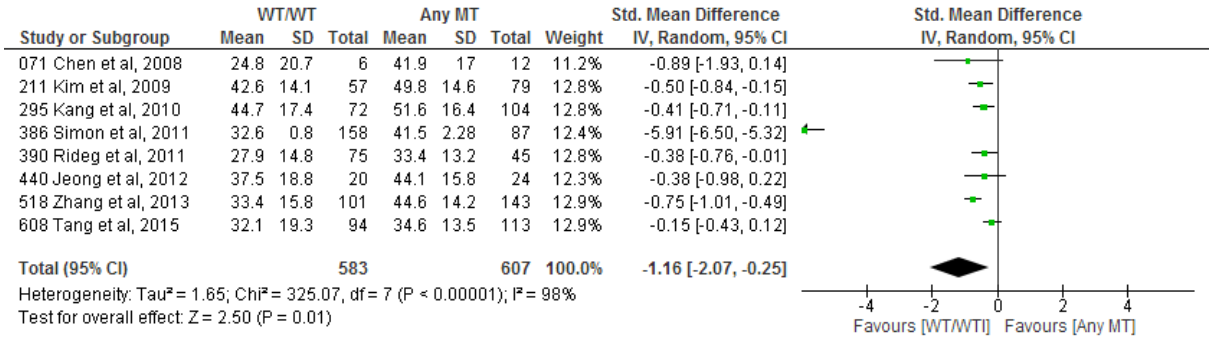


**Figure 2.7 – LTA 5 µmol/L ADP and CYP2C19 Metaboliser Phenotype (*2 and *3 alleles combined)**
WT homozygotes have significantly lower platelet reactivity than carriers of loss-of-function alleles

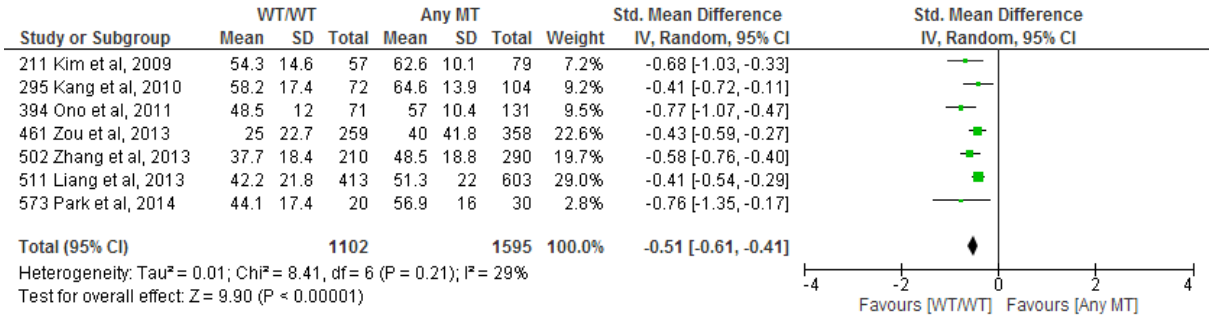


**Figure 2.8 – LTA 20 µmol/L ADP and CYP2C19 Metaboliser Phenotype (*2 and *3 alleles combined)**
WT homozygotes have significantly lower platelet reactivity than carriers of loss-of-function alleles

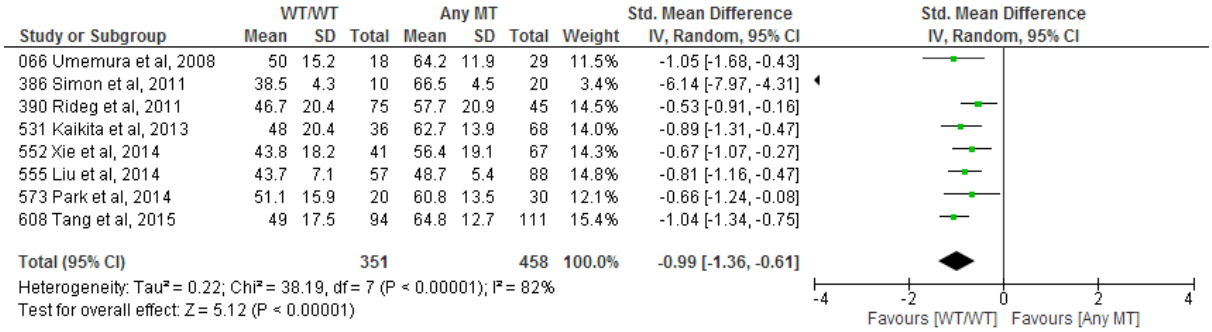


**Figure 2.9 – VASP and CYP2C19 Metaboliser Phenotype (*2 and *3 alleles combined)**
WT homozygotes have significantly lower platelet reactivity than carriers of loss-of-function alleles

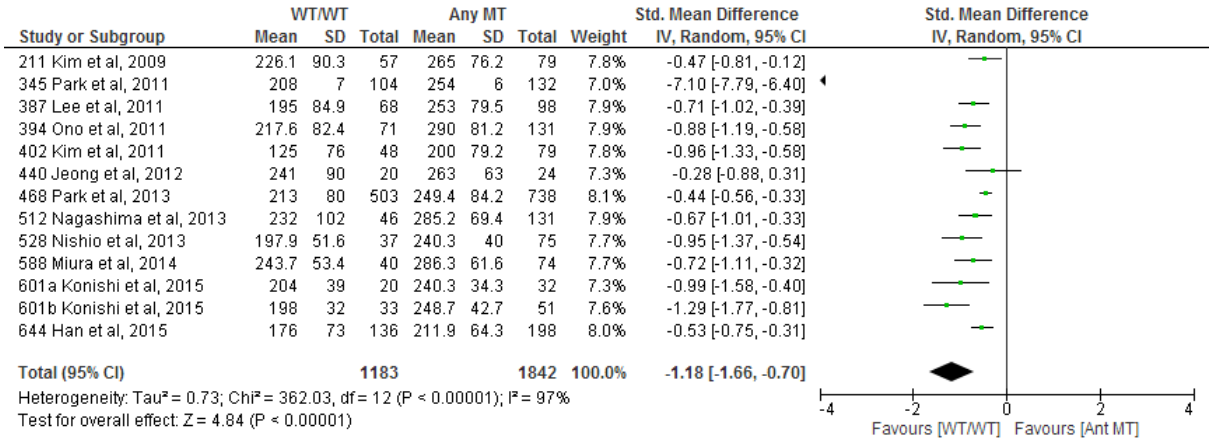


**Figure 2.10 – VerifyNow and CYP2C19 Metaboliser Phenotype (*2 and *3 alleles combined)**

WT homozygotes have significantly lower platelet reactivity than carriers of loss-of-function alleles

### 2.3.4: Other *CYP2C19* variants and platelet reactivity

In addition to the association with *CYP2C19*2*, there is a significant association between the *CYP2C19*3* loss-of-function variant and higher platelet aggregation defined by VerifyNow (3 studies, 1436 participants, Standard Mean Difference -0.34, 95% CI -0.48 to -0.20; P<0.00001 (Hwang et al., 2010, Jeong et al., 2011, Park et al., 2011a)) and LTA 5 µmol/L ADP (2 studies, 324 participants, Standard Mean Difference -0.41, 95% CI -0.72 to -0.09; P=0.01 (Hwang et al., 2010, Jeong et al., 2011)) (**Figures 2.11 and 2.12**). Interestingly, there was no clear association between the gain-of-function variant, CYP2C19*17, and VerifyNow defined platelet aggregation (6 studies, 1813 participants, Standard Mean Difference 0.25, 95% CI -0.13 to 0.64; P=0.25 (Campo et al., 2011, Han et al., 2015, Kassimis et al., 2012, Park et al., 2011a, Oestreich et al., 2014, Rossi et al., 2014)) (**Figure 2.13**).

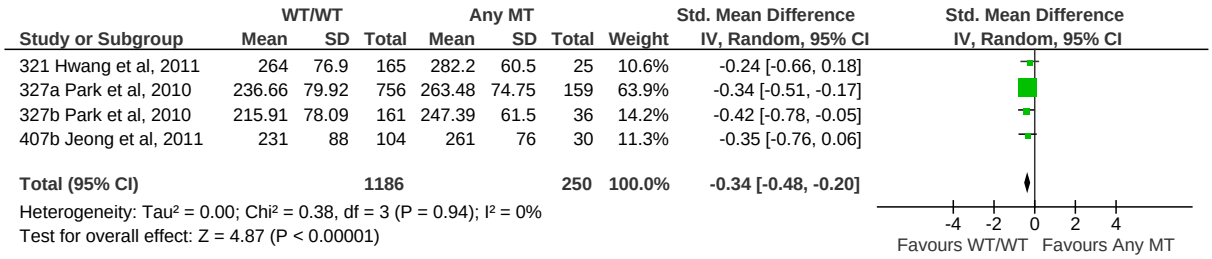


**Figure 2.11 – VerifyNow and CYP2C19*3**
WT homozygotes have significantly lower platelet reactivity than carriers of loss-of-function alleles

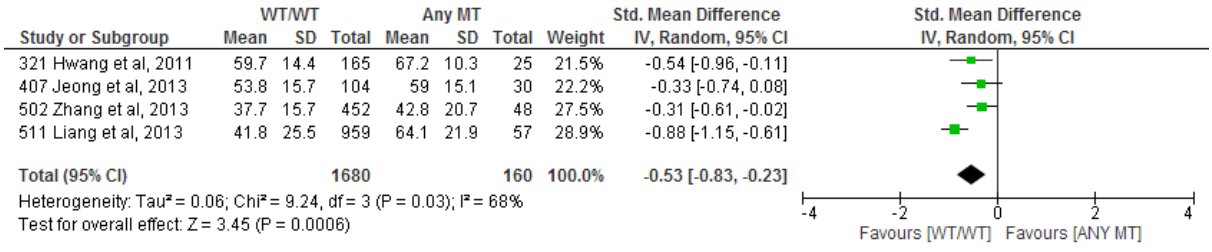


**Figure 2.12 – LTA 20 µmol/L and CYP2C19*3**
WT homozygotes have significantly lower platelet reactivity than carriers of the *3 allele

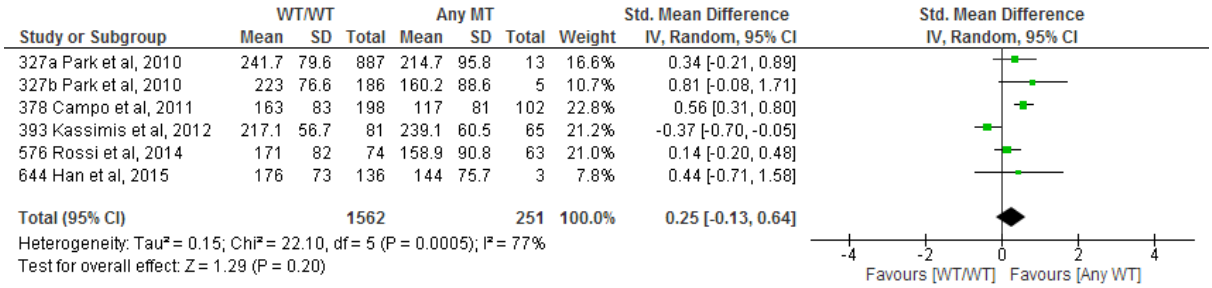


**Figure 2.13 – VerifyNow and CYP2C19*17**
No significant association detected between carriage of the *17, gain-of-function, allele and platelet reactivity

### 2.3.5: *CYP3A5*3* variants and platelet reactivity

In a meta-analysis of five studies and 1802 participants (Campo et al., 2011, Frelinger et al., 2013, Kim et al., 2012, Miura et al., 2014), there was no clear association between *CYP3A5* genotype and VerifyNow defined platelet aggregation (Standard Mean Difference -0.16, 95% CI -0.38 to 0.06; P=0.16). There was no observed heterogeneity between the included studies (I^2: 0%) (**Figure 2.14**).

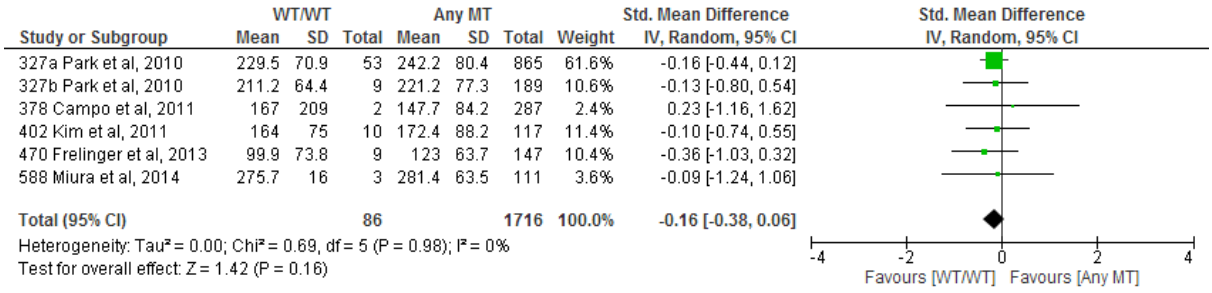


**Figure 2.14 – VerifyNow and CYP3A5*3**
No significant association detected between carriage of the CYP3A5*3 allele and platelet reactivity

### 2.3.6: *CYP2C9*3* variants and platelet reactivity

In a meta-analysis of four studies and 909 participants (Gremmel et al., 2013, Harmsze et al., 2010a, Kassimis et al., 2012, Miura et al., 2014) there was no clear association between carriage of the *CYP2C9*3* allele and VerifyNow defined platelet aggregation (Standard Mean Difference 0.14, 95% CI -0.15 to 0.43; P=0.35) (**Figure 2.15**).

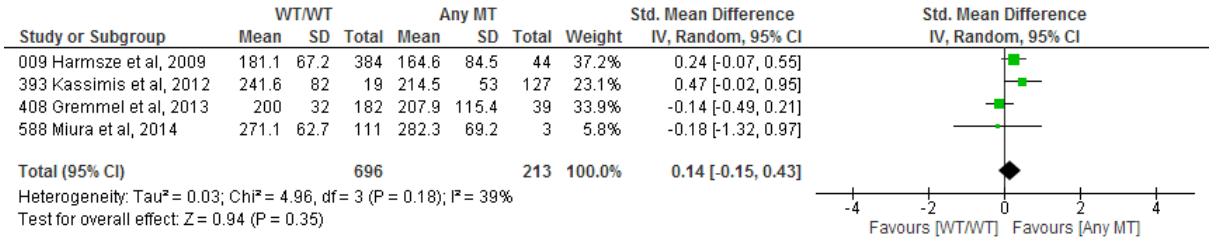


**Figure 2.15 – VerifyNow and CYP2C9*3**
No significant association detected between carriage of the CYP2C9*3 allele and platelet reactivity

### 2.3.7: *ABCB1* C3435T and platelet reactivity

In a meta-analysis of 4 studies and 1031 participants (Frelinger et al., 2013, Harmsze et al., 2010a, Jeong et al., 2011, Rideg et al., 2011) there was no association between LTA 5 µmol/L ADP defined platelet reactivity and *ABCB1* C3435T genotype (Standard Mean Difference 0.05, 95% CI: -0.09 to 0.19, P=0.52) with no heterogeneity detected (**Figure 2.16**). This was replicated with LTA 20 µmol/L ADP (4 studies, 1650 participants, Standard Mean Difference 0.04, 95% CI -0.07 to 0.14; P=0.50 (Frelinger et al., 2013, Harmsze et al., 2010a, Liang et al., 2013, Park et al., 2014)) and VerifyNow (7 studies, 2674 participants, Standard Mean Difference -0.04, 95% CI -0.14 to 0.08; P=0.48 (Campo et al., 2011, Frelinger et al., 2013, Harmsze et al., 2010a, Jeong et al., 2011, Kassimis et al., 2012, Miura et al., 2014, Park et al., 2013b)) (**Figures 2.17 and 2.18**). However, a significant association was detected between higher platelet reactivity and carriage of the T allele using VASP in five studies with 1196 participants (Standard Mean Difference -0.15, 95% CI -0.28 to -0.02; P=0.03 (Frelinger et al., 2013, Latkovskis et al., 2014, Palmerini et al., 2014, Park et al., 2014, Rideg et al., 2011)). The relevance of this finding is unclear given the small magnitude of effect and results from studies using different methodologies for assessment of platelet reactivity (**Figure 2.19**).

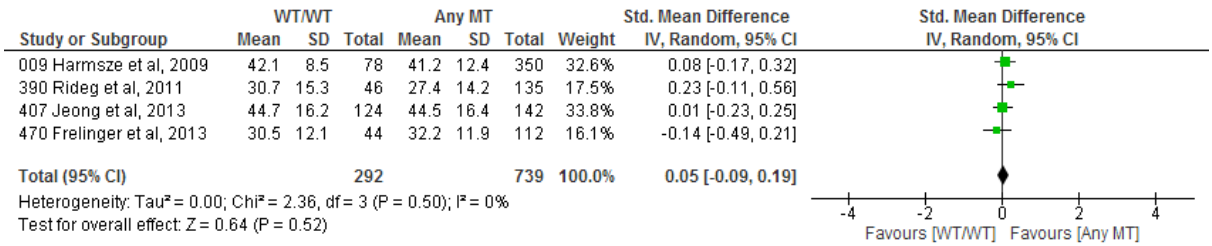


**Figure 2.16 – LTA 5 µmol/L ADP and ABCB1 C3435T**
No significant association detected between carriage pf the ABCB1 3435T allele and platelet reactivity

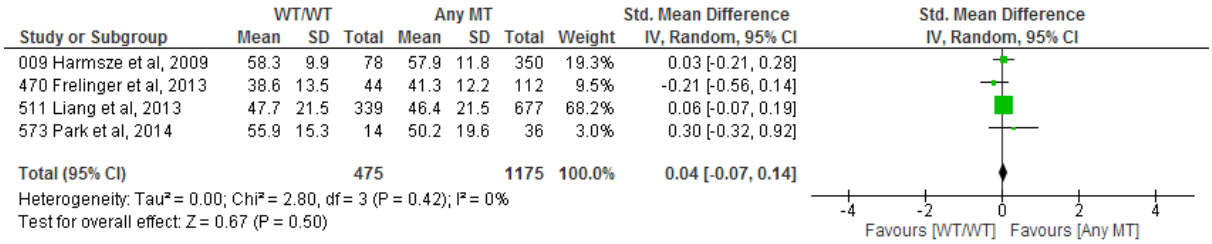


**Figure 2.17 – LTA 20 µmol/L ADP and ABCB1 C3435T**
No significant association detected between carriage of the ABCB1 3435T allele and platelet reactivity

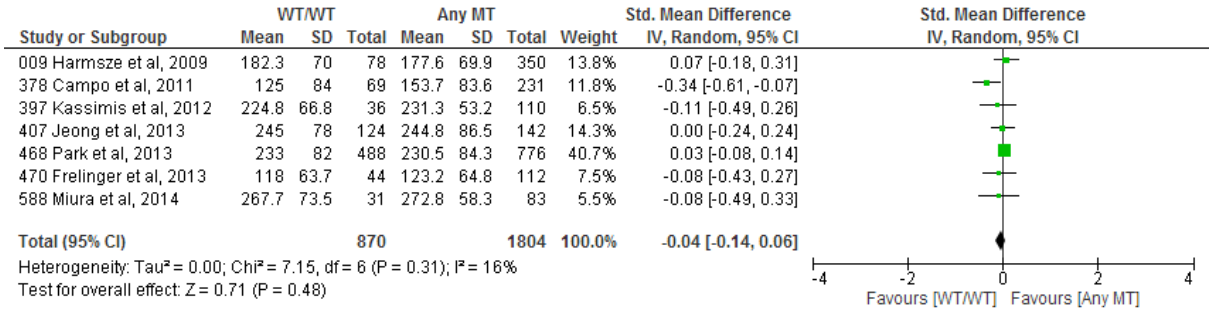


**Figure 2.18 – VerifyNow and ABCB1 C3435T**
No significant association detected between carriage of the ABCB1 3435T allele and platelet reactivity

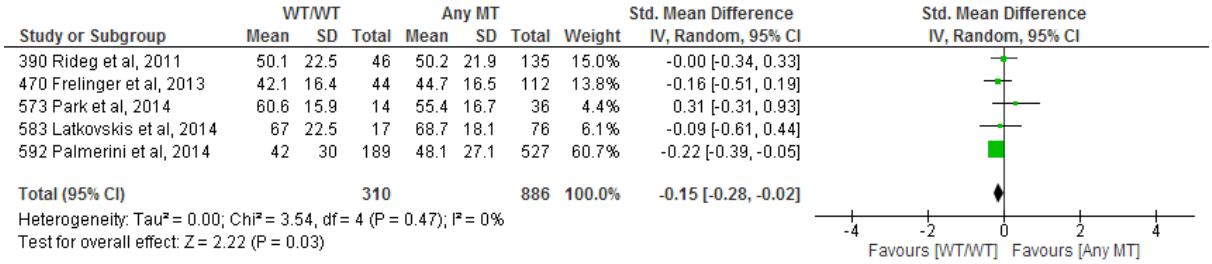


**Figure 2.19 – VASP and ABCB1 C3435T**
ABCB1 3435C homozygotes have significantly lower platelet reactivity than carriers of the ABCB1 3435T allele

### 2.3.8: *PON1* Q192R and platelet reactivity

In a meta-analysis of 4 studies and 537 participants, there was no association between *PON1* Q192R genotype and LTA 20 µmol/L ADP defined platelet aggregation (Standard Mean Difference -0.14, 95% CI -0.34 to 0.07; P=0.20 (Frelinger et al., 2013, Kreutz et al., 2012, Li et al., 2013a, Park et al., 2014)). This was mirrored by meta-analyses using VASP and VerifyNow as outcomes with no significant association demonstrated (Bonello et al., 2012, Chan et al., 2012, Fontana et al., 2011, Frelinger et al., 2013, Kreutz et al., 2012, Miura et al., 2014, Park et al., 2013b, Park et al., 2014, Hulot et al., 2011, Rideg et al., 2011) (**Figures 2.20-2.22**). There was no observed heterogeneity in any of the meta-analyses.

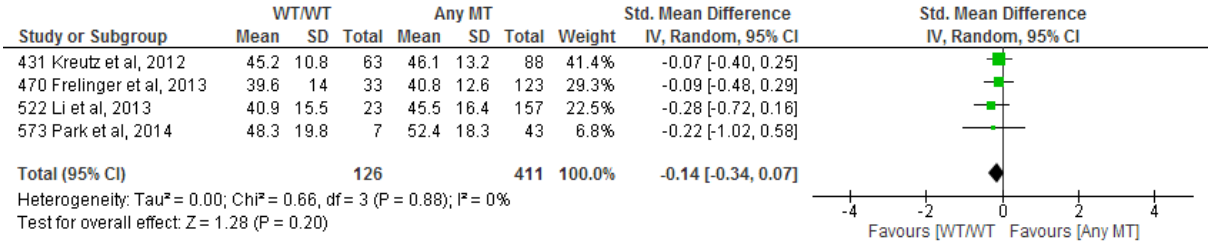


**Figure 2.20 – LTA 20 µmol/L ADP and PON1 Q192R**
No significant association detected between carriage of the PON1 192Q allele and platelet reactivity

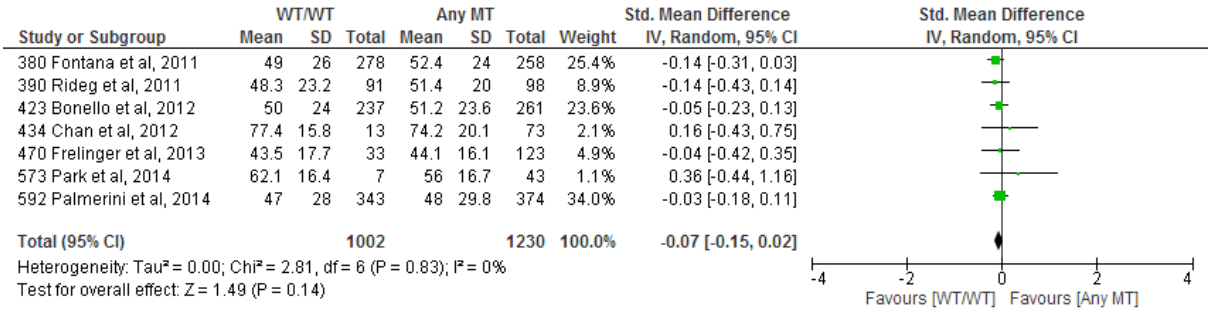


**Figure 2.21 – VASP and PON1 Q192R**
No significant association detected between carriage of the PON1 192Q allele and platelet reactivity

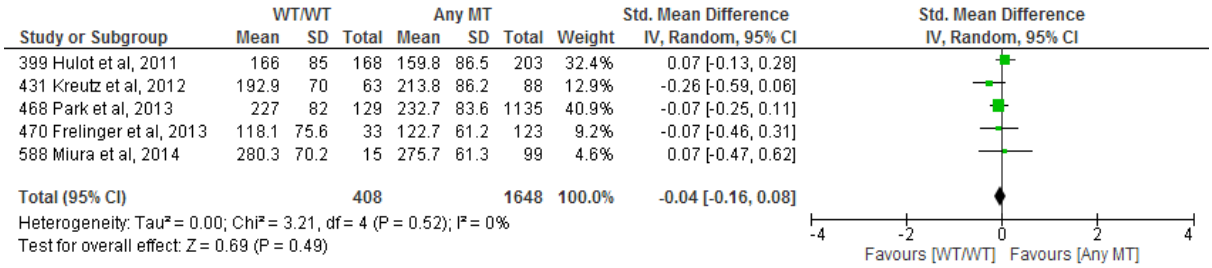


**Figure 2.22 – VerifyNow and PON1 Q192R**

No significant association detected between carriage of the PON1 192Q allele and platelet reactivity

## 2.4: Discussion

The major finding of this meta-analysis is that the *CYP2C19* loss-of-function is consistently associated with higher platelet reactivity across a range of platelet function tests including LTA, VASP and VerifyNow.

The observed association of higher platelet reactivity in *CYP2C19*2* carriers is consistent with a number of published clinical outcome studies that demonstrate an increase in the risk of adverse cardiovascular events in the presence of *CYP2C19* variant alleles. In a collaborative meta-analysis of nine studies and 9685 patients, Mega et al (Mega et al., 2010b) identified a 55% increase in the risk of adverse cardiovascular events (defined as cardiovascular death, MI and ischaemic stroke) in patients carrying the *CYP2C19* variant alleles. This risk is highest in *CYP2C19* LOF variant homozygotes (Overall HR 1.76; 95% CI 1.24-2.50) and lowest in heterozygotes (Overall HR 1.55; 95% CI 1.11-2.17), in keeping with an additive inheritance model. Stent thrombosis, an exquisitely platelet sensitive outcome, showed an even greater association with *CYP2C19* LOF variants, with an overall nearly threefold increase in risk in carriers of the variant alleles. In keeping with the composite outcome, this risk was also higher in variant homozygotes compared to heterozygotes. Importantly, it appears that carriage of the *CYP2C19* LOF allele predicts early events, with significant associations between LOF allele carriage and outcome only being demonstrable in the first 30 days after an event. These data are in keeping with the known complex interplay between ACS, inflammation and anti-platelet drugs as discussed earlier. However, a larger meta-analysis of 15 studies by Bauer et al (Bauer et al., 2011) failed to demonstrate any significant association between the risk of adverse cardiovascular events and carriage of *CYP2C19* variant alleles and only a modest association between carriage of the variant alleles and the risk of stent thrombosis.

The discrepancy between Mega's and Bauer's meta-analyses is most likely driven by different methodologies for the meta-analysis, with Bauer's meta-analysis identifying a number of potential confounders and sources of bias which reduced the level of association between the LOF alleles and outcome in their meta-analysis. Nonetheless, the genome wide association study performed by Shuldiner (Shuldiner et al., 2009) et al demonstrated a single cluster of SNPs significantly associated with platelet reactivity at chromosome 10q24 in the *CYP2C18-CYP2C19-CYP2C9-CYP2C8* gene cluster with the *CYP2C19*2* allele contributing most to the observed association signal. However, the variability in platelet reactivity explained by

the *CYP2C19*2* allele is relatively modest at 12%, although incorporating clinical and other biochemical factors is likely to increase this.

The current meta-analysis demonstrated a significant association between CYP2C19 variant alleles and higher platelet reactivity across three different platelet function tests including one point of care test. Higher on treatment platelet reactivity is associated with ischaemic events. In a meta-regression analysis by Piccolo et al (Piccolo et al., 2014) of 30 randomised trials and 6683 patients, HTPR was significantly associated with a higher risk of adverse clinical outcomes, whilst a strategy of reducing HTPR lowers the risk of adverse clinical outcomes. Interestingly, Piccolo's analysis identified a number of different clinical factors which appear to modify the risk associated with HPR, with the risk of adverse cardiovascular outcomes only observed in studies enrolling patients with unstable coronary artery disease. In addition, other potential modifiers of the relationship between HTPR and outcome were identified, including gender, diabetes and age, in keeping with other known modifiers of platelet reactivity. Importantly, this analysis also identified the type of platelet function test as being a potential modifier of the relationship between HTPR and outcome.

Similarly, a meta-analysis by Wisman et al (Wisman et al., 2014) of 59 studies and 34, 776 patients identified a significant relationship between HTPR on clopidogrel and adverse cardiovascular events. All the platelet function tests included in the current meta-analysis (LTA, VerifyNow and VASP) were associated with adverse cardiovascular events, with broadly similar relative risks across all three. The prevalence of HTPR identified by these tests was variable, with VASP reporting a 56.6% prevalence compared to 27.9% with LTA 10 µmol/L ADP. In addition, and in keeping with Piccolo's meta-regression analysis (Piccolo et al., 2014), several other factors appeared to significantly affect the relationship between HTPR and cardiovascular outcome. This included length of follow up, disease type (unstable versus stable coronary disease) and the outcome measure definition. It is therefore interesting to note that the potential causes of observed heterogeneity in the current meta-analysis appeared to also vary dependent on the platelet function test used. For example, the heterogeneity observed in the LTA meta-analysis appeared to be primarily related to stable versus unstable cardiovascular disease, whilst this was not the case for VerifyNow or VASP. In addition, further sensitivity analyses identified that other factors such as clopidogrel loading dose or proton-pump inhibitors may also be important in determining the degree of heterogeneity observed with VerifyNow and VASP in this meta-analysis.

There was no significant association observed in the current meta-analysis between platelet reactivity and several other genes and polymorphisms, including *CYP3A5*3*, *ABCB1* C3435T and *PON1* Q192R. There are conflicting data suggesting an association between the *CYP3A5*3* variant and platelet reactivity. Whilst CYP3A4 is generally regarded as the most relevant CYP3A enzyme in clopidogrel's bioactivation, CYP3A5 may contribute up to 50% of overall CYP3A function in some patients (Suh et al., 2006) particularly in the presence of substrates or inhibitors of CYP3A4. The *CYP3A5*3* polymorphism is associated with non-expression of CYP3A5, and it is conceivable, therefore, that clopidogrel bioactivation would be substantially lower in carriers of the variant *3 allele. Consistent with this, in a study of 32 healthy volunteers (16 with the *CYP3A5**1*1 genotype and 16 with the *CYP3A5**3*3 genotype), Suh et al demonstrated that carriage of the *3 allele was associated with significantly higher platelet reactivity as compared to the *1 genotype (Suh et al., 2006). Conversely, in a larger, 160 healthy subject, study, carriage of the *CYP3A5*3* allele was not associated with higher platelet reactivity or clopidogrel pharmacokinetics (Frelinger et al., 2013). Given these conflicting results, it seems likely that the *CYP3A5*3* polymorphism exerts an effect on clopidogrel metabolism only in specific circumstances. For example, co-administration of ketoconazole, a potent CYP3A4 and CYP3A5 inhibitor, with clopidogrel in healthy volunteers significantly reduces the exposure to clopidogrel's active metabolite as well as clopidogrel induced platelet inhibition (Farid et al., 2007). However, itraconazole, a selective CYP3A4 inhibitor, does not significantly affect either clopidogrel's pharmacokinetic or pharmacodynamic effects (Suh et al., 2006) in wild type *CYP3A5*1* homozygotes, but in subjects who are *CYP3A5*3* homozygotes, clopidogrel's activation and pharmacodynamic effect is significantly impaired. Similarly, in the CROSS-VERIFY cohort (Park et al., 2012) of 1258 patients genotyped for the *CYP3A5*3* allele, a significant interaction between amlodipine, a CYP3A4 inhibitor, and clopidogrel was only observed in patients who were carriers of the *CYP3A5*3* homozygote genotype. These data suggest that the *CYP3A5* genotype may only be important in specific circumstances such as the co-administration of CYP3A4 inhibitors and clopidogrel.

No significant association was detected between the *ABCB1* C3435T polymorphism and platelet reactivity determined by LTA and VerifyNow. Most published studies assessing the relationship between the *ABCB1* C3435T polymorphism and platelet reactivity do not demonstrate any significant associations between carriage of the variant T allele and clopidogrel induced HTPR. However, for VASP, a significant association between carriage of the T allele and higher platelet reactivity was detected in this meta-analysis which was largely

powered by the GEPRESS study (Palmerini et al., 2014). The GEPRESS study enrolled 1,053 clopidogrel treated NSTEACS patients with a 1 year follow up for clinical outcomes and VASP defined platelet reactivity and HTPR. A comprehensive genotyping strategy, including 13 polymorphisms in 7 genes, was undertaken with only *CYP2C19*2* carriage being associated with VASP PRI values and HTPR but not clinical outcome. The *ABCB1* C3435T polymorphism was not associated with either VASP PRI values or HTPR in the published data, although this is likely to be due to the genotype-phenotype data being analysed in an additive model as opposed to the dominant model utilised in this meta-analysis. However, it is conceivable that *ABCB1* polymorphisms may affect clopidogrel induced platelet reactivity. Clopidogrel is a substrate for P-glycoprotein which is coded for by the *ABCB1* gene, and any loss of function variant, such as the non-coding C3435T polymorphism, could therefore reduce clopidogrel's absorption and consequent bioactivation as demonstrated in a study by Taubert et al (Taubert et al., 2006). In addition, a number of studies have detected a positive association between carriage of the T allele of the *ABCB1* C3435T polymorphism and clopidogrel related HTPR (Harmsze et al., 2010a, Campo et al., 2011), with some clinical outcome studies also detecting an association between the *ABCB1* C3435T polymorphism and adverse cardiovascular events in clopidogrel treated patients (Mega et al., 2010a). Furthermore, several studies have identified that the *ABCB1* C3435T polymorphism is in strong linkage disequilibrium with two coding *ABCB1* polymorphisms, G2677T/A and C1236T, which may better represent the overall effect of *ABCB1* variants on platelet reactivity and clinical outcomes. Future studies should consider using a more comprehensive genotyping strategy to better represent the relationship between *ABCB1* genotype and outcome.

This meta-analysis also did not detect any significant association between the *PON1* Q192R polymorphism and platelet reactivity. These results are not in keeping with Bouman et al's study (Bouman et al., 2011), which demonstrated a significant association between carriage of the QQ genotype and higher platelet reactivity as well as adverse cardiovascular outcomes. However, more recent studies have failed to replicate the association observed by Bouman et al, and the cause of these discordant data remain unclear (Hulot et al., 2011, Trenk et al., 2011). Paraoxonase-1 (*PON-1*) has been postulated as being critical in the production of clopidogrel's active metabolite, with the Q192R polymorphism largely determining the overall activity of PON-1. Furthermore, the importance of *PON-1* to clopidogrel bioactivation was emphasised by the finding that 73% of the variability in clopidogrel induced platelet reactivity was explained by the Q192R polymorphism (Bouman et al., 2011). However, several factors may have contributed to these findings. Firstly,

clopidogrel induced platelet reactivity was not measured in patients until they had completed 12 months of DAPT following stent thrombosis, which may not best represent clopidogrel's pharmacodynamic effect in the context of cardiovascular disease. Secondly, the sample sizes used in Bouman's study were relatively small and therefore may have been subject to confounding from the well characterised inter-individual variability in platelet function. Finally, it is notable that the study did not detect any effect of the *CYP2C19* LOF polymorphism in their pharmacodynamic and clinical cohorts (Trenk et al., 2011), which is not in keeping with the majority of pharmacodynamic or clinical outcome studies.

Finally, several polymorphisms in platelet receptor genes and other cytochrome P450 enzymes could not be combined in this meta-analysis, either due to non-combinable variants or non-combinable outcome measures. In the main, published data on these genes and variants demonstrate inconsistent associations between genotype and outcome (Frere et al., 2008, Giusti et al., 2007, Harmsze et al., 2010a, Lee et al., 2009, Park et al., 2011a), but given their biological plausibility, these polymorphisms merit further investigation, perhaps using large datasets such as TRITON-TIMI-38 and PLATO, to determine their clinical utility and validity.

This meta-analysis clearly demonstrates that the effects of the *CYP2C19* LOF polymorphisms is observed across three major methods of assessing platelet reactivity, including a point of care test (VerifyNow). This is an important finding given the concerns surrounding the poor correlation between individual platelet function tests and whether genotype or phenotype should be used to identify poor responders to clopidogrel and stratify patients. As discussed previously, each platelet function test assesses platelet reactivity in different ways and can be affected by clinical factors that are specific to that assay. For example, light transmittance aggregometry, the most widely used assay in this meta-analysis, is often considered to represent the gold-standard test for platelet function. However, one of LTA's major limitations is the level of operator and interpreter dependence (Michelson, 2004) which often leads to poor reproducibility across different studies. Furthermore, LTA analyses platelet function without other cellular components of blood, and uses different concentrations ADP as a single agonist, which is not reflective of platelet activation in vivo where other cellular components and collagen significantly contribute to overall platelet reactivity (Ohmori et al., 2006). However, given LTA is performed only in platelet-rich or platelet-poor plasma, it is relatively unaffected by clinical variables that may impact on other platelet function tests such as VerifyNow and Multiplate (Choi and Kim, 2018).

VerifyNow, on the other hand, is a point of care platelet function test which reports standardised units (P2Y12 Reaction Units, PRU) which lends itself to standardisation and the development of reference ranges or cut-off values for platelet reactivity. It is a fully point-of-care assay which, unlike LTA, utilises whole blood as the test matrix which may make it more sensitive to other in-vivo factors that affect platelet reactivity. HTPR identified by VerifyNow is robustly associated with adverse cardiovascular outcomes, although this association is not always observed in patients with stable coronary artery disease (Viviani Anselmi et al., 2013, Aradi et al., 2010). However, VerifyNow is sensitive to haematocrit and haemoglobin values with an inverse correlation between Hct/Hb and PRU values (Choi and Kim, 2018). As VerifyNow uses an optical, turbidometric, method to assess platelet reactivity in whole blood, it is conceivable that Hb concentration alters light transmittance with consequent effects on the PRU values.

Finally, VASP phosphorylation utilises flow cytometry to assess platelet reactivity as measured by immunofluorescence to phosphorylated VASP, using whole blood incubated with either PGE1 alone or PGE and ADP together. However, like LTA, it is a laboratory based technique which requires established infrastructure and skilled staff to perform (Kozinski et al., 2014).

Several studies report poor correlation between individual platelet function tests. In a study by Cuisset et al (Cuisset et al., 2010) of 70 patients with cardiovascular disease, the agreement between LTA, VASP and VerifyNow was good, with linear regression coefficients between 0.55 and 0.64 when platelet reactivity was reported as a continuous variable. However, the correlation was weak when the assays were compared on the basis of HTPR, with kappa values varying between only 0.35 and 0.46. Similarly, Lemsele et al (Lemesle et al., 2014) demonstrated good correlation between LTA, VASP and VerifyNow in 100 patients on clopidogrel undergoing PCI. However, despite 45 patients being identified as poor responders by any of the three tests, only 16 patients were defined as poor responders by all three tests using the HPR cut-off values identified by a consensus white paper (Bonello et al., 2010c).

Rapid and reliable demonstration or prediction of clopidogrel non-response has the potential to allow stratification of anti-platelet therapy. Stratification of clopidogrel therapy has been investigated by several studies, with mixed results. In the context of stable coronary artery disease, the GRAVITAS study (Price et al., 2011) failed to demonstrate any benefit from identification of clopidogrel related HTPR with VerifyNow and treatment with clopidogrel

150mg once daily. Similarly, the TRIGGER-PCI study (Trenk et al., 2012), also in stable coronary artery disease, failed to demonstrate any benefit of VerifyNow based stratification and treatment with prasugrel 10mg once daily in clopidogrel non-responders. However, in a comparison between 133 myocardial infarction patients and 67 patients with stable angina, Lee et al (Lee et al., 2014) identified significantly higher platelet aggregation and HTPR rate in unstable patients as compared to stable patients, suggesting that the negative results observed in GRAVITAS and TRIGGER-PCI are due to inclusion of low risk, stable patients. Moreover, in the context of acute coronary syndrome, Aradi et al (Aradi et al., 2014) demonstrated a significant reduction in adverse cardiovascular events when using Multiplate to stratify anti-platelet therapy in a 741-patient study. Similarly, Hazarbasanov (Hazarbasanov et al., 2012) demonstrated a significant reduction in the occurrence of adverse cardiovascular events in patients whose anti-platelet therapy was stratified on the basis of Multiplate defined platelet reactivity compared to those on standard, non-stratified, therapy (5.3% vs 0%, P=0.03). In addition, in the TAILOR randomised study (Dridi et al., 2014), patients randomised to receive either clopidogrel 150mg or prasugrel 10mg had significantly lower rates of HTPR compared to clopidogrel 75mg, with prasugrel 10mg demonstrating significantly better platelet inhibition compared to clopidogrel 150mg. These data from the TAILOR study are in agreement with others that suggest that the optimal alternative anti-platelet treatment in clopidogrel non-responders is either prasugrel or ticagrelor and not double dose clopidogrel. Finally, a cost-effectiveness analysis by Coleman et al (Coleman and Limone, 2013) demonstrated that platelet function test driven anti-platelet drug stratification is cost effective in comparison to the universal use of ticagrelor without stratification. However, it is important to emphasise that stratification of anti-platelet therapy is likely to prove efficacious in certain clinical circumstances only, such as unstable cardiovascular disease or stent thrombosis. Interestingly, this is in keeping with the observed heterogeneity in the *CYP2C19*2* meta-analysis which was removed by analysing separately for stable and unstable cardiovascular disease.

Fundamentally, it remains unclear whether genotype or phenotype should be used to identify clopidogrel poor responders for the purposes of stratification. However, our meta-analysis demonstrates that there is a clear association between *CYP2C19*2* genotype and platelet reactivity, suggesting that either could be potentially used to stratify anti platelet therapy. In a study of 65 patients undergoing PCI for NSTEACS, Ahn et al (Ahn et al., 2013) investigated both platelet function directed (using VerifyNow) and genotype guided (on the basis of the *CYP2C19*2* and **3* allele) stratification, demonstrating that both phenotype and

genotype guided strategies were equally effective in reducing clopidogrel HTPR. Although platelet function tests have the advantage of being a phenotypic test that is sensitive to underlying co-morbidities in the patient, there remains significant concern in relation to inter-test variability and poor consequent poor correlation. Furthermore, several studies have identified clinical factors that may significantly affect individual platelet function tests, such as Hct and VerifyNow, as well as other non-clinical factors such as ethnicity, gender and diet (Miller et al., 2014). Finally, there is little consensus at present on sensitive and specific cut-off values for stratification, with studies continuing to use different definitions for HTPR which are often different to published guidelines. Consequently, genotyping may be a better method of stratification despite the current problems surrounding its lack of consistent association with clinical outcome.

There are several limitations to our meta-analyses. Firstly, for certain gene-test combinations there were only a limited number of papers available for comparison which may affect the generalisability of these analyses. In addition, these meta-analyses were prepared on the basis of data extracted from published papers, with no access to patient-level data from individual studies. It was therefore not possible to adequately adjust for the heterogeneity observed in some of the meta-analyses as either these data were not available in the paper or were reported in a non-extractable format. Finally, there is a risk of publication bias as the meta-analyses were prepared only from published papers. Data extraction from other published data, such as conference abstracts, was attempted but there were insufficient data to adequately assess their quality or, in some cases, their outcome measures and therefore they could not be included. Funnel plots were prepared for all meta-analyses conducted and were generally symmetrical; however, given the low number of studies included in some of the meta-analyses, it is difficult to fully exclude any publication bias. Finally, data were only included up to a cut-off date of November 2015. Since then around 110 additional studies investigating the relationship between genotype and clopidogrel pharmacodynamic or clinical response have been published. However, most papers continue to identify the *CYP2C19* LOF alleles as the primary modifying polymorphisms affecting clopidogrel response and, consequently, it is unlikely that the overall conclusions of this meta-analysis would be different with inclusion of the newer data.

In summary, the current meta-analysis clearly demonstrates that the *CYP2C19*2* and **3* alleles are associated with higher platelet reactivity as defined by three platelet function tests (LTA, VASP and VerifyNow) in patients taking clopidogrel. This provides consistent evidence of the relationship between phenotype and genotyping, demonstrating that

stratification of anti-platelet therapy using either a platelet function test or genotype is possible. Further, well designed and suitably powered stratification studies, incorporating clinical outcomes, adverse events and cost-effectiveness are now clearly required to demonstrate clinical utility.

# Chapter 3 – Influence of genetic polymorphisms on clinical response to clopidogrel: a systematic review and meta-analysis

## 3.1: Introduction

Clopidogrel, an ADP receptor antagonist, has, until recently, been the mainstay of anti-platelet treatment for ACS in combination with aspirin. However, the advent of newer, more potent anti-platelet drugs, such as ticagrelor and prasugrel, has significantly reduced its usage with international guidelines recommending the universal use of ticagrelor as opposed to clopidogrel (Ibanez et al., 2018).

The newer anti-platelet agents have been demonstrated to be superior to clopidogrel in reducing adverse cardiovascular events in unstable cardiovascular disease. For example, in the TRITON – TIMI 38 trial, prasugrel reduced the risk of adverse cardiovascular events by 19% compared to clopidogrel (HR 0.81; 95% CI 0.73 to 0.90, P<0.001) (Wiviott et al., 2007). Similarly, ticagrelor was demonstrated to be superior to clopidogrel in the PLATO trial, with a 16% reduction in risk of adverse cardiovascular events (HR 0.84; 95% 0.77 to 0.92, P<0.001) (Wallentin et al., 2009).

The superiority of the newer anti-platelet agents has been considered to be related to the well-recognised phenomenon of clopidogrel non-response, which has been associated with a significant increase in the risk of adverse cardiovascular events in those deemed to be clopidogrel resistant (Snoep et al., 2007b).

Clopidogrel non-response is likely to be related to clopidogrel's bioactivation. Clopidogrel is a pro-drug, which requires a two-step activation via CYP450 enzymes to its active metabolite. The primary CYP450 isoenzyme responsible for clopidogrel's active metabolite generation is *CYP2C19* which has a number of genetic polymorphisms that alter the activity of the enzyme. A number of loss-of-function alleles have been identified (*2, *3 and others) in addition to a gain-of-function polymorphism (*17). Furthermore, clopidogrel is a substrate for P-gp, and polymorphisms in the *ABCB1* gene (e.g. C3435T) may consequently alter the absorption of clopidogrel. Clopidogrel's pharmacodynamic target, the ADP receptor *P2Y12*, has also been demonstrated to have a number of genetic polymorphisms that could conceivably alter its function, whilst downstream effects of the receptor, such as other platelet receptors or complexes, also demonstrate a number of different genetic polymorphisms (Cuisset et al.,

2007, Staritz et al., 2009). Consequently, a number of different genetic polymorphisms in both clopidogrel's pharmacokinetic and pharmacodynamic pathway may significantly alter its effect and response.

The importance of genetic polymorphisms on the effect of clopidogrel has been investigated by a number of studies. For example, in a genome wide association study, Shuldiner and colleagues identified that the *CYP2C19*2* polymorphism was the primary SNP associated with clopidogrel induced platelet inhibition (Shuldiner et al., 2009). These data are in keeping with clinical outcome studies, with a relative increase of 53% in adverse cardiovascular events demonstrated in *CYP2C19*2* carriers taking clopidogrel in the TRITON-TIMI 38 cohort (Mega et al., 2009). However, data for polymorphisms in clopidogrel's pharmacodynamic pathway, such as the P2Y12 and other platelet receptors are frequently conflicting (Cuisset et al., 2007, Staritz et al., 2009).

The *CYP2C19*2* polymorphism may also be the cause of the observed superiority of the newer anti-platelet agents over clopidogrel. As discussed previously, the genetic sub-studies of both PLATO and TRITON-TIMI 38 (Mega et al., 2010a, Wallentin et al., 2010) have demonstrated that neither ticagrelor nor prasugrel are robustly superior to clopidogrel in patients with a wild-type homozygous *CYP2C19* genotype. Given these data, the *CYP2C19* genotype could be used to identify patients with variant alleles (such as *2) who would benefit from ticagrelor or prasugrel treatment whilst those with a normal, wild-type, genotype could remain on clopidogrel, a significantly less expensive drug.

However, the association between the *CYP2C19*2* allele and poor cardiovascular outcomes in clopidogrel treated patients has not been universally observed. In an IPD meta-analysis of 9 studies and 9685 patients, Mega and colleagues demonstrated (Mega et al., 2010b) a clear association between carriage of the *CYP2C19*2* allele, adverse cardiovascular outcomes and stent thrombosis. However, a further meta-analysis by Bauer and colleagues (Bauer et al., 2011) of fifteen studies failed to demonstrate a consistent association between carriage of the *CYP2C19*2* allele and major adverse cardiovascular events. Furthermore, they could only demonstrate a moderate association between stent thrombosis, considered to be a highly platelet sensitive outcome, and *CYP2C19*2*. Moreover, carriage of the *CYP2C19*2* allele may only explain around 5% of the variability in platelet function in patients with cardiovascular disease, with inclusion of clinical factors only increasing this to 12% (Hochholzer et al., 2010). Finally, the impact of the *CYP2C19*2* polymorphism on clinical outcomes may be substantially modified by the patient's individual clinical situation. For example, in stable

coronary artery disease, several studies have failed to demonstrate any significant effect of *CYP2C19* genotype on adverse cardiovascular outcomes (Pare et al., 2010), whilst the presence of other clinical factors, such as diabetes, hyperlipidaemia and obesity, or interacting medication (such as proton pump inhibitors or calcium channel blockers) may also affect the response to clopidogrel.

Whilst the prospect of stratification of anti-platelet therapy on the basis of genotype appears possible, an alternative strategy would be to use platelet function tests to identify patients with poor response to clopidogrel. However, it remains unclear which platelet function test best represents platelet function in patients, with each testing platelet reactivity in different ways resulting in poor correlation between individual tests. Consequently, genotype, in addition to clinical variables, may be a more reliable strategy for stratification given that it is unambiguous and easily validated for use in a clinical setting.

Given the aforementioned concerns, it is clear that several key unanswered questions remain in relation to the association between genotype, clopidogrel response and clinical outcome. Firstly, is the effect of a particular genetic variant observed across all studies and outcome measures? Secondly, how do clinical covariates and type of cardiovascular disease impact on the observed associations between genotype and outcome? Finally, although most studies have focussed on *CYP2C19* variants and clinical outcomes, are there other genes that may influence the response to clopidogrel? In particular, is there evidence for an effect from other pharmacokinetic modifiers or pharmacodynamic modifiers of clopidogrel response?

In order to address these questions, a comprehensive systematic review and meta-analysis of all published studies investigating the relationship between genetics and clinical outcomes in patients taking clopidogrel was performed. In addition, meta-analyses were prepared only on studies that presented survival data given the discordant conclusions from the Mega and Bauer meta-analyses. As outlined by Tierney et al (Tierney et al., 2007), use of odds ratios and relative risk as summary statistics for time-to-event data are likely to be sub-optimal, given that they report only the number of events rather than the time to event. Consequently, to best represent the relationship between genotype and clinical outcome, the meta-analyses were prepared only on studies that provided survival data or had other data that could be extracted and manipulated to provide survival data, using the methodology proposed by Tierney et al.

## 3.2: Methods

### 3.2.1: Search strategy

Relevant citations were identified using a comprehensive search strategy using PubMed (1966 to November 2015) and Scopus Web of Science. In order to find all relevant citations, a broad search term was used with the following terms in combination or as text words with no language restriction: clopidogrel, thienopyridines, *P2Y12*, *CYP2C19*, *CYP2C9*, *CYP2B6*, *CYP3A4*, *P2Y1*, cytochrome P450, gene, genotype, SNP, allele, polymorphism, variant and haplotype. In addition, manual searching of reference lists was undertaken for each of the extracted papers. Conference abstracts were also identified by searching for supplemental issues of major cardiovascular or clinical pharmacology journals.

### 3.2.2: Data Extraction

Data were extracted by two independent reviewers, with any disagreement discussed and arbitrated by a third reviewer. Initially all citations were reviewed by title and subsequently by abstract. Inclusion criteria were (a) studies that included patients about to commence or already established on clopidogrel and, (b) studies which investigated the effect of genetic variants on the response to clopidogrel. Included data were extracted onto standardised data extraction forms and entered on to a computer spreadsheet. For each study, data were collected on a number of different variables including number of participants, age, setting, risk factors for cardiovascular disease, clopidogrel dose (maintenance and loading), genotype distribution and clinical outcomes. Methodological quality was also assessed (Hardy-Weinberg assessment and genotyping methodology).

### 3.2.3: Outcomes

The primary objective of the meta-analysis was to investigate the relationship between genetic variants and clinical outcomes in patients on clopidogrel. The outcome measures investigated were determined by the clinical outcomes in each paper. Only comparable clinical outcomes were combined in the meta-analysis, and therefore the meta-analyses for some clinical outcomes were broken down by outcome and definition.

### 3.2.4: Statistical Analysis

As the studies frequently reported measurements for the wild type gene along with measurements for both one and two variant types and /or a combined variant type, meta-

analyses were performed using a dominant inheritance model. Where reported separately, mutant type heterozygotes and mutant type homozygotes were combined where possible.

Meta-analyses were performed only on studies that provided data on time to event or survival and were reported as Hazard Ratios. Where studies presented HRs and P-values from a log-rank or Cox proportional hazards model, these were extracted and analysed directly in the meta-analysis. However, where data were not directly available, HRs were indirectly calculated using the methodology provided by Tierney et al (Tierney et al., 2007) wherever possible. Briefly, this included indirect calculation from published ORs, RRs, HRs, 95% CI, number of events in each group and Kaplan-Meier curves using equations provided by Tierney et al in their paper. All analyses were conducted and reported in R statistics (version 3.5.0, R Foundation). Meta-analyses were presented using Forest plots to describe both the individual studies and the overall pooled effect.

Meta-analyses were prepared when more than one study presented survival or time-to-event data.

## 3.3: Results

### 3.3.1: Search Results and Study Characteristics

The initial literature search yielded a total of 652 citations; of those 207 were included on the basis of title, abstract and full-text review. 81 of the papers reported on clinical outcomes and were therefore included in this meta-analysis. A total of 39 studies reported outcomes related to *CYP2C19*2* genotype, 30 reported outcomes related to combined *CYP2C19* metaboliser status, 15 reported outcomes related to *ABCB1* C3435T genotype, 11 reported outcomes related to *PON1* Q192R genotype, 10 reported outcomes related to CYP2C19*17 genotype, 8 reported outcomes related to *CYP3A5*3* genotype and seven reported outcomes related to *CYP2C19*3* genotype. A number of other polymorphisms have been investigated in several studies but these could not be combined in meta-analyses due to incomparable polymorphisms and /or outcomes (**Table 3.1**).

With regard to outcome measures, 49 studies reported on major adverse cardiovascular events (MACE), 32 reported on stent thrombosis, 25 studies reported on MI, 17 studies reported on cardiovascular death, 16 studies reported on any bleeds, 12 studies reported on all-cause mortality and 5 studies reported on target lesion revascularisation (TLR). A number of other clinical outcomes were reported, but these could not be combined in meta-analyses due to small numbers or incomparable polymorphisms (**Table 3.2**).

In total, twenty-four definitions of MACE were used by the 49 studies using MACE as an outcome. The commonest definition was a composite of CV death, non-fatal MI and non-fatal CVA used by 11 studies, followed by six studies using a composite of all-cause mortality, non-fatal MI and non-fatal CVA. Five studies used a composite of CV death, non-fatal MI, target lesion revascularisation (TLR) and stent thrombosis. Most other definitions of MACE were used by only one or two studies (**Table 3.3**).

The characteristics of the included studies are summarised in **Table 3.4**. The studies included a variety of different patient groups. 31 studies were conducted in patients with either ACS or stable coronary artery disease, whilst 27 studies and 12 studies were performed in ACS patients only or stable coronary artery disease patients only respectively. Six studies were performed in stroke patients. The clopidogrel loading dose was also variable: 26 studies reported a loading dose of 300mg, 16 studies reported a loading dose of 600mg and ten studies used either 300 or 600mg loading doses. Only three studies included patients with very high loading doses of clopidogrel (>600mg), whilst 21 studies did not report the loading dose. Most studies utilised a 75mg clopidogrel maintenance dose, with only two studies reporting a greater than 75mg clopidogrel dose. Follow up lengths for clinical outcomes were also variable, with most studies reporting a follow up period of 12 months. The maximum duration of follow up was 96 months and the minimum duration was 24 hours.

### 3.3.2: Methodological Quality

Most studies collected data prospectively, although relatively few reported formal power calculations for their sample size. Several studies reported genetic sub-study data from a larger study, such as TRITON-TIMI 38 (Mega et al., 2010a). Hardy-Weinberg equilibrium was assessed in the majority of the included studies (56 studies), but genotyping quality control was performed in only 30 studies. Most studies reported genotype data in an additive model (38 studies), with 33 studies reporting in a dominant model.

### 3.3.3: Meta-analyses

A total of 14 meta-analyses, incorporating four genes and seven clinical outcomes, were prepared on the basis of the extracted data. A summary of all meta-analyses is provided in **Table 3.5**.

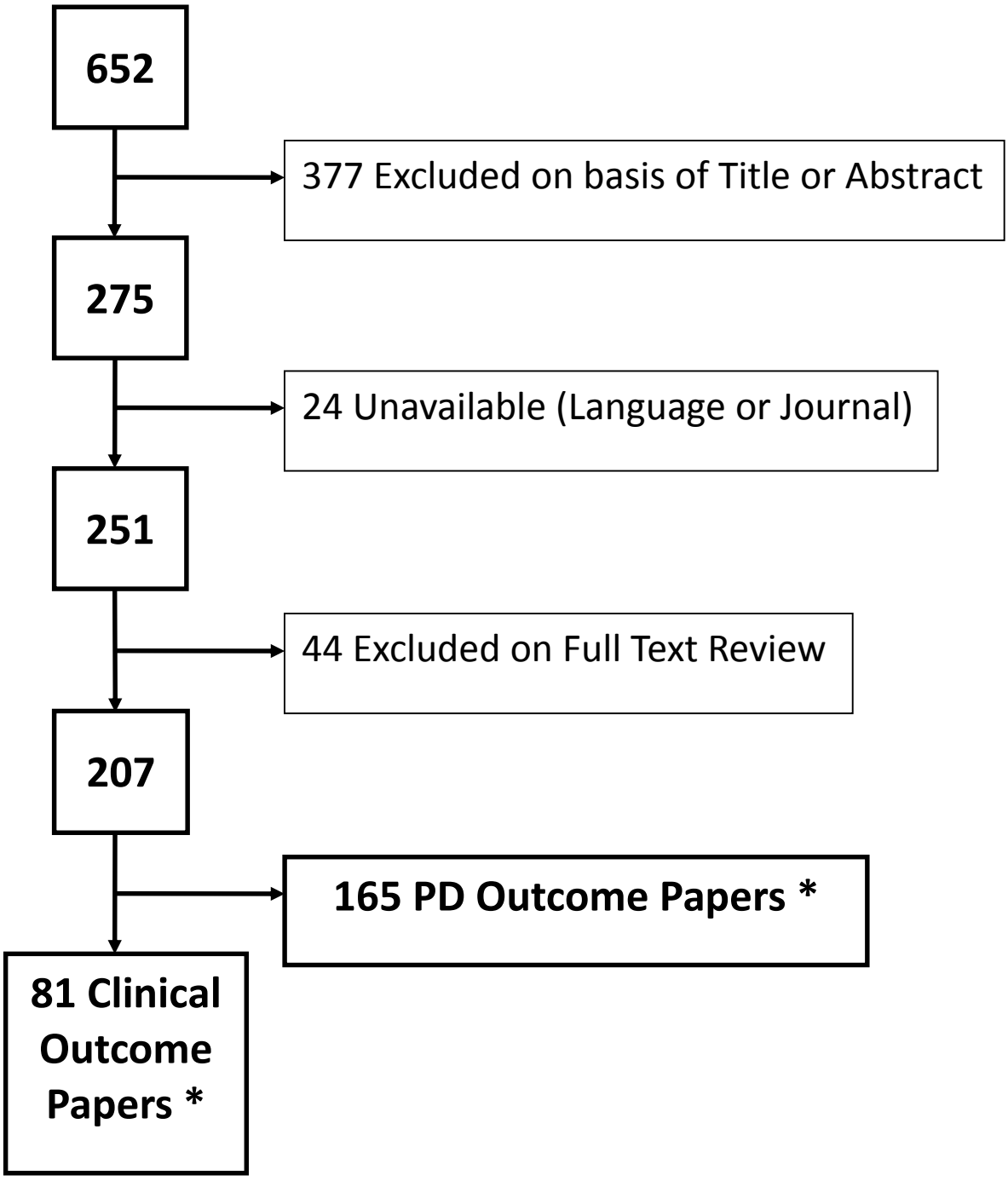


**Figure 3.1 – Literature Search Results** (* 125 papers reported on PD outcomes only, 40 papers reported on both PD and clinical outcomes, 41 papers reported on clinical outcomes only)

| Gene | SNP | Studies | References |
|---|---|---|---|
| *ABCB1* | C1236T | 1 | T18 |
| *ABCB1* | C3435T | 15 | T2, T8, T14, T18, T19, T23, T25, T31, T39, T42, T45, T58, T63, T71, T73 |
| *ABCB1* | G2677T/A | 2 | T18, T39 |
| *ABCB1* | T129C | 1 | T39 |
| *CES1A2* | -816A/C | 2 | T62, T63 |
| *COX1* | -824A/G | 1 | T42 |
| *1A2* | Met | 1 | T7 |
| *1A2* | *1F | 1 | T58 |
| *2B6* | Met | 1 | T7 |
| *2B6* | *3 | 1 | T58 |
| *2B6* | *9 | 1 | T58 |
| *2C19* | Met | 30 | T7, T8, T14, T16, T37, T41, T43, T44, T49, T51, T52, T53, T54, T57, T27, T58, T59, T60, T61, T63, T64, T67, T68, T70, T71, T74, T75, T76, T79, T81 |
| *2C19* | rs1188072C/T | 1 | T42 |
| *2C19* | *17 | 10 | T1, T19, T23, T25, T39, T56, T34, T38, T69, T73 |
| *2C19* | *2 | 39 | T3, T4, T6, T7, T5, T8, T9, T17, T18, T19, T22, T23, T23, T25, T31, T32, T36, T39, T42, T45, T46, T47, T48, T50, T55, T56, T20, T26, T33, T34, T35, T65, T66, T69, T73, T77, T78, T80, T82 |
| *2C19* | *3 | 7 | T8, T31, T39, T45, T56, T66, T73 |
| *2C19* | *4 | 2 | T8, T73 |
| *2C19* | *5 | 1 | T8 |
| *2C9* | Met | 1 | T7 |
| *2C9* | *2 | 3 | T18, T23, T58 |
| *2C9* | *3 | 2 | T18, T23 |
| *3A4* | 1344T/A | 1 | T58 |
| *3A4* | 20239G/A | 1 | T58 |
| *3A4* | C894T | 1 | T59 |
| *3A4* | *1B | 3 | T18, T23, T73 |
| *3A4* | *1G | 2 | T23, T73 |
| *3A5* | Met | 1 | T7 |
| *3A5* | *3 | 8 | T8, T10, T18, T23, T25, T40, T58, T73 |
| *GPIa* | C807T | 1 | T42 |
| *GPIIIa* | PIA1/A2 | 1 | T12 |
| *GPIIIa* | rs8069732T/C | 1 | T42 |
| *IRS1* | A227382808C | 1 | T73 |
| *IRS1* | A227497991G | 1 | T73 |
| *ITGB3* | T196C | 1 | T73 |
| *P2Y1* | A1622G | 1 | T18 |
| *P2Y12* | C34T | 3 | T11, T48, T59 |

| | | | |
|---|---|---|---|
| *P2Y12* | G52T | 1 | T59 |
| *P2Y12* | T744C | 2 | T58, T77 |
| *PON1* | -162A/G | 1 | T50 |
| *PON1* | -108C/T | 2 | T39, T50 |
| *PON1* | -126C/G | 1 | T50 |
| *PON1* | 206T/A | 1 | T58 |
| *PON1* | 672A/G | 1 | T58 |
| *PON1* | A163T | 1 | T73 |
| *PON1* | A575G | 2 | T42, T73 |
| *PON1* | L55M | 3 | T39, T50, T29 |
| *PON1* | Q192R | 11 | T22, T23, T23, T24, T39, T45, T50, T28, T29, T61, T63 |

**Table 3.1 - Number of studies per gene and SNP** (For all T references, please refer to Appendix 2).

| Outcomes | Studies | References |
|---|---|---|
| Bleed (All) | 16 | T7, T12, T25, T30, T50, T53, T15, T37, T45, T48, T49, T33, T34, T38, T71, T72 |
| Minor Bleed | 8 | T1, T14, T16, T37, T47, T48, T38, T81 |
| Major Bleed | 3 | T1, T48, T38 |
| Cardiovascular Death | 17 | T6, T7, T46, T53, T54, T4, T15, T26, T33, T60, T67, T69, T71, T77, T78, T79, T82 |
| Cardiovascular Relapse | 1 | T61 |
| CVA | 17 | T7, T5, T10, T19, T52, T53, T15, T33, T60, T67, T69, T71, T77, T79, T80, T81, T82 |
| Death | 12 | T5, T10, T17, T19, T25, T41, T46, T49, T20, T65, T68, T74 |
| Functional Status | 1 | T51 |
| MACE (All) | 49 | T1, T2, T3, T4, T6, T7, T5, T8, T10, T11, T12, T9, T14, T16, T19, T24, T25, T30, T32, T36, T37, T40, T41, T42, T45, T46, T48, T49, T50, T53, T54, T55, T56, T15, T26, T27, T28, T29, T35, T58, T67, T70, T71, T72, T73, T76, T77, T78, T79 |
| Myocardial Infarction | 25 | T1, T6, T4, T5, T10, T12, T19, T41, T43, T49, T53, T54, T15, T20, T26, T33, T60, T64, T66, T67, T71, T77, T78, T80, T82 |
| NSTEMI | 3 | T5, T33, T69 |
| Perioperative thrombosis | 1 | T68 |
| Prognosis | 1 | T75 |
| Recurrent Angina | 1 | T82 |
| Revascularisation | 4 | T6, T46, T26, T79 |
| Restenosis | 2 | T59, T69 |
| Stable Angina | 1 | T69 |
| Stent Thrombosis | 32 | T1, T4, T6, T7, T5, T9, T14, T18, T19, T22, T23, T23, T24, T25, T39, T41, T44, T47, T49, T53, T57, T15, T20, T26, T33, T34, T62, T63, T74, T77, T78, T82 |
| STEMI | 3 | T5, T33, T69 |
| Target lesion revascularisation | 5 | T41, T57, T20, T74, T78 |
| Target vessel revascularisation | 4 | T20, T60, T67, T74 |
| Unstable angina | 4 | T60, T69, T77, T79 |
| Vascular ischaemia | 1 | T68 |
| Vascular surgery | 1 | T68 |

**Table 3.2 - Number of studies per Clinical Outcome** (For all T references, please refer to Appendix 2).

| Outcomes | Definition | Studies | References |
|---|---|---|---|
| MACE 1 | CV Death, Non-fatal MI | 4 | T14, T36, T42, T35 |
| MACE 2 | CV Death, Non-fatal MI, Non-fatal CVA | 11 | T7, T8, T10, T14, T16, T30, T37, T46, T55, T71 |
| MACE 3 | CV Death, Non-fatal MI, Non-fatal CVA, Recurrent ischaemia, Hospitalisation | 1 | T16 |
| MACE 4 | CV Death, Non-fatal MI, Non-fatal CVA, Stent thrombosis | 2 | T40, T53 |
| MACE 5 | CV Death, Non-fatal MI, Non-fatal CVA, Stent thrombosis, Revascularisation | 1 | T54 |
| MACE 6 | CV Death, Non-fatal MI, PCI, CABG | 1 | T12 |
| MACE 7 | CV Death, Non-fatal MI, Target lesion revascularisation, Stent thrombosis | 5 | T41, T45, T48, T29, T78 |
| MACE 8 | CV Death, Non-fatal MI, Revascularisation | 3 | T6, T26, T27 |
| MACE 9 | CV Death, Stent thrombosis | 1 | T4 |
| MACE 10 | Death, MI, Revascularisation | 1 | T1 |
| MACE 11 | Death, Non-fatal MI | 3 | T9, T19, T24 |
| MACE 12 | Death, Non-fatal MI, Non-fatal CVA | 6 | T2, T5, 378, 410, 527, 396 |
| MACE 13 | Death, Non-fatal MI, Stent thrombosis | 1 | T49 |
| MACE 14 | Death, Non-fatal MI, Target lesion revascularisation | 1 | T19 |
| MACE 15 | Death, Non-fatal CVA, Target lesion revascularisation | 1 | T19 |
| MACE 16 | CV Death, MI, CVA, Stent thrombosis, Readmission | 1 | T19 |
| MACE 17 | Neurological Events | 1 | T11 |
| MACE 18 | Unstable angina, Transient ischaemic attack, Revascularisation | 1 | T37 |
| MACE 19 | CV Death, Non-fatal MI, Stent thrombosis | 2 | T26, T58 |
| MACE 20 | CV Death, Non-fatal MI, Readmission | 1 | T35 |
| MACE 21 | CV Death, Non-fatal MI, Non-fatal CVA, Target vessel revascularisation, Periprocedural MI | 2 | T67, T70 |
| MACE 22 | CV Death, Non-fatal MI, Non-fatal CVA, Unstable angina, Target vessel revascularisation | 2 | T60, T79 |
| MACE 23 | CV Death, Non-fatal MI, Target vessel revascularisation, Stent thrombosis | 3 | T72, T73, T76 |
| MACE 24 | CV Death, Non-fatal MI, Non-fatal CVA, Unstable angina, Stent thrombosis | 1 | T77 |

**Table 3.3 – MACE definitions per study** (For all T references, please refer to Appendix 2).

| Author | Year | Type | Outcome | Gene | N | Clop LD | Clop MD | Setting | FU | Cohort |
|---|---|---|---|---|---|---|---|---|---|---|
| Anselmi C et al [T58] | 2014 | PC | MACE | 2C19*2, *3; ABCB1 C3435T; 1A2*1F; 2B6*9, *3; 2C9*2; 3A4 20239G>A, 1344T>A; 3A5*3; P2Y12 T744C; PON1 206T>A, PON1 672A>G | 1432 | 600 | 75 | PCI (SCAD) | > 12 months | |
| Arima Y et al [T70] | 2015 | PC | MACE | 2C19*2/*3 | 518 | 300 | 75 | PCI (ACS+SCAD) | >12 months | |
| Bhatt DL et al [T37] | 2012 | RCT | MACE, Bleed | 2C19*2 | 2266 | NS | 75 | SCAD | 800 days | CHARISMA |
| Bouman HJ et al [T23] | 2011 | CC+PC | ST | 2C19*2; 2C9*2, *3; 3A4*1B, *1G (IVS10); 3A5*3; PON1 Q192R; ABCB1 C3435T | 112+1982 | 300-600 | 75 | PCI (ACS+SCAD) | 12 months | |
| Campo G et al [T25] | 2011 | PC | Death, ST, Bleed, MACE | 2C19*2, *17; 3A5*3; ABCB1 C3435T | 300 | 600 | 75 | PCI (ACS+SCAD) | 12 months | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cayla G et al [T30] | 2011 | CC | Bleed, MACE | 2C19*2, *3, *17; 2C9*2, *3; 2B6*5, *9; 3A5*3; POR C1508T; PON1 Q192R, L55M; ABCB1 C3435T; P2Y12 T744C; ITGB3 T196G; MTHFR C677T | 369 | VAR | 75 | PCI (ACS+SCAD) | NA | ONASSIST |
| Chen DY et al [T39] | 2012 | CC | ST | 2C19*2, *3, *17; PON1 Q912R, L55M, C108T; ABCB1 C3435T, T129C, G2677T/A | 4964 | NS | NS | PCI (ACS+SCAD) | NA | CAPTAIN |
| Collet JP et al [T6] | 2009 | PC | CV Death, ST, MACE, MI, Revasc | 2C19*2 | 259 | VAR | 75 | ACS | Up to 8 years | AFIJI |
| Cresci S et al [T65] | 2014 | PC+CROSS | Death | 2C19*2 | 2062 | NS | NS | ACS | 12 months | TRIUMPH |
| Dai Z et al [T38] | 2012 | PC | Bleed, Maj Bleed, Min Bleed | 2C19*17 | 520 | 300 | 75 | PCI (BSS+ACS) | 1 month | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Depta J et al [T69] | 2015 | PC | STEMI, NSTEMI, UA, SA, Restenosis, CVA, CV Death | 2C19*2, *17 | 2062 | NS | NS | ACS | 12 months | TRIUMPH |
| Giusti B et al [T4] | 2009 | PC | ST, MACE | 2C19*2 | 772 | 600 | 75 | PCI (ACS+SCAD) | 6 months | RECLOSE |
| Guo B et al [T68] | 2014 | PC | Vasc surg, thrombosis, death, ischaemia | 2C19*2/*3 | 50 | NS | 75 | PAD | 12 months | |
| Harmsze AM et al [T18] | 2010 | CC | ST | 2C19*2, *3; 2C9*2, *3; 3A4*1B; 3A5*3; ABCB1 C1236T, G2677T/A, C3435T; P2Y1 A1622G | 596 | NS | 75 | PCI (ACS+SCAD) | 12 months | |
| Hokimoto S et al [T60] | 2014 | PC+RCT | CV Death, MACE, CVA, UA, MI, Revasc | 2C19*2/*3 | 174 | 300 | 75 | PCI (ACS+SCAD) | 18 mths | |
| Hulot JS et al [T29] | 2011 | PC | MACE | 2C19*2; PON1 L55M, Q192R | 371 | 300-900 | 75 | ACS | Up to 6 years | CLOVIS-2, AFIJI |
| Jeong YH et al [T31] | 2011 | PC | MACE, Bleed | 2C19*2, *3; ABCB1 C3435T | 266 | 600 | 75 | PCI (ACS) | >12 months | ACCEL-AMI |
| Jia DM et al [T51] | 2013 | PC | Funct Status | 2C19*2/*3; 3A4 C894T; | 259 | NS | 75 | CVA | 6 months | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | P2Y12 C34T, G52T | | | | | |
| Kang YH et al [T50] | 2013 | CC | MACE, Bleed | 2C19*2; PON1 -108C>T, -126C>G, -162A>G, L55M, Q192R | 538 | 300 | 75 | PCI (ACS) | 12 months |
| Kim HS et al [T56] | 2013 | PC | MACE | 2C19*2, *3, *17 | 2188 | 300-600 | NS | PCI (ACS+SCAD) | 12 months |
| Konishi A et al [T74] | 2015 | PC | Death, ST, TLR, TVR | 2C19*2/*3 | 196 | NS | 75 | PCI (ACS+SCAD) | >450 days |
| Li S et al [T78] | 2015 | PC | MACE, CV Death, MI, ST, TLR | 2C19*2 | 198 | NS | 75 | PCI (ACS+SCAD) | 12 months |
| Liang ZY et al [T53] | 2013 | PC | CV Death, ST, Bleed, CVA, MI, MACE | 2C19*2, *3, *17; 3A4 rs2242480C>T, rs2404955G>A, rs2246709A>G, rs4646437C>T; 3A5 rs3800959T>C, 15524T>C; P2Y12 34C>T, 52G>T, 744T>C; ABCB1 C3435T | 1106 | 600 | 75 | PCI (ACS) | 12 months |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lin Y et al [T59] | 2014 | PC | In-stent restenosis | P2Y12 C34T, G52T; 3A4 C894T; 2C19*2, *3 | 90 | NS | 75 | VAS | > 12 months | |
| Malek LA et al [T17] | 2010 | PC | Death | 2C19*2 | 261 | 300-600 | 75 | PCI (ACS) | 4 years | |
| Marcucci R et al [T36] | 2012 | PC | MACE | 2C19*2 | 1187 | 600 | 75 | PCI (ACS) | 6 months | |
| Martinez-Quintana E et al [T61] | 2014 | RC | CV Relapse | 2C19*2/*3 | 263 | NS | 75 | ACS+SCAD | 12 months | |
| McDonough CW et al [T81] | 2015 | PC | CVA, Bleed | 2C19*2/*17 | 522 | NA | 75 | CVA | VAR, median 3.2 yrs | SPS3-GENES |
| Mega JL et al [T15] | 2010 | PC | MACE, Bleed, ST, CV Death, MI, CVA | ABCB1 C3435T, G2677T/A, C1236T | 1471 | 300 | 75 | PCI (ACS) | 450 days | TRITON-TIMI 38 |
| Mega JL et al [T32] | 2011 | RCT | MACE | 2C19*2 | 333 | NS | 75-300 | SCAD | 30 days | ELEVATE-TIMI 56 |
| Mega JL et al [T7] | 2009 | PC | MACE, Bleed, ST, CV Death, MI, CVA | 2C19; 2C9; 2B6; 3A5; 3A4; 1A2 | 1459+162 | 300 | 75 | PCI (ACS)+HV | 450 days | TRITON-TIMI 38 |
| Mizobe M et al [T67] | 2014 | PC | CV Death, MACE, MI, CVA, Revasc, Interproc event | 2C19*2/*3 | 519 | 300 | 75 | PCI (ACS+SCAD) | > 12 months | |
| Nagashima Z et al [T54] | 2013 | PC | CV Death, MI, MACE | 2C19*2/*3 | 177 | 300 | 75 | PCI (ACS) | 12 months | |
| Nishio R et al [T41] | 2012 | PC | Death, ST, MI, MACE, TLR | 2C19 | 160 | 300 | 75 | PCI (ACS+SCAD) | 3 years | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Nishio R et al [T57] | 2013 | PC | ST, TLR | 2C19*2, *3 | 112 | 300 | 75 | PCI (ACS+SCAD) | 12 months | |
| Oh IY et al [T26] | 2012 | PC | MACE, Revasc, MI, CV Death, ST | 2C19*2 | 2146 | 300-600 | 75 | PCI (ACS+SCAD) | 12 months | SKY |
| Palmerini T et al [T73] | 2014 | PC | MACE | 2C19*2, *3, *4, *17; 3A4*1G, *1B; 3A5*3; ABCB1 C3435T; IRS1 A227382808C, A227497991G; PON1 A163T, A575G; ITGB3 T196C | 750 | 300-600 | 75 | PCI (ACS) | 12 months | GEPRESS |
| Pare G et al [T16] | 2010 | PC | MACE, Bleed | 2C19 | 2549+570 | 300 | 75 | ACS+AF | 12 months | ACTIVE-A, CURE |
| Park KW et al [T40] | 2012 | PC | MACE | 2C19*2; 3A5*3 | 1258 | 300-600 | 75 | PCI (ACS+SCAD) | 12 months | CROSS-VERIFY |
| Peng Y et al [T46] | 2013 | PC | Death, Revasc, MACE, CV Death | 2C19*2 | 506 | 300 | 75 | ACS+SCAD | 12 months | |
| Qiu L et al [T75] | 2015 | PC | Poor prognosis | 2C19*2/*3 | 211 | NS | 75 | CVA | 6 months | |
| Rideg O et al [T27] | 2011 | RCT | MACE | 2C19*2, *3, *17; ABCB1 C3435T, G2677T/A; PON 1 Q192R | 189 | 600 | 75-150 | PCI (SCAD) | 12 months | DOSER |

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|---|---|---|---|---|---|---|---|---|---|---|
| Rothenbacher D et al [T55] | 2013 | EPID | MACE | 2C19*2 | 1050 | NS | 75 | SCAD | 8 years | |
| Sawada T et al [T20] | 2011 | PC | Death, MI, TVR, TLR, ST(OCT) | 2C19*2 | 100 | 300 | 75 | PCI (ACS+SCAD) | VAR - over 200 days | |
| Sen HM et al [T80] | 2014 | PC | CVA | 2C19*2 | 51 | NA | 75 | CVA | 12 months | |
| Shetkar S et al [T64] | 2014 | PC | MACE | 2C19*2/*3/*17 | 110 | NS | NS | PCI (ACS+SCAD) | NS | |
| Shuldiner AR et al [T3] | 2009 | PC | MACE | 2C19*2 | 227+429 | 300-600 | 75 | PCI (SCAD) +HV | 12 months | AMISH PAPI, SINAI, CLEAR PLATELETS |
| Sibbing D et al [T22] | 2011 | CC+PC | ST | 2C19*2; PON1 Q192R | 1439 | 600 | 75 | PCI (ACS+SCAD) | 30 days | |
| Sibbing D et al [T1] | 2010 | PC | ST, Bleed, MI, MACE | 2C19*17 | 1524 | 600 | 75 | PCI (ACS+SCAD) | 30 days | |
| Sibbing D et al [T5] | 2009 | PC | Death, ST, CVA, MACE, MI, STEMI, NSTEMI | 2C19*2 | 2485 | 600 | 75 | PCI (ACS) | 30 days | ISAR (REACT, SMART2, SWEET, REACT2) |
| Siller-Matula JM et al [T34] | 2011 | PC | ST, Bleed | 2C19*2, *17 | 416 | 600 | 75 | PCI (ACS+SCAD) | 12 months | PEGASUS-PCI |
| Simon T et al [T21] | 2011 | NA | NA | NA | NA | NA | NA | NA | NA | FAST-MI |
| Simon T et al [T28] | 2011 | PC | MACE, MI, CVA, Death, Bleed | PON1 Q192R | 2432 | 300-900 | 75 | ACS | 12 months | FAST-MI |
| Simon T et al [T8] | 2009 | PC | MACE | 2C19*2, *3, *4, *5, *17; 3A5*3; P2Y12 C34T; ITGB3; ABCB1 C3435T | 2208 | 300-900 | 75 | ACS | 12 months | FAST-MI |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Spiewak M et al [T2] | 2009 | PC | MACE | ABCB1 C3453T | 98 | 300-600 | 75 | PCI (ACS) | 24 months |
| Spokoyny I et al [T52] | 2013 | RC | CVA | 2C19 | 43 | NS | 75 | CVA | NS |
| Suh JW et al [T10] | 2006 | PC | MACE, Death, MI, CVA | 3A5*3 | 348 | 300 | 75 | PCI (ACS+SCAD) | 6 months |
| Sun B et al [T77] | 2015 | PC | MACE | 2C19*2; 3A5*3; P2Y12 T744C | 118 | 300 | 75 | PCI (ACS) | 6 months |
| Sun W et al [T71] | 2014 | PC | CV Death, Bleed, CVA, MI, MACE | 2C19*2/*3, *17 | 625 | NS | 75 | CVA | 12 months |
| Syros G et al [T12] | 2009 | PC | MACE, Bleed, MI | P1A1/A2 | 200 | NS | NS | PCI (SCAD) | 12 months |
| Tabata N et al [T79] | 2015 | PC | MACE, CV Death, MI, CVA, UA, Revasc | 2C19*2/*3 | 434 | 300 | 75 | PCI+SCAD | 1-3 years |
| Tang N et al [T76] | 2015 | PC | MACE | 2C19*2/*3 | 178 | 300 | 75 | PCI (ACS) | 6 months |
| Tang XF et al [T45] | 2013 | PC | Bleed, MACE | 2C19; PON1 Q192R; ABCB1 C3435T | 670 | 300 | 75 | PCI (ACS+SCAD) | 12 months |
| Tang XF et al [T48] | 2013 | PC | Bleed, MACE | 2C19*2; P2Y12 C34T | 577 | 300 | 75 | PCI (ACS) | 12 months |
| Teixera R et al [T35] | 2012 | PC | MACE | 2C19*2 | 95 | VAR | 75 | ACS | VAR - median 136 days |
| Tiroch KA et al [T19] | 2010 | PC | Death, ST, MACE, MI, CVA | 2C19*2, *17; ABCB1 rs1045642 | 928 | 600 | 75 | PCI (ACS) | 12 months |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Tousoulis D et al [T47] | 2013 | PC | ST, Bleed | 2C19*2 | 353 | NS | 75 | SCAD | 2 years | |
| Trenk D et al [T24] | 2011 | PC | ST, MACE | PON1 Q192R | 760 | 600 | 75 | PCI (SCAD) | 12 months | EXCELSIOR |
| Trenk D et al [T9] | 2008 | PC | MACE, ST | 2C19*2 | 797 | 600 | 75 | PCI (SCAD) | 12 months | EXCELSIOR |
| Verschuren JJW et al [T42] | 2013 | CC | MACE | COX1 -824A>G; P2Y1 893C>T; GP1a 807C>T; GPIIIa rs8069732T>C; 2C19*2, rs11188072; ABCB1 C3435T; PON1 576A>G | 1327 | 600 | 75 | PCI (ACS) | 12 months | MISSION-AMI |
| Wallentin L et al [278] | 2010 | PC | MACE, ST Bleed | 2C19*2; ABCB1 C3435T | 5148 | 300-600 | 75 | ACS | 12 months | PLATO |
| Wei Y et al [T82] | 2015 | PC | Rec Angina, MI, CVA, ST, CV Death | 2C19*2 | 110 | 300 | 75 | PCI+ACS | 12 months | |
| Wu H et al [T43] | 2012 | PC | MI | 2C19*2/*3 | 233 | 300 | 75 | PCI (ACS) | Peri-procedure | |
| Xie C et al [T63] | 2014 | PC+CC | ST | CES1A2 -816A/C; 2C19*2/*3; PON1 Q192R; ABCB1 C3435T | 104 | 300-600 | 75 | PCI (ACS+SCAD) | 12 months | |
| Xie X et al [T49] | 2013 | PC | Death, ST, Bleed, MI, MACE | 2C19 | 1068 | 600 | 75 | PCI (ACS +SCAD) | 12 months | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Yoshimura H et al [T66] | 2014 | CC | MI | 2C19*2/*3 | 121 | 300 | 75 | PCI (SCAD) | 28 days |
| Yuo L et al [T33] | 2011 | PC | ST, CV Death, MI, NSTEMI, CVA, Bleed, STEMI | 2C19*2 | 1738 | 300 | 75 | PCI (ACS+SCAD) | 180 days |
| Zhang JH et al [T72] | 2014 | PC | MACE, Bleed | ABCB1 C3435T, multiple SNPs | 452 | 300 | 75 | PCI (ACS) | 12 months |
| Ziegler S et al [T11] | 2005 | PC | MACE | P2Y12 C34T, G52T | 473 | NS | 75 | PAD | 2 years |
| Zou JJ et al [T44] | 2013 | PC | ST | 2C19 | 617 | 300 | 75 | PCI (ACS+SCAD) | 12 months |
| Zou JJ et al [T62] | 2014 | PC | ST | CES1A2 -816A/C | 249 | 300 | 75 | PCI (ACS+SCAD) | 12 months |

**Table 3.4 - Characteristics of the studies included in meta-analysis** (For all T references, please refer to Appendix 2).

| MA | Outcome | Gene | Potential Studies | Combinable Studies | References | N | HR | 95% CI | I^2 |
|---|---|---|---|---|---|---|---|---|---|
| **1** | CV DEATH | 2C19*2 | 9 | 2 | T6, T26 | 2405 | 3.64 | 0.99 to 0.75 | 0% |
| **2** | DEATH | 2C19*2 | 7 | 2 | T5, T17 | 2746 | 1.35 | 0.61 to 3.00 | 40.20% |
| **3** | MACE | 2C19*2 | 24 | 10 | T3, T5, T6, T9, T25, T26, T35, T36, T42, T50 | 9347 | 1.80 | 1.33 to 2.43 | 62.78% |
| **4** | MI | 2C19*2 | 10 | 2 | T5, T6 | 2744 | 2.33 | 0.50 to 10.84 | 87.16% |
| **5** | REVASC | 2C19*2 | 3 | 3 | T5, T26, T46 | 2911 | 1.13 | 0.87 to 1.46 | 18.40% |
| **6** | ST | 2C19*2 | 19 | 6 | T4, T5, T6, T7, T26, T34 | 7429 | 3.20 | 1.79 to 5.72 | 30.19% |
| **7** | BLEED | 2C19 | 6 | 4 | T7, T37, T49, T71 | 5413 | 0.81 | 0.70 to 0.93 | 0% |
| **8** | CV DEATH | 2C19 | 7 | 2 | T7, T71 | 2085 | 5.62 | 1.92 to 13.30 | 0% |
| **9** | MACE | 2C19 | 17 | 11 | T7, T8, T14, T16, T37, T56, T58, T60, T70, T71, T79 | 19288 | 1.39 | 1.14 to 1.71 | 69.48% |
| **10** | MAJ BLEED | 2C19 | 4 | 2 | T16, T37 | 5364 | 1.04 | 0.80 to 1.35 | 57.80% |
| **11** | ST | 2C19 | 10 | 2 | T7, T49 | 2457 | 3.20 | 1.72 to 5.98 | 0% |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **12** | MACE | ABCB1 C3435T | 11 | 3 | T8, T15, T25 | 3959 | 1.39 | 0.99 to 1.95 | 39.80% |
| **13** | MACE | 3A5*3 | 7 | 2 | T7, T25 | 1581 | 1.06 | 0.59 to 1.91 | 16.12% |
| **14** | MACE | PON1 Q192R | 8 | 4 | T24, T28, T29, T50 | 4393 | 1.00 | 0.75 to 1.31 | 20.45% |

**Table 3.5 – Summary of all meta-analyses** (For all T references, please refer to Appendix 2).

### 3.3.4: *CYP2C19*2* Polymorphism and Clinical Outcome Assessment

*CYP2C19*2* and Cardiovascular Death

A total of 9 studies (Collet et al., 2009, Depta et al., 2015, Giusti et al., 2009, Li et al., 2015b, Luo et al., 2011, Oh et al., 2012, Peng et al., 2013, Sun et al., 2015a, Wei et al., 2015) were eligible to be included in this meta-analysis. Only three studies (Collet et al., 2009, Oh et al., 2012, Peng et al., 2013) reported sufficient data for inclusion and extraction or calculation of hazard ratios. Study 471 (Oh et al., 2012) presented hazard ratios for three categorical groups and therefore could not be included in a dominant model. Consequently, a meta-analysis of two studies (Collet et al., 2009, Oh et al., 2012), including 2,405 patients, was conducted which did not detect a significant association between cardiovascular death and carriage of the *CYP2C19*2* allele (HR 3.64; 95% CI 0.99 to 7.05). No significant heterogeneity was detected in this meta-analysis, with similar patient groups and clopidogrel loading doses used in the studies included in this meta-analysis (**Figure 3.2**).

*CYP2C19*2* and all-cause mortality

Seven studies (Campo et al., 2011, Cresci et al., 2014, Malek et al., 2010, Peng et al., 2013, Sawada et al., 2011, Sibbing et al., 2009, Tiroch et al., 2010) were potentially suitable for inclusion in this meta-analysis. However, only three studies (Malek et al., 2010, Peng et al., 2013, Sibbing et al., 2009) reported sufficient data for extraction or calculation of hazard ratio. Peng et al (Peng et al., 2013) presented hazard ratios for three categorical groups and therefore could not be included in a dominant model. Consequently, a meta-analysis of the two remaining studies (Sibbing et al., 2009, Malek et al., 2010) and 2,746 patients demonstrated no significant association between the *CYP2C19*2* polymorphism and risk of all-cause mortality (HR 1.35; 95% CI 0.61 to 3.00). Moderate heterogeneity was detected (I^2=40.2) which could be explained by the difference in follow up periods between the studies (Study 56 (Sibbing et al., 2009) – 30 days; Study 287 (Malek et al., 2010) – maximum of 50 months) (**Figure 3.3**).

*CYP2C19*2* and major adverse cardiovascular events (MACE)

A total of 24 studies (Campo et al., 2011, Collet et al., 2009, Giusti et al., 2009, Kang et al., 2013, Marcucci et al., 2012, Kim et al., 2013, Li et al., 2015b, Mega et al., 2009, Mega et al., 2011, Oh et al., 2012, Palmerini et al., 2014, Peng et al., 2013, Rideg et al., 2011, Rothenbacher et al., 2013, Shuldiner et al., 2009, Sibbing et al., 2009, Simon et al., 2009, Sun et al., 2015a, Tang et al., 2013a, Tang et al., 2013b, Teixeira et al., 2012, Tiroch et al., 2010,

Trenk et al., 2008, Verschuren et al., 2013) were identified as potentially eligible for inclusion into this meta-analysis. Six studies were excluded as they did not report survival analysis, and a further eight studies were excluded as they presented survival data for three categorical genotype groups which were not combinable into a dominant model. Consequently, a meta-analysis of the remaining 10 studies, which included 9,347 patients, demonstrated a significant association between carriage of the *CYP2C19*2* allele and risk of MACE (HR 1.80; 95% CI 1.33 to 2.43) (Campo et al., 2011, Collet et al., 2009, Kim et al., 2013, Marcucci et al., 2012, Oh et al., 2012, Shuldiner et al., 2009, Sibbing et al., 2009, Teixeira et al., 2012, Trenk et al., 2008, Verschuren et al., 2013). There was significant heterogeneity observed in this meta-analysis, with a number of different patient types (ACS, stable disease) and clopidogrel loading doses included. In addition, this meta-analysis included all studies reporting MACE, irrespective of its definition. Importantly, six different definitions of MACE were used by the studies included in the meta-analysis (**Figure 3.4**)

*CYP2C19*2* and myocardial infarction

Ten studies were potentially eligible for inclusion in this meta-analysis (Collet et al., 2009, Li et al., 2015b, Luo et al., 2011, Oh et al., 2012, Sawada et al., 2011, Sibbing et al., 2009, Sun et al., 2015a, Tiroch et al., 2010, Wei et al., 2015, Wu et al., 2012). Of these ten, eight studies were excluded as no survival analysis was reported. Consequently, only two studies, which included 2,744 patients, were included in this meta-analysis (Collet et al., 2009, Sibbing et al., 2009). No significant association between carriage of the *CYP2C19*2* allele and myocardial infarction was detected (HR 2.33; 95%CI 0.50 to 10.84) although significant heterogeneity was present (I^2 = 87.16%). Similar to previous meta-analyses, there was a significant difference in the length of follow up between the two studies which may explain the degree of heterogeneity (**Figure 3.5**).

*CYP2C19*2* and revascularisation

A total of three studies were potentially eligible for inclusion in this meta-analysis (Collet et al., 2009, Oh et al., 2012, Peng et al., 2013) and all three reported data on survival in a total of 2,911 patients. However, no clear association was detected between carriage of the *CYP2C19*2* and the risk of revascularisation (HR 1.13; 95% CI 0.87 to 1.46). No significant heterogeneity was detected in this meta-analysis (**Figure 3.6**).

*CYP2C19*2* and stent thrombosis

Nineteen studies were assessed as being potentially suitable for this meta-analysis (Bouman et al., 2011, Campo et al., 2011, Chen et al., 2012b, Collet et al., 2009, Giusti et al., 2009, Li et al., 2015b, Luo et al., 2011, Mega et al., 2009, Oh et al., 2012, Sawada et al., 2011, Sibbing et al., 2009, Sibbing et al., 2011, Siller-Matula et al., 2012, Sun et al., 2015a, Tiroch et al., 2010, Tousoulis et al., 2013, Trenk et al., 2008, Wei et al., 2015). Twelve studies were excluded as they did not report survival data, and a further study was excluded due to genotype data being incomplete. Consequently, a total of six studies, including 7,429 patients, demonstrated a significant association between carriage of the *CYP2C19*2* allele and the risk of stent thrombosis (HR 3.20; 95% CI 1.79-5.72). Heterogeneity was relatively low with an I^2 value of 30.19%. (**Figure 3.7**).

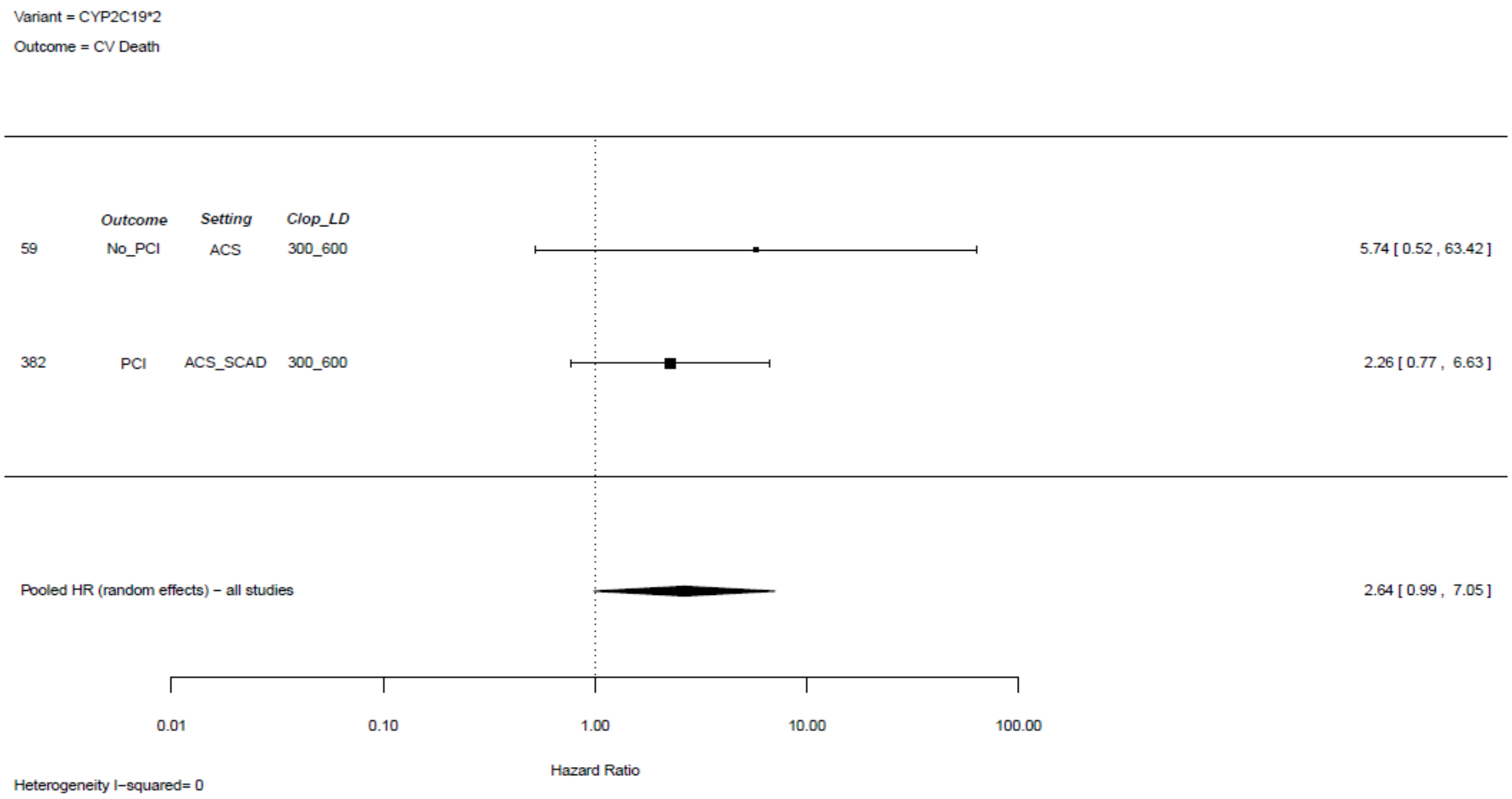


**Figure 3.2 – Meta-analysis of CYP2C19*2 and cardiovascular death** (59 - Collet et al, 2009; 382 - Oh et al, 2012)
No significant association detected between carriage of the CYP2C19*2 allele and cardiovascular death

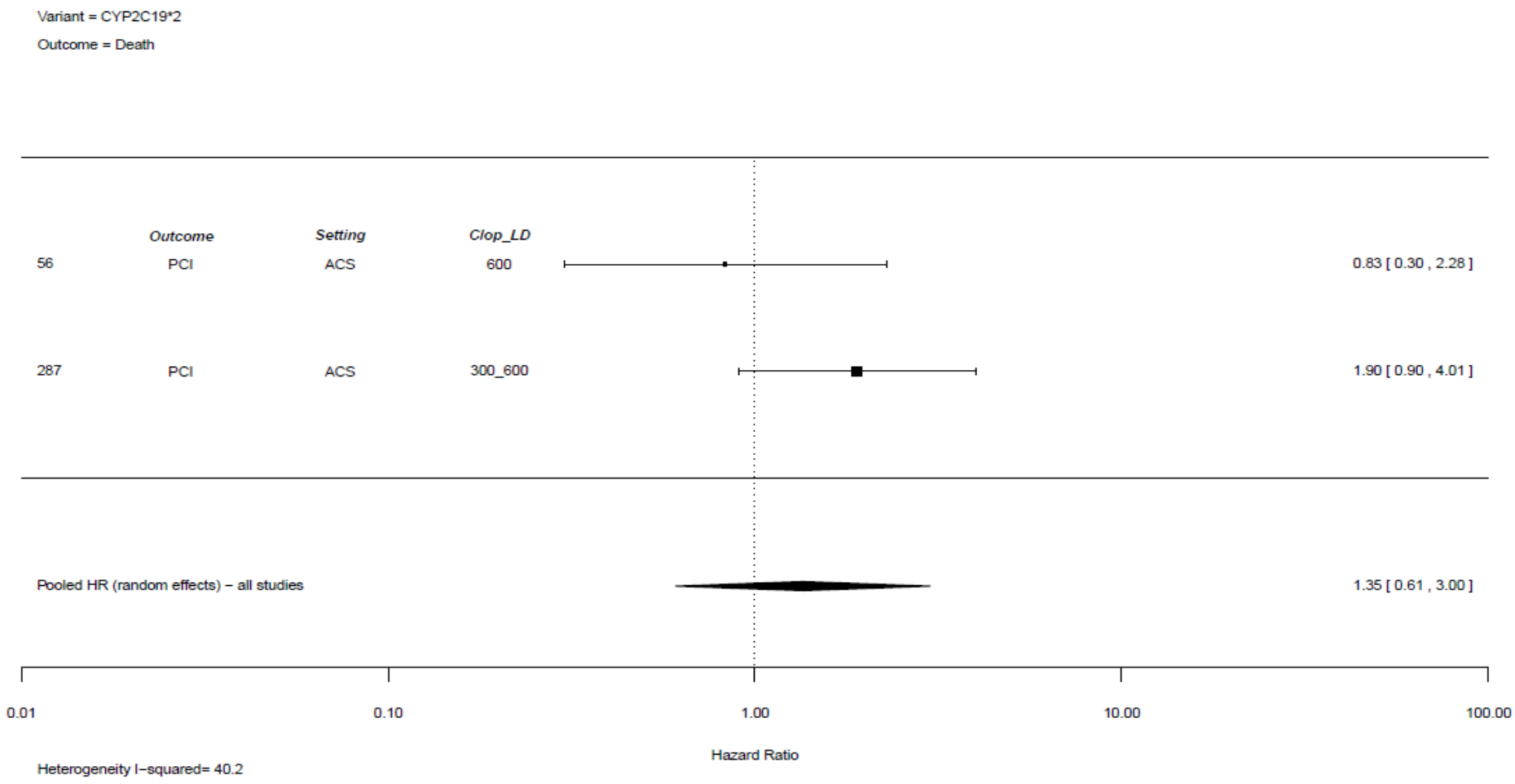


**Figure 3.3 – Meta-analysis of CYP2C19*2 and all-cause mortality** (56 – Sibbing et al, 2009; 287 – Malek et al, 2010)
No significant association detected between carriage of the CYP2C19*2 allele and all-cause mortality

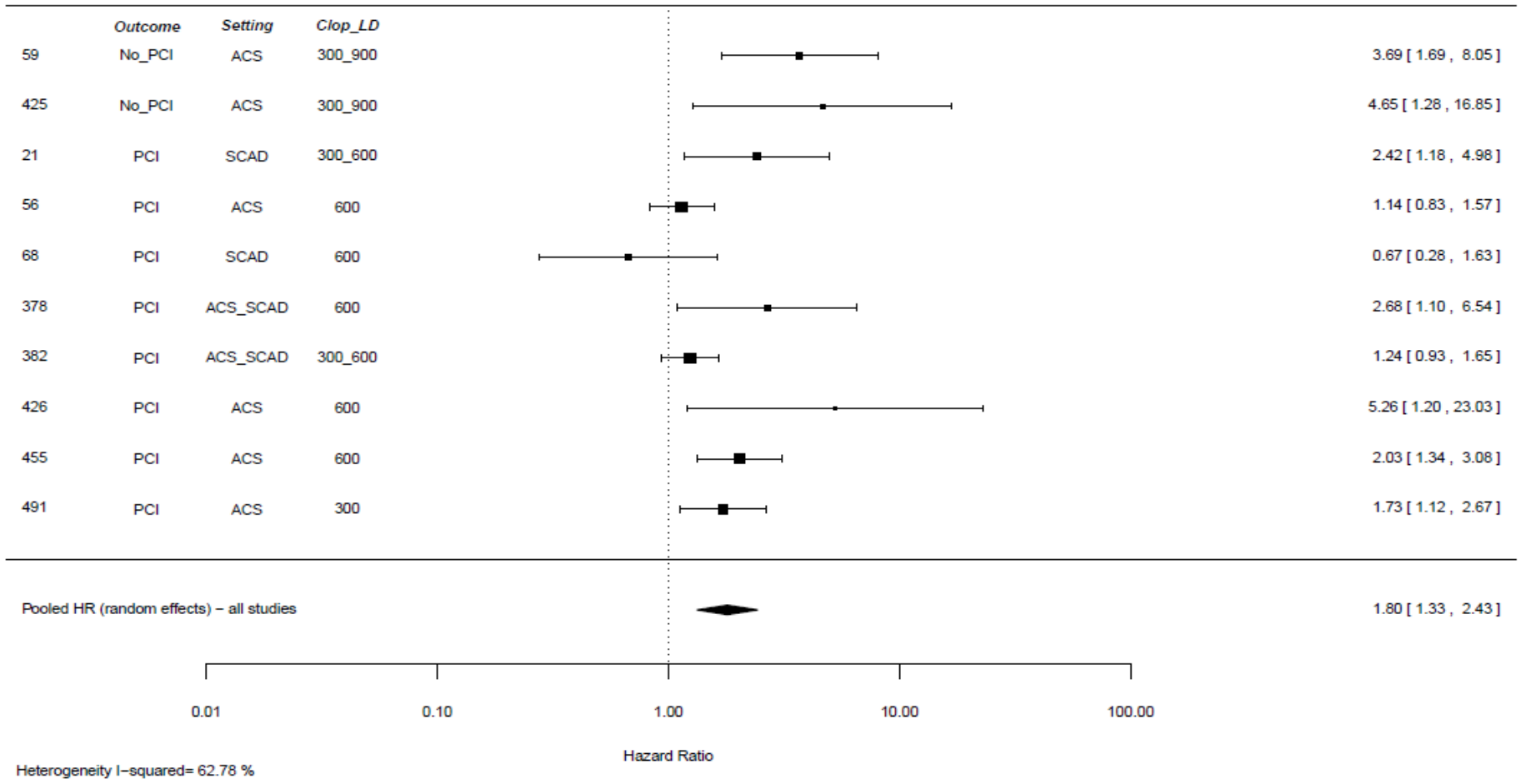


**Figure 3.4 – Meta-analysis of CYP2C19*2 and MACE** (59 – Collet et al, 2009; 425 – Teixera et al, 2012; 21 – Shuldiner et al, 2009; 56 – Giusti et al, 2009; 68 – Trenk et al, 2008; 378- Campo et al, 2011; 382 – Oh et al, 2012; 426 – Marcucci et al, 2012; 455 – Verschuren et al, 2013; 491 – Kang et al, 2013) CYP2C19*2 allele carriers have a significantly higher risk of MACE

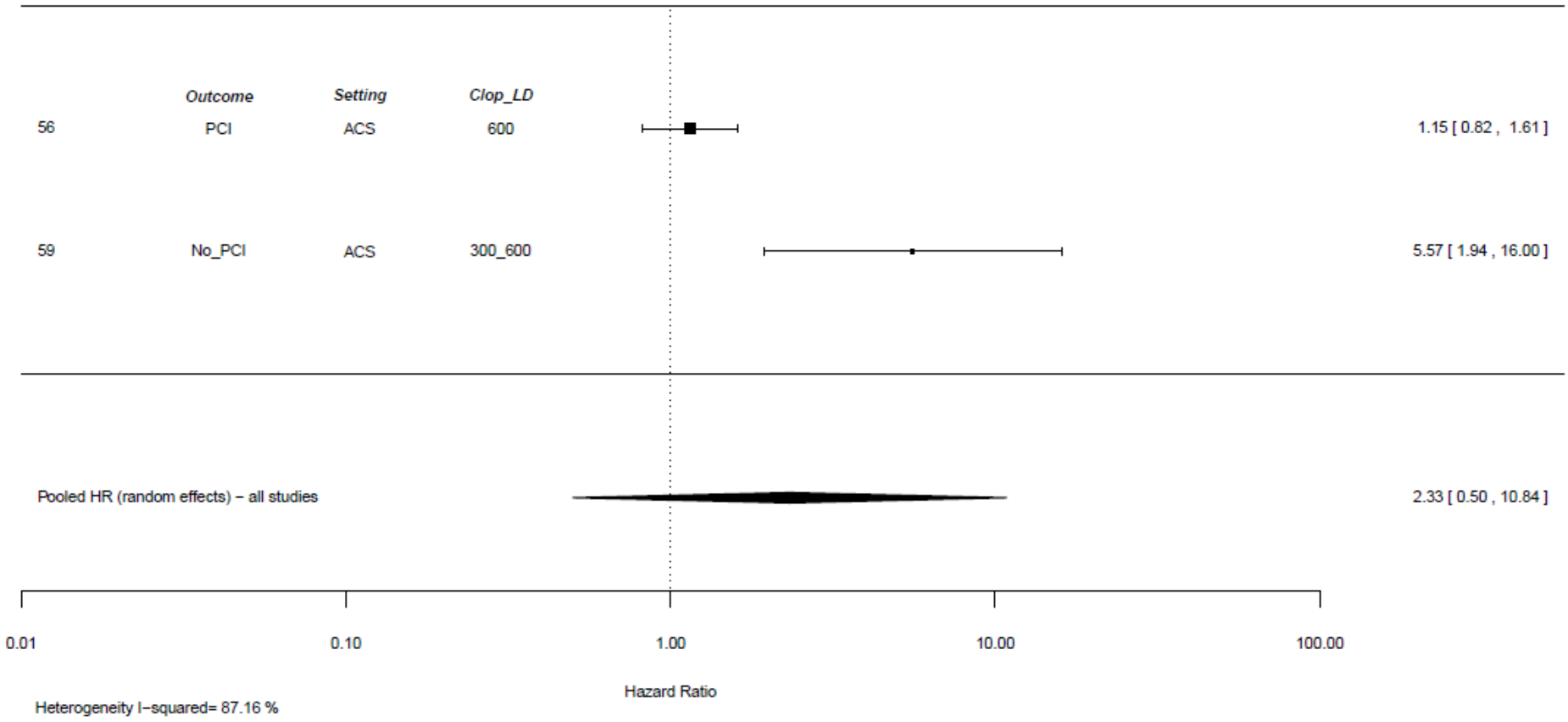


**Figure 3.5 – Meta-analysis of CYP2C19*2 and myocardial infarction** (56 – Sibbing et al, 2009; 59 – Collet et al, 2009)
No significant association detected between carriage of the CYP2C19*2 allele and myocardial infarction

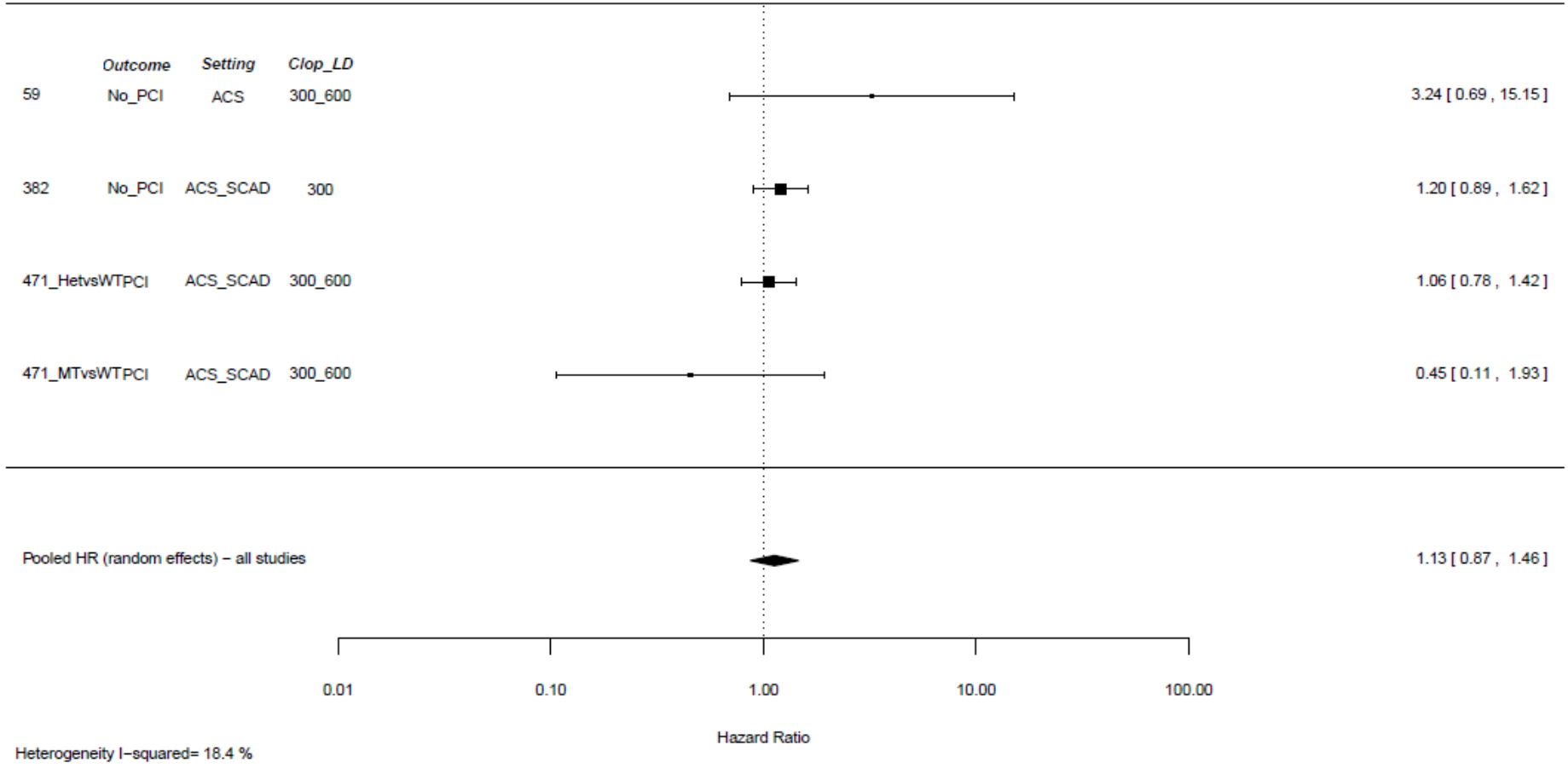


**Figure 3.6 – Meta-analysis of CYP2C19*2 and revascularisation** (59 – Collet et al, 2009; 382 – Oh et al, 2012; 471 – Peng et al, 2013)
No significant association detected between carriage of the CYP2C19*2 allele and revascularisation

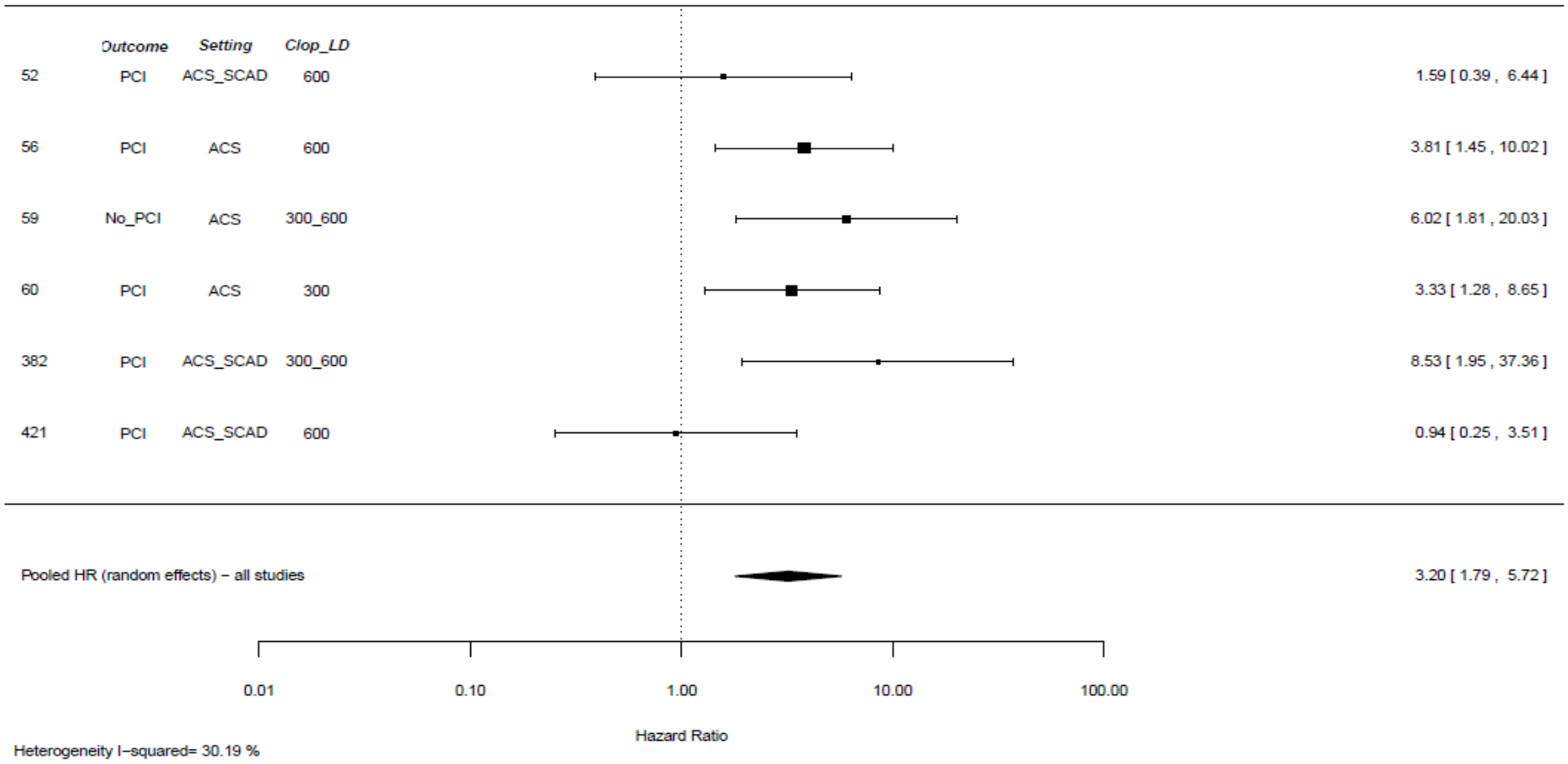


**Figure 3.7 – Meta-analysis of CYP2C19*2 and stent thrombosis** (52 – Giusti et al, 2009; 56 – Sibbing et al, 2009; 59 – Collet et al, 2009; 60 – Mega et al, 2009; 382 – Oh et al, 2012 421 – Siller-Matula et al, 2011)
CYP2C19*2 allele carriers have a significantly higher risk of stent thrombosis

### 3.3.5 *CYP2C19* metaboliser phenotype and Clinical Outcome Assessment

Studies that combined the loss-of-function CYP2C19*2 and *3 alleles together in a single analysis were defined as *CYP2C19* metaboliser phenotype and reported in the meta-analyses reported below.

*CYP2C19* and bleeding

Six studies were potentially combinable in this meta-analysis (Bhatt et al., 2012, Liang et al., 2013, Mega et al., 2009, McDonough et al., 2015, Sun et al., 2015b, Xie et al., 2013). However, two studies were not combinable either due to incomplete genotype data for the LOF alleles or not reporting survival data. Consequently, four studies (Bhatt et al., 2012, Mega et al., 2009, Sun et al., 2015b, Xie et al., 2013), which included 5,413 patients, were included in a meta-analysis which detected a significant reduction in the risk of bleeding events in carriers of any *CYP2C19* LOF alleles (HR 0.81; 95% CI 0.70 to 0.93). No heterogeneity was observed in this meta-analysis (I^2 = 0%) (**Figure 3.8**).

*CYP2C19* and cardiovascular death

A total of seven studies were identified as potentially combinable in this meta-analysis (Hokimoto et al., 2014, Liang et al., 2013, Mega et al., 2009, Mizobe et al., 2014, Nagashima et al., 2013, Sun et al., 2015b, Tabata et al., 2016a). Five studies were excluded as they did not report survival analysis data. Consequently, only two studies (Mega et al., 2009, Sun et al., 2015b), which included 2,085 patients, could be combined in this meta-analysis. A significant association between carriage of any *CYP2C19* LOF allele and the risk of cardiovascular death was detected (HR 5.62; 95% CI 1.92 to 13.30) with no significant heterogeneity detected (I^2 = 0%) (**Figure 3.9**).

*CYP2C19* and major adverse cardiovascular events

Seventeen studies were assessed as potentially eligible for inclusion in this meta-analysis (Arima et al., 2015, Bhatt et al., 2012, Hokimoto et al., 2014, Kim et al., 2013, Liang et al., 2013, Mega et al., 2009, Mizobe et al., 2014, Nagashima et al., 2013, Nishio et al., 2012, Pare et al., 2010, Simon et al., 2009, Sun et al., 2015b, Tabata et al., 2016a, Tang et al., 2015, Viviani Anselmi et al., 2013, Wallentin et al., 2010, Xie et al., 2013). Six studies were excluded due to either reporting three genotype groups that could not be combined in the survival analysis or not reporting survival data. Consequently, a total of 11 studies, representing 19,288 patients, were included in the final meta-analysis (Arima et al., 2015, Bhatt et al., 2012, Kim et al., 2013, Mega et al., 2009, Mizobe et al., 2014, Pare et al., 2010, Simon et al.,

2009, Sun et al., 2015b, Tabata et al., 2016a, Viviani Anselmi et al., 2013, Wallentin et al., 2010). A significant association between carriage of any *CYP2C19* LOF allele and the risk of MACE was observed (HR 1.39; 95% CI 1.14 to 1.71), although a significant degree of heterogeneity was also demonstrated (I^2 = 69.48%). As described in the *CYP2C19*2* MACE meta-analysis, this meta-analysis also included studies with any definition of MACE. Indeed, eight different definitions of MACE were used in the studies included in this meta-analysis, which may explain the observed heterogeneity (**Figure 3.10**).

*CYP2C19* and Major bleeding

A total of four studies were potentially combinable in this meta-analysis (Bhatt et al., 2012, McDonough et al., 2015, Pare et al., 2010, Wallentin et al., 2010). Two studies were excluded due to not reporting survival data, with the remaining two studies (Bhatt et al., 2012, Pare et al., 2010) combined in this meta-analysis. No significant association between carriage of any *CYP2C19* LOF allele and the risk of major bleeding was detected (HR 1.04; 95% CI 0.80 to 1.35) in this meta-analysis of 5,364 patients. Significant heterogeneity was detected (I^2 = 57.8%) despite both studies including similar types of patients as well as utilising the same loading dose of clopidogrel (**Figure 3.11**).

*CYP2C19* and stent thrombosis

Ten studies were assessed as being potentially eligible for inclusion in this meta-analysis (Harmsze et al., 2010b, Konishi et al., 2015, Liang et al., 2013, Mega et al., 2009, Nishio et al., 2012, Nishio et al., 2013, Wallentin et al., 2010, Xie et al., 2013, Xie et al., 2014, Zou et al., 2013). Eight studies were excluded due to not reporting survival data or presenting data in three genotype groups that were not combinable in a dominant model. Consequently, only two studies, which included 2,457 patients, were included in the final meta-analysis (Mega et al., 2009, Xie et al., 2013). A significant association was detected between carriage of any *CYP2C19* LOF allele and risk of stent thrombosis with no observed heterogeneity (I^2 = 0%) (**Figure 3.12**).

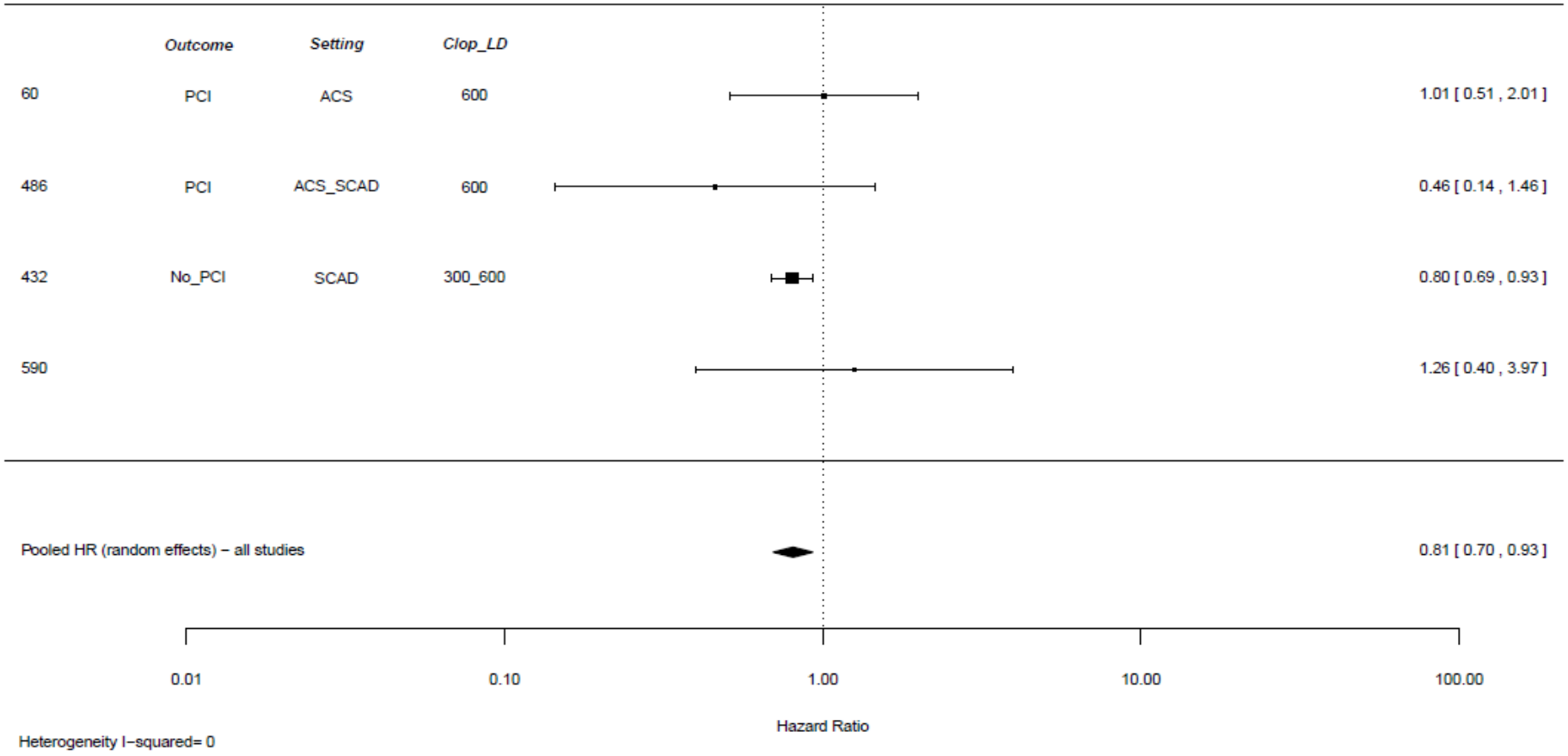


**Figure 3.8 – Meta-analysis of CYP2C19 metaboliser status and bleeding** (60 – Mega et al, 2009; 486 – Xie et al, 2013; 432 – Bhatt et al, 2012; 590 – Sun et al, 2014)
Carriers of CYP2C19 loss-of-function alleles (*2 and *3) have a lower risk of bleeding

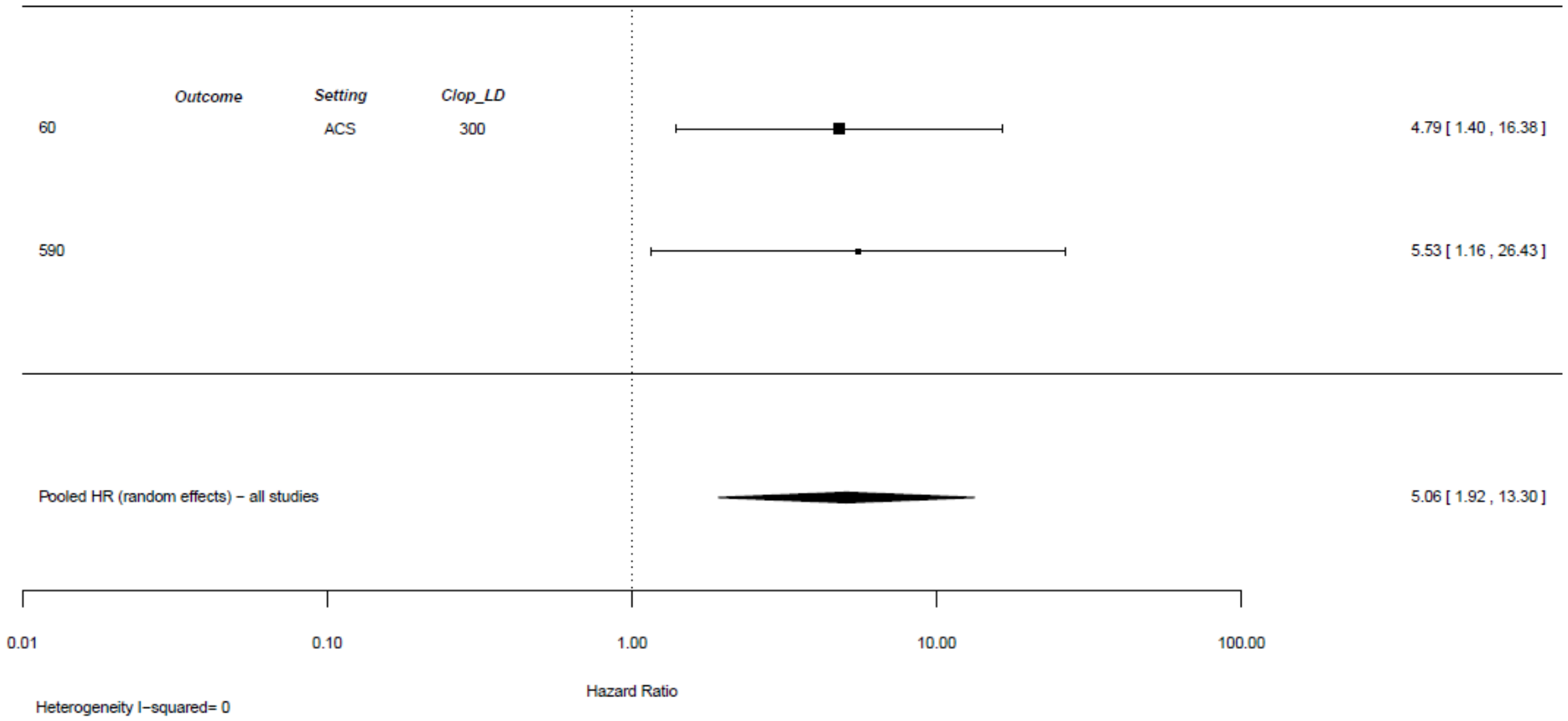


**Figure 3.9 – Meta-analysis of CYP2C19 metaboliser status and cardiovascular death** (60 – Mega et al, 2009; 590 – Sun et al, 2014)
Carriers of CYP2C19 loss-of-function alleles (*2 and *3) have a higher risk of cardiovascular death

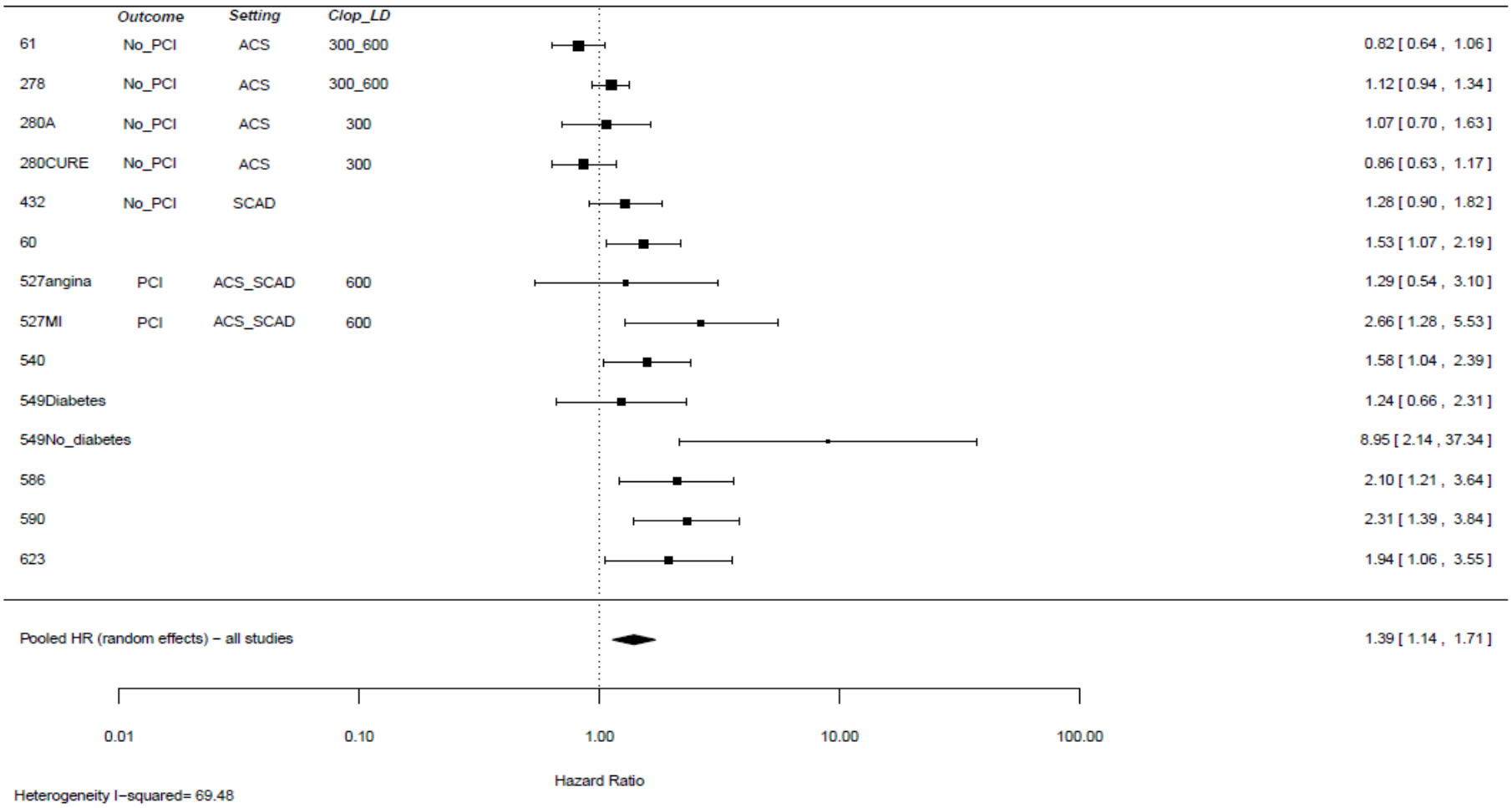


**Figure 3.10 – Meta-analysis of CYP2C19 metaboliser status and MACE** (61 – Simon, 2009; 278 – Wallentin, 2010; 280 – Pare, 2010; 432 – Bhatt, 2012; 60 – Mega, 2009; 527 – Kim, 2013; 540 – Anselmi, 2014; 549 – Hokimoto, 2014; 586 – Arima, 2015; 590 - Sun, 2014; 623 – Tabata, 2015)
Carriers of CYP2C19 loss-of-function alleles (*2 and *3) have a higher risk of MACE

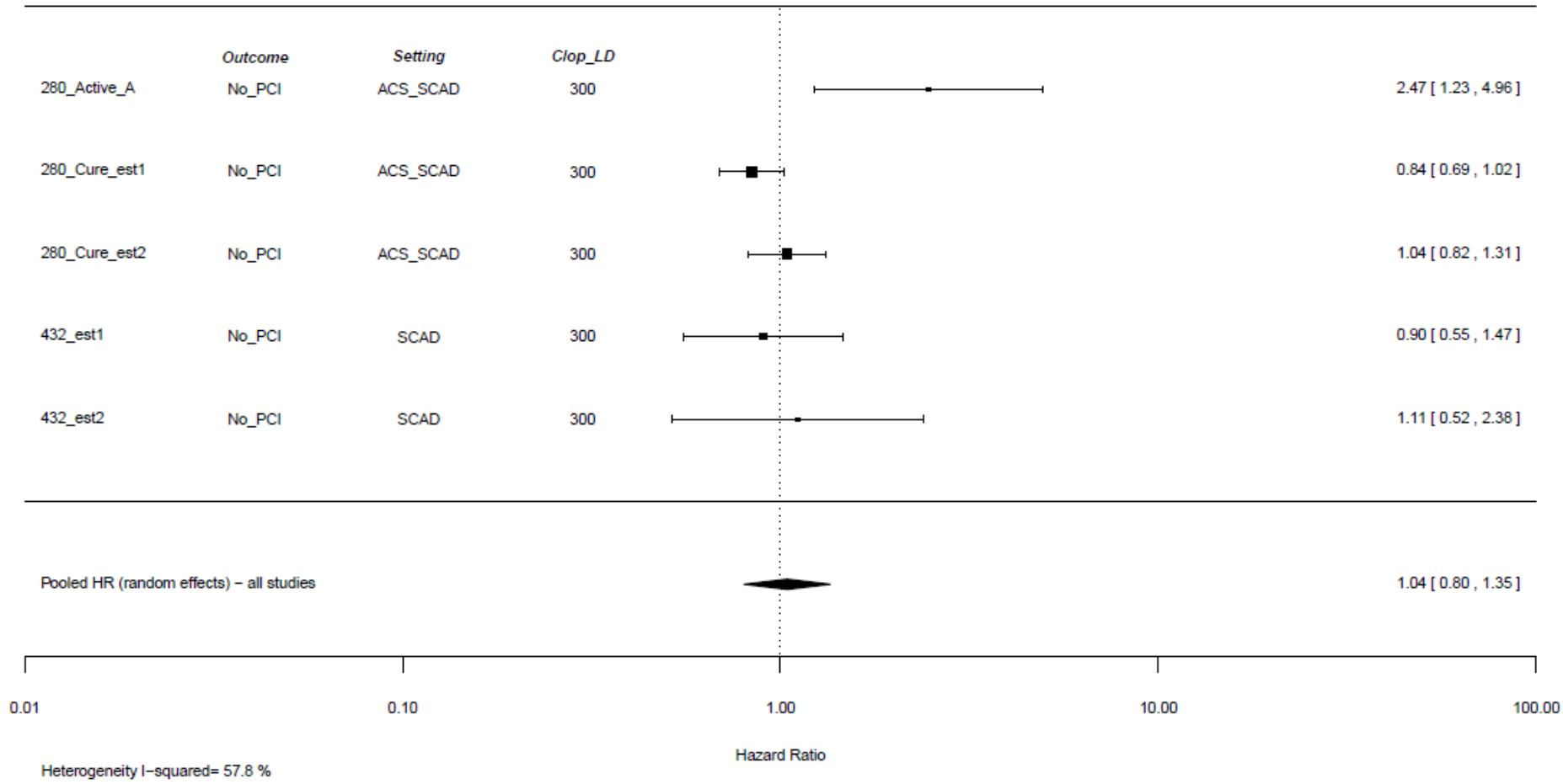


**Figure 3.11 – Meta-analysis of CYP2C19 metaboliser status and major bleeding** (280 – Pare et al, 2010; 432 – Bhatt et al, 2012)
No significant association detected between carriage of the CYP2C19 loss-of-function alleles (*2 and *3) and major bleeding

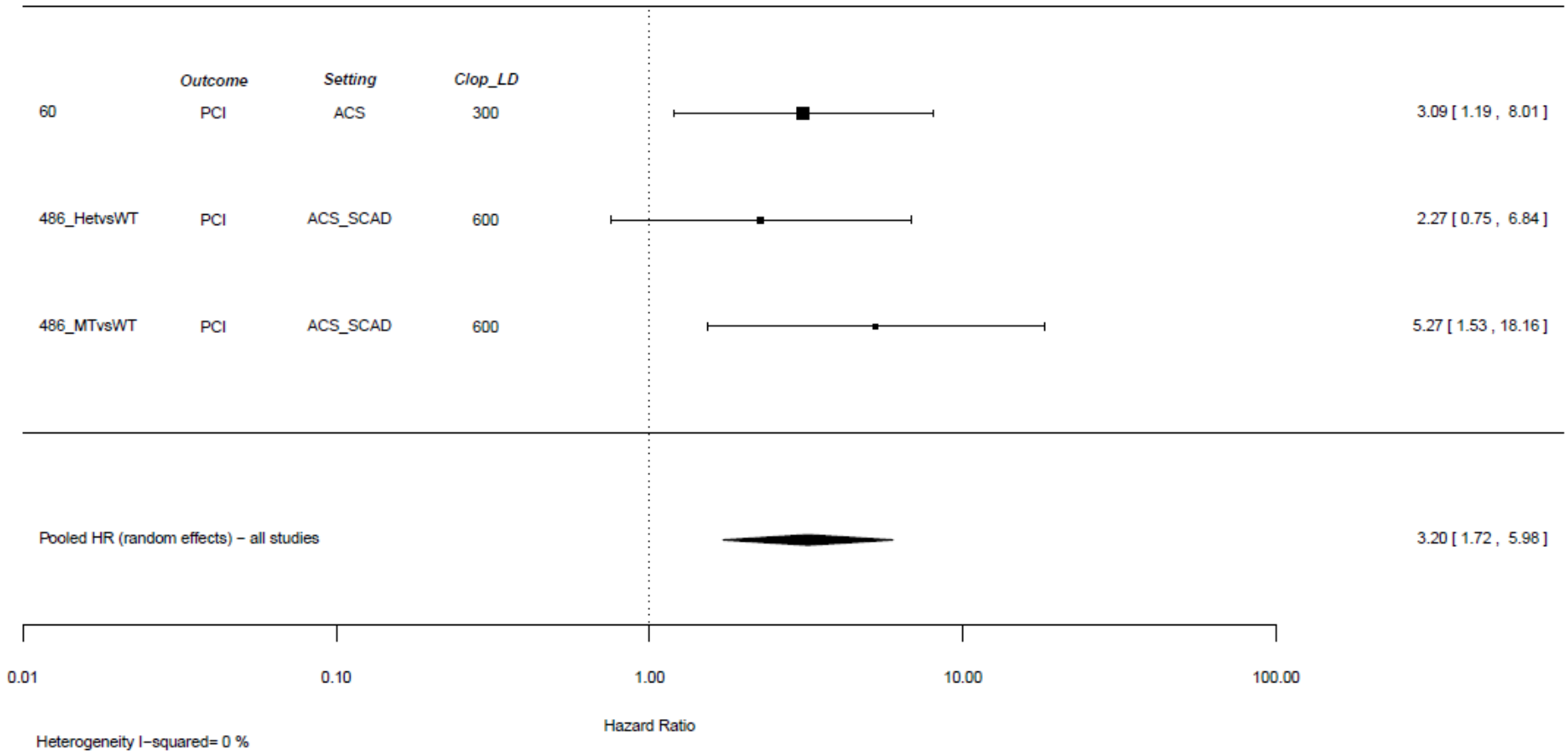


**Figure 3.12 – Meta-analysis of CYP2C19 metaboliser status and stent thrombosis** (60 – Mega et al, 2009; 486 – Xie et al, 2013)
Carriers of CYP2C19 loss-of-function alleles (*2 and *3) have a higher risk of MACE

### 3.3.6: Other Polymorphisms and Clinical Outcome Assessments

*ABCB1* C3435T and major adverse cardiovascular events

A total of twelve studies were assessed as being potentially combinable in this meta-analysis (Campo et al., 2011, Mega et al., 2010a, Palmerini et al., 2014, Simon et al., 2009, Spiewak et al., 2009, Sun et al., 2015b, Tang et al., 2013a, Tiroch et al., 2010, Verschuren et al., 2013, Viviani Anselmi et al., 2013, Wallentin et al., 2010, Zhang et al., 2014b). However, nine of these studies were excluded as a result of either not reporting survival data or reporting genotype groups that could not be combined in a dominant model. Consequently, only three studies, representing 3,959 patients, could be combined in this meta-analysis (Campo et al., 2011, Mega et al., 2010a, Simon et al., 2009). No significant association was observed between the risk of MACE and the *ABCB1* C3435T genotype. Between study heterogeneity was relatively low with an I^2 value of 39.8% (**Figure 3.13**).

*CYP3A5*3* and major adverse cardiovascular events

Seven studies were identified as potentially eligible for inclusion in this meta-analysis (Campo et al., 2011, Mega et al., 2009, Palmerini et al., 2014, Park et al., 2012, Simon et al., 2009, Suh et al., 2006, Viviani Anselmi et al., 2013). Five studies were excluded due to not reporting survival data or presenting data in groups that could not be combined into a dominant model. Two studies (Campo et al., 2011, Mega et al., 2009), which included 1,581 patients, were combined into this meta-analysis. No significant association was detected between the risk of MACE and carriage of the *CYP3A5*3* allele, with no significant heterogeneity observed (I^2 = 16.12%) (**Figure 3.14**).

*PON1* Q192R and major adverse cardiovascular events

A total of eight studies were assessed as being potentially combinable in this meta-analysis (Hulot et al., 2011, Kang et al., 2013, Palmerini et al., 2014, Simon et al., 2011b, Tang et al., 2013a, Trenk et al., 2011, Verschuren et al., 2013, Viviani Anselmi et al., 2013). Four studies were excluded as they did not report survival data. Consequently, four studies (Hulot et al., 2011, Kang et al., 2013, Simon et al., 2011b, Trenk et al., 2011), representing 4,393 patients, were included in the final meta-analysis. No significant association between the *PON1* Q192R genotype and risk of MACE was detected (HR 1.00; 95% CI 0.75 to 1.31). Again, heterogeneity was demonstrated to be very low, with a reported I^2 value of 20.45% (**Figure 3.15**).

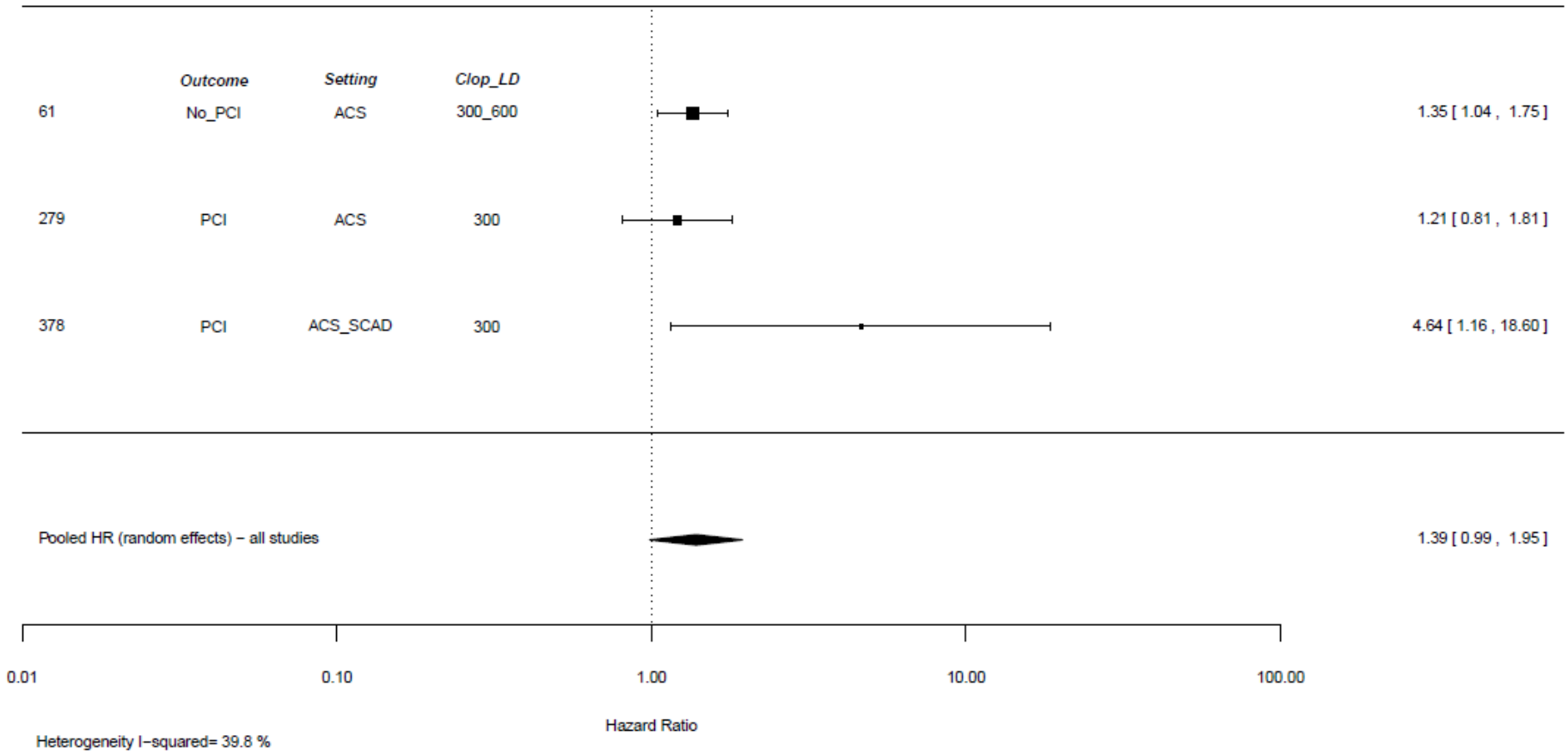


**Figure 3.13 – Meta-analysis of ABCB1 C3425T and MACE** (61 – Simon et al, 2009; 279 – Mega et al, 2010; 378 – Campo et al, 2011)
No significant association detected between carriage of the ABCB1 3435T allele and MACE

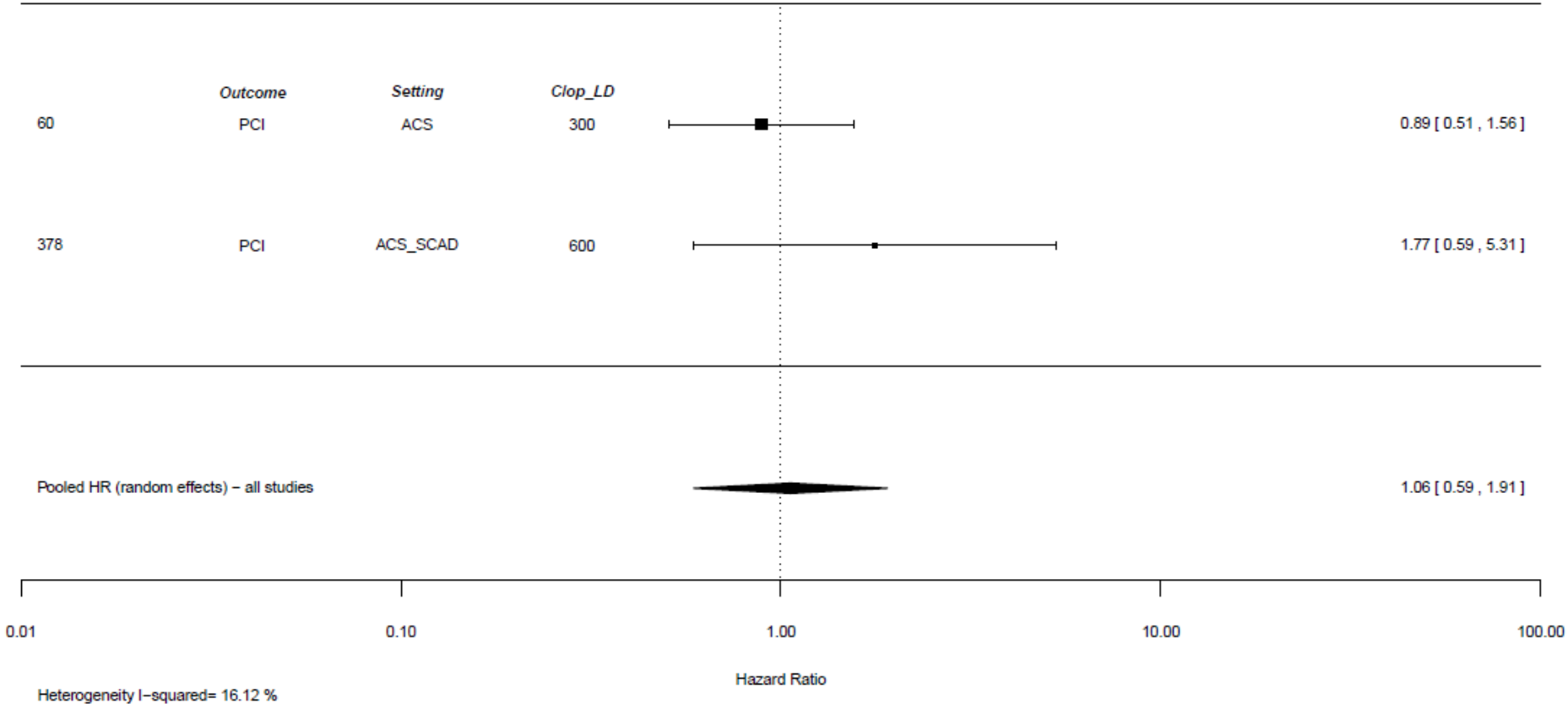


**Figure 3.14 – Meta-analysis of CYP3A5*3 and MACE** (60 – Mega et al, 2009; 378 – Campo et al, 2011)
No significant association detected between carriage of the CYP3A5*3 allele and MACE

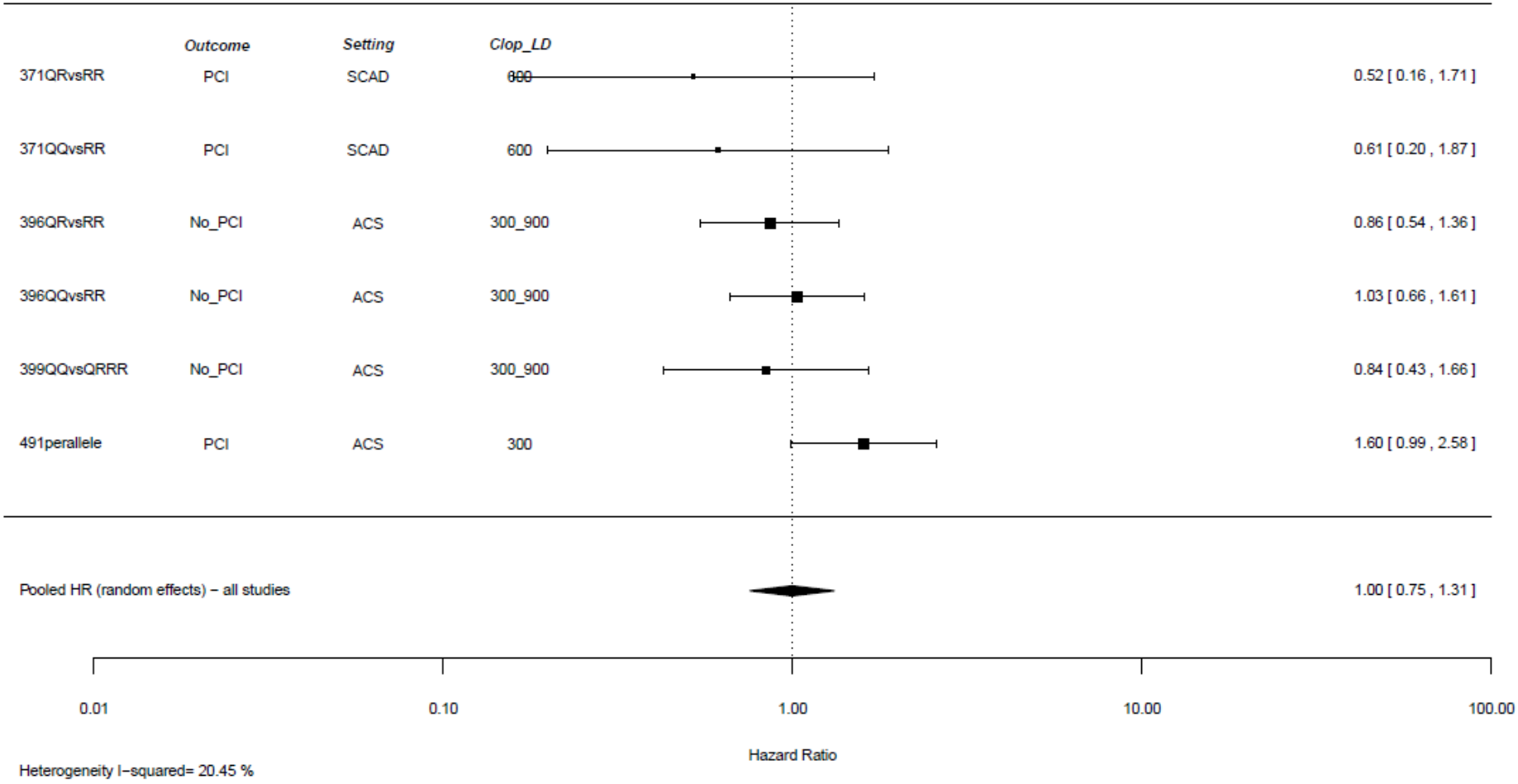


**Figure 3.15 – Meta-analysis of PON1 Q192R and MACE** (371 – Trenk et al, 2011; 396 – Simon et al, 2011; 399 – Hulot et al, 2011; 491 – Kang et al, 2013)
No significant association detected between carriage of the PON 192Q allele and MACE

### 3.4: Discussion

The major finding of this meta-analysis is that the *CYP2C19* loss-of-function alleles are associated with adverse clinical outcomes in the context of cardiovascular disease. However, in this meta-analysis, the effect of the polymorphism was not uniform with some outcomes being associated with the variant alleles and others not.

The observed association between the *CYP2C19*2* allele and clinical outcome in this meta-analysis is consistent with a number of other studies. In a genetic sub-study of the TRITON-TIMI 38 study, Mega and colleagues (Mega et al., 2009) demonstrated a 53% relative increase in the risk of MACE in carriers of the *CYP2C19*2* allele in a cohort of 1477 patients (HR 1.53; 95% CI 1.07 to 2.19, P=0.01) and a threefold increase in the risk of stent thrombosis (HR 3.09; 95%CI 1.19 to 8.00). These data have been mirrored by other large studies (Simon et al., 2009). However, in the genetic sub-study of the PLATO trial, the effect of the *CYP2C19*2* polymorphism on outcome was less clear (Wallentin et al., 2010). In the 4,904 clopidogrel treated patients with genotype data, only the rate of MACE at 30 days post randomisation was significantly higher in the *CYP2C19*2* allele carriers compared to wild type *CYP2C19* homozygotes (HR 1.37; 95%CI 1.04-1.82, P=0.028). The rate of MACE beyond 30 days was not significantly different between the two genotype groups in clopidogrel treated patients, and this was replicated across other outcome measures such as stent thrombosis, cardiovascular death and myocardial infarction. Similarly, in the CURE trial genetic sub-study (Pare et al., 2010), no significant effect of the *CYP2C19*2* polymorphism on cardiovascular outcomes was demonstrated in 5059 ACS patients. Interestingly, subjects with the gain-of-function *CYP2C19*17* polymorphism reported significantly lower rates of cardiovascular events compared to the wild-type genotype, in keeping with an increased rate of clopidogrel bio-activation (HR 0.55; 95% CI 0.42 to 0.73, P=0.02). However, it should be noted that the rate of PCI and stenting was very low in the CURE study which may explain the lack of effect of the *CYP2C19*2* polymorphism. Conversely, the rate of PCI in clopidogrel treated patients in the PLATO study was around 60%, significantly higher than the 14% in the CURE trial. However, the PLATO genetic sub-study does not report the PCI rate in genotyped patients and consequently it is unclear whether the PCI rate was similar to the main trial.

These conflicting data are replicated in the published meta-analyses. In Mega's collaborative meta-analysis (Mega et al., 2010b), nine studies were included representing 9,685 patients. Whilst this meta-analysis demonstrated clear and consistent associations between the carriage of the *CYP2C19*2* allele and adverse cardiovascular outcomes, it did not include data from either the PLATO or CURE genetic sub-studies. Given the size of those studies, it is

likely that they would have substantially reduced the effect size observed in Mega's meta-analysis. Indeed, the later meta-analysis from Bauer et al (Bauer et al., 2011) did include both the PLATO and CURE genetic sub-studies and failed to detect a significant association between carriage of the *CYP2C19*2* allele and most cardiovascular outcomes apart from a moderate association with stent thrombosis. In addition, Bauer's meta-analysis also identified, and commented on, the poor methodological quality of a large proportion of the published data on clopidogrel response, clinical outcome and *CYP2C19*2* genotype, which are mirrored by the findings from the present meta-analysis. Specifically, these include lack of adequate genotyping quality control, failure to assess compliance with Hardy-Weinberg equilibrium, unclear duration of follow-up, failure to assess adherence to clopidogrel and potential influence from industry or other healthcare institution funding. Importantly, the present meta-analysis also identified that outcome measure definitions were very variable between individual studies. For example, this meta-analysis identified twenty-four separate definitions of MACE in the included studies. Analysis under a single, universal, definition of MACE revealed widely different effect sizes from the included studies, with high levels of heterogeneity observed as a result. Consequently, this limits the generalisability of the detected associations between MACE and the *CYP2C19* genotype in the present meta-analysis and, given the small numbers of studies in each separate MACE definition, it was not possible to perform individual meta-analyses for each definition. Importantly, this finding is likely to affect other published meta-analyses which have included MACE as an outcome.

The present meta-analysis also demonstrated a significant association between stent thrombosis and carriage of *CYP2C19* LOF alleles. This is in keeping with the published data including both Mega's and Bauer's meta-analyses. Stent thrombosis is a largely platelet driven outcome measure and it is therefore the most sensitive clinical outcome measure to clopidogrel response, in keeping with the observed higher effect sizes seen in previous pharmacogenetic studies compared to other clinical outcomes, outlined in the Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines for CYP2C19 polymorphism testing and clopidogrel therapy (Scott et al., 2013).

The present meta-analysis failed to demonstrate a consistent association between carriage of *CYP2C19* LOF alleles and cardiovascular death, with the meta-analysis using only the *CYP2C19*2* allele failing to show an association whereas the *CYP2C19* metaboliser phenotype meta-analysis demonstrated a significant association. It should be noted that only two studies were included in both of these individual meta-analyses and therefore any associations detected should be treated with caution. Furthermore, the *CYP2C19*

metaboliser phenotype meta-analysis included stroke patients only which may limit the applicability of this meta-analysis to patients with coronary artery disease. Finally, the present meta-analysis failed to detect any association between carriage of the *CYP2C19*2* allele and all-cause mortality, myocardial infarction and revascularisation. This is likely to be due to the low number of studies included in the individual meta-analyses, as well as significant differences between the individual studies such as length of follow-up, clopidogrel doses and clinical context.

In keeping with the pharmacodynamic meta-analysis, no significant association were observed between *ABCB1* C3435T genotype and MACE. As discussed previously, it is conceivable that ABCB1 genotype could affect clopidogrel response given that clopidogrel is a substrate for P-gp. Interestingly, a number of clinical outcome studies have suggested that the *ABCB1* C3435T polymorphism may be associated with clinical outcomes. In a further genetic sub-study of the TRITON-TIMI 38 study, Mega and colleagues (Mega et al., 2010a) identified that *ABCB1* 3435 TT homozygotes had an increased risk of major adverse cardiovascular events compared to CT or CC genotypes (HR 1.69; 95% CI 1.05-2.72). However, the rates of stent thrombosis were no different in the two genotype groups, although the event rates were low in both groups. Furthermore, there appeared to be an additive effect of the *CYP2C19*2* polymorphism in *ABCB1* 3435TT homozygotes, with the relative risk of adverse cardiovascular events increasing by around 30% when both variants were combined (HR 1.97; 95% CI 1.38-2.82). Similarly, in a study of 300 patients with either stable or unstable cardiovascular disease undergoing PCI, carriage of the *ABCB1* 3435T allele carriers significantly increased the occurrence of a composite endpoint comprised of death, myocardial infarction and stroke (1.5 vs 8.6%, P=0.02) (Campo et al., 2011). However, other studies have failed to demonstrate a significant association between outcome and the *ABCB1* C3435T polymorphism, including in the genetic sub-study of the PLATO trial (Wallentin et al., 2010). As discussed previously, these inconsistent data may be related to the observed linkage disequilibrium between the *ABCB1* C3435T polymorphism and the G2677T/A and C1236T polymorphism. However, the genetic sub-study of the TRITON-TIMI 38 trial also analysed for the combined haplotype of the three polymorphisms but this did not significantly affect the association between genotype and clinical outcome. Importantly, most studies did not routinely report the use of other drugs that are P-gp substrates or inhibitors, such as amiodarone, which are likely to be used in this patient group (Simon et al., 2009).

Similarly, this meta-analysis failed to demonstrate an association between clinical outcome and the *CYP3A5*3* polymorphism. As discussed previously, CYP3A5 may only impact clopidogrel response in specific circumstances such as CYP3A4 inhibition (Farid et al., 2007, Park et al., 2012). In a study by Suh et al (Suh et al., 2006) of 348 patients undergoing stent implantation, a significant association between carriage of the CYP3A5*3 allele and 6 month adverse cardiovascular events was observed (OR 4.89; 95% CI 1.28-18.7). Notably, the number of CYP3A metabolised drugs taken by the patient was also identified as an independent risk factor for adverse cardiovascular events, in keeping with the relationship between the polymorphism and co-administration of CYP3A4 metabolised drugs or inhibitors. Furthermore, in a study of 1258 patients undergoing PCI (Park et al., 2012), no significant association was observed between *CYP3A5*3* genotype and adverse cardiovascular outcome in patients not taking amlodipine, whereas in patients taking amlodipine, carriage of the *CYP3A5*3* allele was strongly associated with adverse cardiovascular outcomes. Neither study included in the present meta-analysis for *CYP3A5*3* reported any data on use of CYP3A4 metabolised drugs, and therefore it is unclear whether the use of these medications impacted on its result.

The present meta-analysis also did not report any association between the *PON1* Q192R polymorphism and MACE. Again, the generalisability of this finding is limited due to the low number of studies included. However, as discussed previously, several studies (Hulot et al., 2011, Trenk et al., 2011) have failed to replicate the initial associations reported by Bouman et al (Bouman et al., 2011) for reasons that remain unclear currently.

A number of other polymorphisms have been investigated in relation to clopidogrel response and clinical outcomes, including its pharmacodynamic target, *P2Y12* (Lin et al., 2014, Simon et al., 2009, Sun et al., 2015a). Simon et al (Simon et al., 2009) failed to demonstrate an association between two *P2Y12* polymorphisms (C34T and H1/H2) and a composite outcome of cardiovascular death, non-fatal MI and non-fatal stroke. However, Ziegler et al (Ziegler et al., 2005), in their cohort of peripheral artery disease patients, demonstrated a fourfold higher risk of neurological events in patients carrying the 34T allele who were taking clopidogrel (HR 3.96; 95%CI 1.02 to 17.84; P=0.048). However, more recent studies investigating common *P2Y12* polymorphisms (Lin et al., 2014, Sun et al., 2015a) have failed to demonstrate any significant effect on clinical outcomes.

Given the findings of the present meta-analysis, it is clear that *CYP2C19* genotype could be used as a marker for stratification for anti-platelet drugs, with wild-type homozygotes

continuing to receive clopidogrel whilst the more potent anti-platelet drugs could be used only in carriers of the *CYP2C19* LOF alleles. Several studies have investigated the use of genotype guided stratification for anti-platelet drugs. In a cohort of 628 patients undergoing PCI, Shen and colleagues (Shen et al., 2016) randomised patients to either a 'routine group' (clopidogrel 75mg daily) or an 'individual group'. All patients received a loading dose of clopidogrel (600mg) prior to PCI, and in the individual group, patients were divided into metaboliser groups determined by their *CYP2C19* genotype. Patients with an extensive metaboliser (EM, wild-type homozygotes) genotype continued to receive clopidogrel 75mg once daily whilst the intermediate metabolisers (IM, LOF heterozygotes) received clopidogrel 150mg once daily. Finally, the poor metaboliser group (PM, LOF homozygotes) received ticagrelor 90mg twice daily. All patients were followed up at 1 month, 6 months and 12 months for MACE (all-cause mortality, MI, target vessel revascularisation) and bleeding. At 12 months, the occurrence of MACE was significantly lower in the individual group in comparison to the routine group (4.2% vs 9.4%, P=0.01) with no observed increase in the rate of bleeding (8.1% vs 6.0%, P=0.29). In addition, there were no significant differences in MACE occurrence in the EM, IM and PM groups (5.3%, 2.9%. 5.3% respectively, P=0.59) and no significant differences in the rate of bleeding between those groups. These findings suggest that genotyped guided stratification has the potential to significantly reduce the risk of MACE in clopidogrel treated patients, whilst also establishing that the efficacy of clopidogrel in EM patients is similar to the more potent ticagrelor in PM patients. Several other studies have also identified that genotype guided dosing is effective in reducing platelet reactivity, with one study demonstrating that using either clopidogrel or prasugrel on the basis of genotype achieved similar levels of platelet inhibition to universal usage of ticagrelor (Malhotra et al., 2015).

One critical consideration in utilising a genotype stratified approach is cost-effectiveness. A cost-effectiveness analysis by Wang et al (Wang et al., 2018e) established that genotype guided dosing of anti-platelets was cost-effective in comparison to either universal ticagrelor or universal clopidogrel usage. In addition, inclusion of prasugrel into a cost-effectiveness model did not alter the superior cost-effectiveness of a genotype guided approach (Jiang and You, 2017). However, an older cost-effectiveness analysis by Sorich et al (Sorich et al., 2013) identified that universal ticagrelor may be more effective than a genotype guided approach albeit at a higher cost that was likely to be within acceptable limits for funding. However, Sorich's analysis reported only a small difference in the cost of clopidogrel treatment compared to ticagrelor treatment, which is not in keeping with price differential used in

Wang's or Jiang's analyses or the current UK market price for both drugs. In addition, both Wang's and Jiang's cost-effectiveness analyses demonstrate that genotype guided dosing may be more cost-effective than platelet function test guided dosing. A cost-effectiveness analysis by Coleman et al (Coleman and Limone, 2013) demonstrated that the incremental cost-effectiveness ratio (ICER) for platelet reactivity driven anti-platelet therapy compared with universal clopidogrel were between USD 40,100 and USD 49,143 per quality-adjusted life year (QALY), which compares to USD 2560/QALY and USD 10,153/QALY for genotype guided dosing in Wang et al's and Liang et al's cost-effectiveness analyses respectively.

There are several limitations to the current meta-analysis. Firstly, whilst a large number of studies have been published investigating the relationship between genetic polymorphisms, clopidogrel response and clinical outcome, only a small number could be combined in meta-analyses. This was also compounded by the decision to combine only studies that reported survival data, which resulted in only very small numbers in some meta-analyses which severely limits their generalisability. In addition, it was notable that several different definitions were utilised for studies reporting MACE as an outcome which resulted in high reported heterogeneity in all the MACE meta-analyses. Despite identifying several different definitions for MACE, separate meta-analyses could not be prepared for each definition given the low number of studies reporting each definition.

Secondly, this meta-analysis was performed only on published data. This limited the ability to correct for any heterogeneity observed in the individual meta-analyses and also prevents analysis of separate outcomes that were reported as composites within the published studies. Where possible, particular characteristics of studies that may affect outcome, such as the type of patients included in the study or the clopidogrel loading dose, have been identified and reported in each meta-analysis. Consequently, future meta-analyses should be conducted using individual patient data where possible. In addition, it was not possible to conduct meta-regression analysis to investigate the source of heterogeneity given the low number of studies included in most meta-analyses.

Thirdly, as is common to most meta-analyses, there is a risk of publication bias given that only published studies were included in the meta-analyses. To overcome this, an attempt was made to include published conference abstracts; however these could not be included since there was insufficient data available on outcomes or to adequately assess their quality.

Fourthly, as discussed in Chapter 2, the cut-off date for the data included in this meta-analysis was November 2015. Since then, a number of additional papers have been

published, of which around 70 report on clinical outcomes. However, following review of these studies, most have continued to report a positive association between carriage of the *CYP2C19* LOF alleles and adverse cardiovascular outcomes and, therefore, are unlikely to affect the conclusions of this meta-analysis. Moreover, most newly published studies have not reported survival data and therefore the numbers of includable papers in this meta-analysis is likely to be very low.

Finally, it should be noted that a dominant mode of inheritance was assumed for each meta-analysis, as this was the case for a significant proportion of the included studies. Statistical methods for genetic meta-analyses are available which estimate the mode of inheritance from the data, thus removing the need to make a specific assumption. However, since they rely on information from studies reporting on three genotype groups separately, they were not applied in this current meta-analysis as most included studies did not provide data for all genotype groups. Although power is not lost due to incorrect assumptions regarding the mode of inheritance, it is recommended that future studies report data for all three groups. However, the frequency of variant homozygotes is often low and therefore large studies will be required to ensure a sufficient number of mutant-type homozygotes.

In summary, the current meta-analyses suggest that carriage of the *CYP2C19*2* polymorphism increases the risk of adverse cardiovascular outcomes in patients treated with clopidogrel. These data suggest that utilising the *CYP2C19*2* polymorphism as a marker for stratification is possible, with existing published data demonstrating that this is likely to be a clinically and cost effective strategy. Further well designed and suitably powered stratification studies, investigating clinical outcomes and cost-effectiveness, are required to demonstrate clinical utility.

# Chapter 4 – The influence of genetic polymorphisms in aspirin's pharmacokinetic and pharmacodynamic pathway on platelet reactivity in aspirin treated patients with acute coronary syndrome

## 4.1: Introduction

Aspirin remains one of the most widely used anti-platelet drugs worldwide, with indications ranging from treatment of acute coronary syndromes and ischaemic strokes through to secondary prevention of cardiovascular disease. It is an effective drug, with clear evidence of significant risk reduction in cardiovascular events across a range of patient populations and disease indications. In the pivotal Antithrombotic Triallists' Collaboration (ATC) (Antithrombotic Trialists, 2002) meta-analysis of 287 studies and 212,000 patients, aspirin treated patients demonstrated a consistent 25% reduction in serious vascular events compared to controls or placebo. This translates to an absolute risk reduction of a serious vascular event by 36 per 1000 treated for two years, which substantially out-weighs the increased risk of bleeding from aspirin use.

However, the response to aspirin can be variable across different patients and diseases whilst non-response to aspirin has been associated with an increased risk of adverse cardiovascular outcomes. In a study of 465 patients with stable coronary artery disease, the prevalence of aspirin non-response was 20% with an associated fourfold increase in risk of adverse cardiovascular outcomes in the five year follow up period (OR 4.28; 95% CI 1.64 to 11.20, P=0.03) (Chen and Chou, 2018b). However, a 900-patient study, again in stable cardiovascular disease, failed to demonstrate any significant prognostic effect of aspirin non-response over a three year follow up (Larsen et al., 2017). Nonetheless, a large meta-analysis from Krasopoulos et al, (Krasopoulos et al., 2008) including 20 studies and 2930 patients, demonstrated a fourfold increase in the risk of adverse cardiovascular events in patients deemed to have aspirin non-response compared to those with normal aspirin responsiveness (OR 3.85; 95% CI 3.08 to 4.08, P < 0.001). These findings are in keeping with a meta-analysis from Snoep et al (Snoep et al., 2007a), which also demonstrated a similar increase in the risk of adverse cardiovascular events across 15 studies and 1800 patients.

The underlying causes of aspirin non-response remain unclear. Like other anti-platelet drugs, various clinical factors may affect aspirin response either directly or indirectly. However, several studies have suggested that the response to aspirin may be a heritable trait. In a

study (Faraday et al., 2007) of 1880 asymptomatic subjects from families with premature coronary artery disease, aspirin response was determined to be highly heritable based on a number of COX-1 and non-COX-1 dependent platelet function tests. Only 1-13% of variation in aspirin response could be explained by clinical factors, with age and gender being the most important identified variables. Heritable factors explained 27% to 77% of the observed variance in platelet function, with adjustment for the clinical variables having a minimal effect on the observed heritability. These findings suggest that genetic factors may have a critical role in determining aspirin response.

Aspirin's primary pharmacodynamic effect is the irreversible acetylation of COX-1 at serine-529, thereby reducing the conversion of arachidonic acid (AA) to thromboxane A2, a potent platelet activator and vasoconstrictor. However, platelet activation is a complex process, with several inter-relating pathways that involve multiple enzymes, mediators and platelet receptors. Consequently, studies investigating the role of genetic polymorphisms in determining aspirin responses have focussed on a number of different genes controlling different platelet receptors and enzymes.

Several studies have focussed on investigating the relationship between common *COX-1* polymorphisms and aspirin response. The most common *COX-1* polymorphism, C50T, has been demonstrated to be associated with aspirin non-response (Lepantalo et al., 2006), whereas several studies have failed to detect any association between the C50T polymorphism and aspirin response (Li et al., 2013b, Yi et al., 2013). Similar results have been observed for other *COX-1* variants, although it should be noted that COX-1 phenotype, and therefore aspirin response, may be better represented by a haplotype of five *COX-1* SNPs rather than the individual variants. In addition, other components of the arachidonic acid pathway demonstrate inconsistent associations with aspirin response as exemplified by SNPs in thromboxane synthase and the TXA2 receptor (Lordkipanidze et al., 2011, Postula et al., 2011, Wang et al., 2013).

Like clopidogrel, considerable focus has been placed on the relationship between aspirin response and platelet receptors or other platelet surface proteins. A number of studies have investigated common polymorphisms in the ADP receptor, *P2Y12*, and response to aspirin with largely negative results (Bernardo et al., 2006, Bierend et al., 2008, Isordia-Salas et al., 2012, Lev et al., 2007b, Ulehlova et al., 2014). Interestingly, some studies have demonstrated positive associations between *P2Y1* receptor polymorphisms (Li et al., 2007, Timur et al., 2012) and aspirin response, although a number of negative studies have also been published

(Lev et al., 2007b, Lordkipanidze et al., 2011, Wang et al., 2013). Similarly, the *GPIIIa* PIA1/A2 polymorphism has been demonstrated to potentially affect aspirin response, although this may be dependent on the type of platelet function test utilised for determining aspirin response. In a meta-analysis of 16 studies investigating the *GPIIIa* PIA1/A2 polymorphism, Floyd and colleagues demonstrated an association between the PIA2 variant and aspirin response, but only in studies that used the PFA-100 as the measure of platelet inhibition (Floyd and Ferro, 2014).

The impact of genetic polymorphisms on aspirin's pharmacokinetic pathway have not been robustly assessed. Aspirin is rapidly metabolised to salicylic acid in the portal circulation following oral administration. Further metabolism within the liver by glucuronidation and hydroxylation may be dependent on UGT1A6 and CYP2C9, respectively. Consequently, polymorphisms within those enzymes may significantly affect aspirin response. Studies in healthy volunteers demonstrated a significant effect of the *UGT1A6*2* polymorphisms, with faster generation of metabolites in *2 allele carriers compared to wild-type homozygotes (Chen et al., 2007, van Oijen et al., 2009). However, in patients, there appears to be little significant effect on platelet reactivity, as demonstrated by Postula and colleagues in a study of 287 diabetic patients (Postula et al., 2013).

Finally, the GeneSTAR study (Mathias et al., 2010) conducted a genome wide association study in over 2000 healthy volunteers following 14 days of aspirin treatment. Whilst a number of novel SNPs were identified at genome-wide significance level, some were only associated with certain platelet function tests and not others. However, in agreement with other genome-wide studies (Lewis et al., 2013), variants in platelet endothelial aggregation receptor-1 (*PEAR-1*) were identified as potential modifiers of aspirin response (Keramati et al., 2018). However, like other variants investigated for association with aspirin response, conflicting data exist with some studies demonstrating a clear association between *PEAR1* variants and outcome and others not (Lewis et al., 2013, Peng et al., 2016).

In summary, despite the high heritability of aspirin response, it remains unclear whether specific genetic variants are associated with aspirin response. To date, studies have mostly focussed on investigating single polymorphisms rather than utilising a comprehensive pathway analysis in order to identify other genes that may impact on aspirin's pharmacokinetic or pharmacodynamic response. Furthermore, findings in healthy volunteers may not necessarily reflect response in patients where concomitant disease acts as an important confounder. Additionally, the patient groups included in studies may not

represent the patient group most likely to suffer harm from poor response to aspirin, with most studies focusing primarily on patients with stable disease. As discussed previously, response to aspirin can be assessed using a variety of different assays, some of which test COX-1 specific pathways and others not, with consequent poor agreement and high variability between different assays.

In order to address this question, a study investigating the association between genetic variants, chosen on the basis of aspirin's PK and PD pathway, and platelet function was conducted in a cohort of patients with non-ST elevation acute coronary syndromes (NSTEACS).

## 4.2: Methods

### 4.2.1: Patient Cohort

Patients included in this study were recruited from the prospective 'Pharmacogenetics of Acute Coronary Syndrome' (PhACS) study. This was a prospective study which recruited 1470 patients with an index admission diagnosis of NSTEACS, across multiple UK hospital sites.

Patients were included in the study if they were in hospital with a primary diagnosis of an acute coronary syndrome. ACS was defined as either a positive troponin or ECG changes with a history consistent with an ACS. ECG changes were further defined as ST-segment depression, transient ST-segment elevation, T-wave inversion or ST-segment elevation. Specific exclusion criteria included ST elevation MI, diagnosis or other pathology likely to account for symptoms or troponin rise and being unwilling or unable to consent.

Subjects were followed up for a minimum of 12 months from recruitment, with physical visits at month 1 (visit 2) and month 12 (visit 3) to collect laboratory samples and clinical data. Data were collected on recurrent cardiovascular events, changes to medications, changes to diagnoses and occurrence of PCI or CABG. Patient defined outcomes, such as medication adherence and functional status were also assessed. After visit 3, subjects were contacted every 12 months and a case-note review was undertaken to collect data on cardiovascular events, changes to medications, changes to diagnoses and interventions such as PCI and CABG. Follow up continued annually until the final patient recruited had completed 12 months of follow up.

Blood and urine samples were taken at each physical visit (baseline, visit 1 and visit 2). A sample for genotyping was taken at the baseline visit only. Two samples for platelet function (4mL hirudin and 3.6mL citrate), 1 sample for RNA (9mL), 1 serum sample (9mL) and 1 urine sample (plain tube) was taken at all visits. Platelet function was assessed using the PFA-100 and Multiplate analysers. Assessment of platelet function was only performed at three sites (Royal Liverpool University Hospital (PFA-100 and Multiplate), Liverpool Heart and Chest Hospital (PFA-100 and Multiplate) and Blackpool Victoria Hospital (Multiplate only)).

The primary outcome measure for the PhACS study was a composite outcome of cardiovascular mortality, non-fatal myocardial infarction and non-fatal stroke. Secondary outcome measures were all cause mortality, bleeding and development of left ventricular failure. Outcome measures used in the PhACS study were defined by outcome measures utilised in other cardiovascular outcome trials (cardiovascular mortality defined by the PLATO trial criteria (Wallentin et al., 2009), non-fatal MI defined by the TRITON-TIMI 38 trial criteria (Wiviott et al., 2007), non-fatal stroke and bleeding defined by the HORIZONS-AMI study (Mehran et al., 2008). All outcome measures were adjudicated by a panel of cardiologists.

The study received ethical approval from the Liverpool Adult Research Ethics Committee and was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki. Site Specific Approval was obtained for all participating hospital sites.

### 4.2.2: Patient Selection

Patients were selected for this study from the main PhACS cohort. Participants were included if they had data on platelet function at month 1 and had sufficient quantity and quality of DNA to ensure successful genotyping.

### 4.2.3: Outcome measures

The outcome measure for this study was platelet function. Platelet function was assessed by either the PFA-100 or Multiplate platforms.

### 4.2.4: Platelet Function Testing

PFA-100

Blood was collected in a 3mL citrate tube and analysed using the PFA-100 platform (Dade-Behring International, Miami, Florida) in compliance with the manufacturer's instruction. All samples were tested within two hours of the specimen collection. Briefly, the PFA-100

assesses platelet function under high shear conditions utilising a 150-micrometre aperture coated with collagen and epinephrine (CEPI). Whole, citrated, blood is injected through the aperture at a constant flow rate, and the time taken for the aperture to close is measured, with results presented as closure times in seconds. Samples were processed locally at the recruitment site by research nurses. A cut-off value of <193 seconds was used as the definition of aspirin non-response in this study, in line with the manufacturer's instructions and published data (Reny et al., 2008). PFA-100 data are presented as categorical data (responder or non-responder).

Multiplate

Blood was collected in a 3mL hirudin tube (Verum Diagnostica GmbH, Munich, Germany) and analysed using the Multiplate platform (Verum Diagnostica GmbH, Munich, Germany) in compliance with the manufacturer's instructions. Briefly, the Multiplate platform assesses platelet reactivity using the principal of impedance aggregometry where activated platelets adhere to electrodes, increasing the overall resistance measured across a circuit. Whole blood (300 microL) was pipetted into test chambers followed by the addition of 300 microL of 0.9% saline. These samples were allowed to incubate for 3 minutes with stirring provided by magnetic stirrers. After three minutes, arachidonic acid was added (to a final concentration of 0.5mM). Platelet aggregation was determined over six minutes with final values reported as aggregation units (AU) per minute. Samples were processed locally at the recruitment site by research nurses. Multiplate data are presented as continuous data in order to represent overall platelet reactivity.

**4.2.5: Selection of genetic polymorphisms**

Genes and polymorphisms were selected following review of the Platelet Aggregation Inhibitor Pathway, Pharmacodynamics from the PharmGKB website (www.pharmgkb.org/pathway/PA154444041/overview). In addition, a literature review on aspirin's pharmacokinetic pathway was also undertaken to identify additional genes and polymorphisms. Following identification, genes and polymorphisms were selected for genotyping if the minor allele frequency was greater than 0.05 and the SNP had been identified to be functional or non-synonymous.

A total of 16 polymorphisms in ten genes were chosen for genotyping on the basis of these criteria. They included genes involved in aspirin's pharmacokinetic pathway (*UGT1A6*, *CYP2C9*, *CES2*) and pharmacodynamic pathway (*PTGS-1* (*COX-1*), *TBXA2R*, *PTGDR*, *PTGER3*,

*PTGER4*, *PTGFR*, *TBXAS1* and *TBXA2R*). A summary of the included genes and polymorphisms is provided in **Table 4.1**.

### 4.2.6: Genotyping

DNA extraction was performed using the Chemagic Magnetic Module 1 system in compliance with the manufacturer's instructions and standard procedures.

Genotyping for the included SNPs was performed using commercially available TaqMan real-time PCR genotyping assays following addition of 1x Genotyping Master Mix (Applied Biosystems, Carslbad, USA). A total of 20ng of genomic DNA per reaction was genotyped using an ABI7900HT real-time PCR system (Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions and standard procedure. Genotyping was performed in duplicate for ten percent of samples to ensure quality control of the genotyping process. Genotyping was conducted in the Wolfson Centre for Personalised Medicine by Dr Dan Carr and myself.

### 4.2.7: Statistical analysis

Data were presented as mean and standard deviation for continuous variables, and frequencies or percentages for categorical variables. For continuous variables, data were compared using ANOVA with categorical data being compared using the Chi-squared test. Correction for multiple testing was performed using the Bonferroni method. A P value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 24 (SPSS, Chicago, USA).

## 4.3: Results

### 4.3.1: Patient Characteristics

A total of 263 patients were included in this analysis from the PhACS study, all of whom had platelet function data at the month 1 visit (visit 2) and sufficient DNA for genotyping. PFA-100 platelet function data were available in 179 patients and Multiplate platelet function data were available in 108 patients. A summary of patient demographics, clinical risk factors and medications is provided in **Table 4.2**. Notably, statistically significant differences were present between the PFA-100 and Multiplate patient groups, with Multiplate group patients having lower rates of chronic kidney disease, diabetes and previous MI compared to the PFA-100 group. Platelet function summary data are presented in **Table 4.3**.

| Gene | Rs# | AmA Change | MAF | Allele | Successful? |
|---|---|---|---|---|---|
| ***CYP2C9*** | rs1799853 | p.R144C | 0.089 | *2 | Yes |
| ***CYP2C9*** | rs1057910 | p.I359L | 0.061 | *3 | Yes |
| ***UGT1A6*** | rs6759892 | p.S7A | 0.381 | *2,*3,*4 | Yes |
| ***UGT1A6*** | rs1105879 | p.R184S | 0.308 | *2,*4 | Yes |
| ***UGT1A6*** | rs2070959 | p.T181A | 0.271 | *2,*5 | Yes |
| ***PEAR1*** | rs12041331 | NA | 0.08 | | Yes |
| ***COX-1*** | rs10306114 | c.-842A>G | 0.153 | | Yes |
| ***TBXA2R*** | rs4523 | p.X308Y | 0.5 | | Yes |
| ***CES2*** | rs62057932 | p.H6R | 0.5 | | No |
| ***COX-1*** | rs3842787 | c.50C>T/p.L17P | 0.104 | | No |
| ***PTGDR*** | rs41311442 | p.C17R | 0.07 | | No |
| ***PTGER1*** | rs7249305 | p.R256H | 0.093 | | No |
| ***PTGER2*** | rs77558975 | p.P226H | 0.054 | | No |
| ***PTGER4*** | rs77448213 | p.P211L | 0.198 | | No |
| ***PTGFR*** | rs1123153 | p.X7K | 0.196 | | No |
| ***TBXA2R*** | rs5749 | p.T160A | 0.095 | | No |
| ***TBXAS1*** | rs13306050 | p.L512P | 0.069 | | No |

**Table 4.1 – Genes and polymorphisms included in this analysis**

| | | All Patients | PFA Patients | Multiplate Patients | P-Value* |
|---|---|---|---|---|---|
| | N | **263** | **179** | **108** | |
| **Males** | N | 184 | 126 | 76 | >0.99 |
| | % | 69.96 | 70.39 | 70.37 | |
| **Age** | Mean | 64.01 | 65.01 | 62.31 | 0.18 |
| | SD | 12.55 | 12.96 | 11.68 | |
| | Median | 64.08 | 64.94 | 62.89 | |
| | Q1 | 54.21 | 54.55 | 53.80 | |
| | Q3 | 72.99 | 74.66 | 70.36 | |
| | IQR | 18.79 | 20.11 | 16.56 | |
| **BMI** | Mean | 28.86 | 28.66 | 29.33 | 0.27 |
| | SD | 6.03 | 6.06 | 5.94 | |
| | Median | 28.11 | 27.73 | 28.40 | |
| | Q1 | 24.62 | 24.57 | 25.46 | |
| | Q3 | 32.20 | 31.60 | 32.89 | |
| | IQR | 7.58 | 7.03 | 7.43 | |
| **Hypertension** | N | 158 | 107 | 64 | 0.93 |
| | % | 60.08 | 59.78 | 59.26 | |
| **Hyperlipid** | N | 137 | 100 | 53 | 0.26 |
| | % | 52.09 | 55.87 | 49.07 | |
| **Peripheral arterial disease** | N | 19 | 16 | 5 | 0.17 |
| | % | 7.22 | 8.94 | 4.63 | |
| **Chronic Kidney Disease** | N | 23 | 21 | 2 | **<0.01** |
| | % | 8.75 | 11.73 | 1.85 | |
| **Diabetes** | N | 56 | 43 | 15 | **0.04** |
| | % | 21.29 | 24.02 | 13.89 | |
| **Prior MI** | N | 83 | 63 | 22 | **0.01** |
| | % | 31.56 | 35.20 | 20.37 | |
| **Prior PCI** | N | 33 | 21 | 13 | 0.94 |
| | % | 12.55 | 11.73 | 12.04 | |
| **Prior CABG** | N | 27 | 17 | 10 | 0.95 |
| | % | 10.27 | 9.50 | 9.26 | |
| **Current Smoker** | N | 64 | 38 | 30 | 0.21 |
| | % | 24.33 | 21.23 | 27.78 | |
| **Previous Smoker** | N | 110 | 83 | 34 | 0.01 |
| | % | 41.83 | 46.37 | 31.48 | |
| **Non Smoker** | N | 84 | 57 | 40 | 0.37 |
| | % | 31.94 | 31.84 | 37.04 | |
| **Aspirin** | N | 263 | 179 | 108 | >0.99 |
| | % | 100.00 | 100.00 | 100.00 | |
| **Clopidogrel** | N | 231 | 158 | 94 | 0.76 |

| | | | | | |
|---|---|---|---|---|---|
| | % | 87.83 | 88.27 | 87.04 | |
| **Warfarin** | N | 9 | 7 | 5 | 0.77 |
| | % | 3.42 | 3.91 | 4.63 | |
| **LMWH** | N | 9 | 8 | 3 | 0.47 |
| | % | 3.42 | 4.47 | 2.78 | |
| **Beta-Blocker** | N | 222 | 148 | 93 | 0.44 |
| | % | 84.41 | 82.68 | 86.11 | |
| **CCB** | N | 46 | 40 | 13 | **0.03** |
| | % | 17.49 | 22.35 | 12.04 | |
| **Nitrate** | N | 147 | 88 | 69 | **0.02** |
| | % | 55.89 | 49.16 | 63.89 | |
| **Statin** | N | 247 | 167 | 103 | 0.47 |
| | % | 93.92 | 93.30 | 95.37 | |
| **ACE-I** | N | 196 | 133 | 77 | 0.58 |
| | % | 74.52 | 74.30 | 71.30 | |
| **ARB** | N | 20 | 12 | 12 | 0.19 |
| | % | 7.60 | 6.70 | 11.11 | |
| **PPI** | N | 95 | 62 | 39 | 0.80 |
| | % | 36.12 | 34.64 | 36.11 | |

**Table 4.2 – Demographics of included patients** (*P-value relates to the comparison between the PFA-100 tested patients and the Multiplate tested patients)

| **PFA Closure Time** | **N** | **%** |
|---|---|---|
| **>193s** | 103 | 57.54 |
| **<193s** | 76 | 42.46 |
| | | |
| **Multiplate ASPI Test AUC** | **Mean** | **SD** |
| | 25.13 | 21.43 |

**Table 4.3 – Summary of platelet function test data**

### 4.3.2: Genotyping

Due to technical reasons, a number of polymorphisms could not be reliably genotyped either due to low call rates, unreliable assays or undetectable variant alleles. These included polymorphisms in *CES2*, *PTGER2*, *PTGER4*, *PTGFR*, *PTGER1*, *PTGDR* and *TBXAS1*. Consequently, data are presented only for 8 SNPs in five genes (CYP2C9, UGT1A6, PEAR1, TBXA2R and COX-1).

### 4.3.3: Allele and genotype frequencies

Allelic and genotype frequencies are reported in the individual gene and outcome tables (**Tables 4.4 and 4.5**). One polymorphism (*UGT1A6* rs2070959 T181A) was noted to deviate from Hardy-Weinberg equilibrium (P<0.01) which is likely to be related to the lower call-rate for this SNP. No other polymorphism deviated from HWE (P>0.05).

### 4.3.4: *CYP2C9*2* and aspirin related platelet inhibition

No significant association was detected between PFA-100 defined aspirin non-responders and *CYP2C9*2* genotype using an additive model. Using the < 193s cut-off for aspirin non-response, 74 patients were determined to be a non-responder and the frequency of the *2 variant allele was not significantly different in responders compared to non-responders (13% vs 14% respectively, uncorrected P=0.737, corrected P >0.999) (**Table 4.4**).

In addition, no significant association was observed between Multiplate defined platelet reactivity and *CYP2C9*2* genotype. Mean ASPI test AUC was reported as 24.7 +/- 23.2 AU*min in *1/*1 genotypes, 16.4 +/-8.0 AU*min in *1*2 genotypes and 19.8 +/-9.2 AU*min in *2*2 genotypes (uncorrected P=0.181, corrected P>0.999) (**Table 4.5**).

### 4.3.5: *CYP2C9*3* and aspirin related platelet inhibition

No significant association was detected between *CYP2C9*3* genotype and aspirin non-response as defined by PFA-100. For the 193s cut-off value, there was no significant differences in carriage of the variant *3 allele between responders (N=103) and non-responders (N=74) (7% vs 10% respectively, uncorrected P=0.342, corrected P>0.999) (**Table 4.4**).

For Multiplate defined platelet inhibition, there was no significant difference in AU*min values in *CYP2C9* *1*1 genotypes in comparison to the *1*3 genotypes (22.0 +/- 20.8 AU*min vs 25.7 +/- 13.5 AU*min respectively, uncorrected P=0.561, corrected P>0.999) (**Table 4.5**).

### 4.3.6: *PEAR1* rs12041331 and aspirin related platelet inhibition

No significant association was detected between the allelic or genotype frequencies and PFA-100 defined aspirin response. For the 193s cut-off values, 73 patients were categorised as non-responders with no difference in the variant A allelic frequencies between the responders and non-responders observed (8% vs 10%, uncorrected P=0.414, corrected P>0.999) (**Table 4.4**).

In keeping with the PFA-100 data, no significant association was noted between the Multiplate ASPI test values and *PEAR1* rs12041331 genotype (uncorrected P=0.728, corrected P>0.999) (**Table 4.5**).

### 4.3.7: *COX-1* rs10306114 (-824A>G) and aspirin related platelet inhibition

For PFA-100 defined aspirin response, no significant association was observed between genotype and allelic frequencies in responders compared to non-responders. For the 193s cut-off value, the frequency of the variant G allele was 8% in responders and 6% in non-responders (uncorrected P=0.541, corrected P>0.999) (**Table 4.4**).

No significant associations were detected between Multiplate ASPItest values and *COX-1* rs10306114 genotype, although it should be noted that only 1 patient carried the GG genotype in this group. Multiplate values were similar across all three genotype groups, with no significant association detected (uncorrected P=0.111, corrected P=0.888) (**Table 4.5**).

### 4.3.8: *TBXA2R* rs4523 (X308Y) and aspirin related platelet inhibition

A potential association between both genotype and allelic frequency was observed for this polymorphism, although statistical significance was lost following correction for multiple testing. A higher frequency of the variant T allele was observed in non-responders compared to responders, using the PFA-100 <193s cut-off value (47% vs 31%, uncorrected P=0.008, corrected P=0.064). CT and TT genotypes were also more frequent in non-responders when compared to responders (70% vs 51% of patients, uncorrected P=0.039, corrected P=0.312) (**Table 4.4**).

However, no significant association was observed between *TBXA2R* rs4523 genotype and Multiplate ASPI test values, with similar results observed across all genotype groups (uncorrected P=0.458, corrected P>0.999) (**Table 4.5**).

### 4.3.9: *UGT1A6* rs6759892 (S7A) and aspirin related platelet inhibition

No clear association was detected between carriage of the variant G allele of the rs6759892 SNP and PFA-100 defined aspirin response, although both the variant genotype and allelic frequencies were higher in responders compared to non-responders. The GT and GG genotype frequency was not significantly different between the responders and non-responders (77% vs 57%, uncorrected P=0.137 and corrected P>0.999 across all three genotype groups). Similarly, no significant difference was observed for the allelic frequencies between responders and non-responders (47% vs 37%, uncorrected P= 0.059, corrected P=0.472) (**Table 4.4**).

No significant association was detected between *UGT1A6* rs6759892 genotype and Multiplate ASPItest values, with similar results noted across all three genotype groups (**Table 4.5**).

### 4.3.10: *UGT1A6* rs2070959 (T181A) and aspirin related platelet inhibition

There was no clear association between PFA-100 defined aspirin response and *UGT1A6* rs2070959 genotype. For the 193s cut-off value, the carriage of the variant AG and GG genotypes was not significantly different in responders and non-responders (33% vs 19%, uncorrected P=0.155 and corrected P>0.999 across all three genotype groups). In addition, no significant difference in allelic frequency was observed between responders and non-responders (22% vs 13%, uncorrected P=0.064, corrected P=0.512) (**Table 4.4**).

For Multiplate, an initially significant association was detected between *UGT1A6* rs2070959 genotype and ASPItest AUC values but this became non-significant following correction for multiple testing. Platelet reactivity appeared to be significantly higher in variant homozygotes at 42.0+/-39.2 AU*min compared to 21.0 +/- 16.1 AU*min in wild type homozygotes (uncorrected P=0.03 and corrected P=0.24 across all three genotype groups) (**Table 4.5**).

However, it should also be noted that this SNP appeared to deviate from Hardy-Weinberg equilibrium (P <0.01) which may be related to the lower number of patients that were successfully genotyped for this SNP compared to the other SNPs including in this analysis.

### 4.3.11: *UGT1A6* rs1105879 (R184S) and aspirin related platelet inhibition

No significant association was detected between the rs1105879 polymorphism and PFA-100 determined aspirin response. No significant differences were noted in allelic or genotype frequencies between responders and non-responders for the 193s cut-off (**Table 4.4**).

Similarly, no clear association was detected between Multiplate ASPItest determined platelet reactivity and genotype. Platelet reactivity was numerically higher in variant CC genotypes (35.9 +/-36.9) compared to wild-type AA genotypes (22.7 +/- 18.5) but this was not statistically significant (uncorrected P=0.063, corrected P=0.504) (**Table 4.5**).

| | | | | Genotype frequency | | | | | | | | Allelic frequency | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | A1/A1 | | A1/A2 | | A2/A2 | | | | A1 | | A2 | | | |
| **Gene/SNP** | **Alleles (A1/A2)** | **PFA-100 CT (s)** | **Total** | **n** | **%** | **n** | **%** | **n** | **%** | **P-value (Uncorr)** | **P-value (Corr)** | **n** | **%** | **n** | **%** | **P-value (Uncorr)** | **P-value (Corr)** |
| ***CYP2C9*2*** | ***1/*2*** | **<193** | 74 | 56 | 75.7 | 17 | 23.0 | 1 | 1.4 | 0.397 | >0.999 | 129 | 87.2 | 19 | 12.8 | 0.737 | >0.999 |
| | | **>193** | 103 | 79 | 76.7 | 19 | 18.4 | 5 | 4.9 | | | 177 | 85.9 | 29 | 14.1 | | |
| ***CYP2C9*3*** | ***1/*3*** | **<193** | 74 | 60 | 81.1 | 13 | 17.6 | 1 | 1.4 | 0.42 | >0.999 | 133 | 89.9 | 15 | 10.1 | 0.342 | >0.999 |
| | | **>193** | 103 | 88 | 85.4 | 15 | 14.6 | 0 | 0.0 | | | 191 | 92.7 | 15 | 7.3 | | |
| ***UGT1A6* S7A (rs6759892)** | **T/G** | **<193** | 73 | 31 | 42.5 | 30 | 41.1 | 12 | 16.4 | 0.137 | >0.999 | 92 | 63.0 | 54 | 37.0 | 0.059 | 0.472 |
| | | **>193** | 103 | 29 | 28.2 | 51 | 49.5 | 23 | 22.3 | | | 109 | 52.9 | 97 | 47.1 | | |
| ***UGT1A6* T181A (rs2070959)** | **A/G** | **<193** | 67 | 54 | 80.6 | 8 | 11.9 | 5 | 7.5 | 0.155 | >0.999 | 116 | 86.6 | 18 | 13.4 | 0.064 | 0.512 |
| | | **>193** | 88 | 59 | 67.0 | 20 | 22.7 | 9 | 10.2 | | | 138 | 78.4 | 38 | 21.6 | | |
| ***UGT1A6* R184S (rs1105879)** | **A/C** | **<193** | 74 | 35 | 47.3 | 33 | 44.6 | 6 | 8.1 | 0.377 | >0.999 | 103 | 69.6 | 45 | 30.4 | 0.173 | >0.999 |
| | | **>193** | 103 | 39 | 37.9 | 51 | 49.5 | 13 | 12.6 | | | 129 | 62.6 | 77 | 37.4 | | |
| ***PEAR1* rs12041331** | **G/A** | **<193** | 73 | 59 | 80.8 | 13 | 17.8 | 1 | 1.4 | 0.501 | >0.999 | 131 | 89.7 | 15 | 10.3 | 0.414 | >0.999 |
| | | **>193** | 103 | 89 | 86.4 | 12 | 11.7 | 2 | 1.9 | | | 190 | 92.2 | 16 | 7.8 | | |
| ***COX-1* - 842A>G (rs10306114)** | **A/G** | **<193** | 74 | 65 | 87.8 | 9 | 12.2 | 0 | 0.0 | 0.665 | >0.999 | 139 | 93.9 | 9 | 6.1 | 0.541 | >0.999 |
| | | **>193** | 103 | 88 | 85.4 | 14 | 13.6 | 1 | 1.0 | | | 190 | 92.2 | 16 | 7.8 | | |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***TBXA2R* X308Y (rs4523)** | **C/T** | **<193** | 59 | 18 | 30.5 | 27 | 45.8 | 14 | 23.7 | **0.039** | 0.312 | 63 | 53.4 | 55 | 46.6 | 0.008 | 0.064 |
| | | **>193** | 88 | 43 | 48.9 | 35 | 39.8 | 10 | 11.4 | | | 121 | 68.8 | 55 | 31.3 | | |

**Table 4.4 – Summary of PFA-100 defined aspirin response and genotype** (*Corrected P-values for multiple testing)

| | | | Genotype Frequency | | | Mean (SD) Multiplate AUC | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **SNP** | **Alleles (A1/A2)** | **N** | **A1/A1** | **A1/A2** | **A2/A2** | **A1/A1** | **A1/A2** | **A2/A2** | **P-value** | **P-value*** |
| ***CYP2C9*2*** | ***1/*2** | 108 | 0.69 | 0.25 | 0.06 | 24.7±23.2 | 16.4±8.0 | 19.8±9.2 | 0.181 | >0.999 |
| ***CYP2C9*3*** | ***1/*3** | 108 | 0.90 | 0.10 | 0.00 | 22.0±20.8 | 25.7±13.5 | - | 0.561 | >0.999 |
| ***UGT1A6* S7A (rs6759892)** | **T/G** | 107 | 0.31 | 0.48 | 0.21 | 22.5±20.6 | 21.1±17.0 | 25.2±26.2 | 0.714 | >0.999 |
| ***UGT1A6* T181A (rs2070959)** | **A/G** | **94** | 0.63 | 0.29 | 0.08 | 21.0±16.1 | 18.0±8.8 | 42.0±39.2 | **0.030** | 0.24 |
| ***UGT1A6* R184S (rs1105879)** | **A/C** | 108 | 0.38 | 0.53 | 0.09 | 22.7±18.5 | 19.8±16.5 | 35.9±36.9 | 0.063 | 0.504 |
| ***PEAR1* rs12041331** | **G/A** | 107 | 0.84 | 0.11 | 0.05 | 22.0±20.3 | 26.8±23.3 | 20.8±8.0 | 0.728 | >0.999 |
| ***COX-1* -842A>G (rs10306114)** | **A/G** | 107 | 0.83 | 0.16 | 0.01 | 20.7±14.5 | 31.8±37.9 | 18.0 | 0.111 | 0.888 |
| ***TBXA2R* X308Y (rs4523)** | **C/T** | 101 | 0.45 | 0.40 | 0.15 | 26.7±27.7 | 18.6±9.4 | 18.7±13.2 | 0.139 | >0.999 |

**Table 4.5 – Summary of Multiplate ASPI test defined platelet reactivity and genotype** (*Corrected P-values for multiple testing)

## 4.4: Discussion

In this analysis, a potential association was detected between two polymorphisms, one in aspirin's pharmacokinetic pathway (*UGT1A6*) and one in aspirin's pharmacodynamic pathway (*TBXA2R*).

*TBXA2R* codes for the thromboxane A2 receptor, which promotes platelet activation and subsequent platelet aggregation. Higher levels of thromboxane A2 are associated with more severe atherosclerotic disease, with cardiovascular risk factors such as diabetes, smoking and obesity also associated with higher thromboxane levels (Gleim et al., 2013). In our data, a possible association was demonstrated between carriage of the variant T allele of the rs4523 (C924T) polymorphism and aspirin non-response which is in keeping with other published data. In a case-control study of 210 patients undergoing off-pump CABG and 210 patients with stable coronary artery disease as a control, Wang et al (Wang et al., 2013) demonstrated a significant association between carriage of the variant T allele in the *TBXA2R* rs4523 polymorphism and high on-aspirin platelet reactivity (OR 4.5; 95% CI 1.8 to 11.1). In addition, the *TXBA2R* rs4523 polymorphism has been associated with Multiplate defined aspirin non-response. In a study of 55 patients undergoing carotid endarterectomy, Roullet et al (Roullet et al., 2018) identified a number of polymorphisms, including *TXBA2R* rs4523, which significantly increased platelet reactivity as determined by the Multiplate platform. Conversely, a recent study by De Iuliis et al (De Iuliis et al., 2018) demonstrated a strong association between carriage of the C allele of the *TBXA2R* rs4523 polymorphism and higher PFA-100 derived platelet aggregation, a finding at odds with the data in the current analysis and previous studies. Interestingly, this study also assessed whether the expression of *TXBA2R* was altered by rs4523 genotype, with C allele homozygotes expressing higher levels of the receptor compared to T allele homozygotes. Furthermore, T allele homozygotes appeared to express a less stable receptor which, in tandem with lower expression of the receptor in T allele homozygotes, led to lower platelet aggregation in patients with the TT genotype.

Several studies have also identified other variants in the *TXBA2R* genes that are associated with platelet function and, in some studies, an increased risk of adverse vascular outcomes. Postula et al demonstrated a significant association between *TXBA2R* rs1131882 genotype and PFA-100 defined aspirin non-response in a cohort of 295 diabetic patients treated with aspirin for primary prevention (Postula et al., 2011). Similarly Peng et al (Peng et al., 2016), in a study of 283 ischaemic stroke patients, detected a significant association between

*TXBA2R* rs1131882 genotype, serum thromboxane B2 levels and aspirin non-response. Importantly, *TXB2AR* polymorphisms may also increase the risk of significant vascular events. In a study of 407 patients who had a cerebral infarction and 270 controls, Zhao et al (Zhao et al., 2013) demonstrated a significant association between the *TXBA2R* rs768963 polymorphism and risk of CVA in a Chinese population. Notably, no association was observed for the rs4523 polymorphism, although the number of C allele carriers was very low in both the CVA and control group which is not in keeping with other studies. These data are in keeping with a mechanistic study by Yi and colleagues (Yi et al., 2017a) which demonstrated that the *TBXA2R* rs1131882 polymorphism is independently associated with the risk of carotid plaque instability, which may lead with to an increased risk of ischaemic stroke.

Clearly, our data did not withstand correction for multiple testing. Taken together with the contradictory data in the literature on the association between *TBXAR2* polymorphisms and either platelet function tests or clinical events, it is not clear whether variation in this gene is important. However, there is biological plausibility for the importance of this gene. *TBXA2R* is expressed in several tissues, including platelets, leucocytes and atherosclerotic plaques and it is conceivable that *TBXA2R* polymorphisms may increase expression of the receptor or sensitivity of the receptor to TXA2 (Wang et al., 2013). As previously discussed, COX-2 expression is increased in atherosclerotic plaques and is less sensitive to aspirin-induced inhibition than COX-1. Conceivably, increased *TBXA2R* expression or sensitivity could increase platelet reactivity to non-COX-1 generated TXA2 in aspirin treated patients, with a consequent increase in platelet reactivity independent of aspirin's inhibition of COX-1. Furthermore, given the widespread expression of *TBXA2R* in atherosclerotic plaques, increased expression or sensitivity of *TBXA2R* may significantly affect the pathogenesis and development of atherosclerosis and vascular disease (Zhao et al., 2013) with a consequent increase in the risk of adverse cardiovascular outcomes. Therefore, further studies in larger patient cohorts may be warranted.

Our analysis also demonstrates a potential association between polymorphisms in the *UGT1A6* gene and aspirin response, although this was not significant following correction for multiple testing. As discussed previously, aspirin is metabolised rapidly into salicylic acid which then undergoes either hydroxylation or glucuronidation in the liver. Multiple UDP-glucuronyltransferase (UGT) enzymes are involved in the metabolism of salicylic acid (Kuehl et al., 2006) although several studies have identified *UGT1A6* SNPs as being primarily involved in determination of aspirin effect (van Oijen et al., 2009). In a study by Chen et al (Chen et al., 2007), conducted in 28 healthy volunteers (19 *UGT1A6*1* homozygotes and nine

*UGT1A6*2* homozygotes), urinary excretion of aspirin and its metabolites were significantly lower in volunteers homozygous for the *1 allele compared to the *2 allele homozygotes. In addition, the *1 homozygotes excreted aspirin and its associated metabolites over a longer period than the *2 homozygotes, with a greater percentage excretion 12 hours post aspirin dose compared to the first 12 hours. These data suggest that *UGT1A6*2* allele carriers and homozygotes have more rapid glucuronidation of salicylic acid than *1 homozygotes, which could potentially lead to lower aspirin-induced platelet inhibition. Similarly, in a study of nine female healthy volunteers (five *UGT1A6*1* homozygotes and four *UGT1A6*2* homozygotes), Van Oijen et al (van Oijen et al., 2009) demonstrated a significantly lower plasma level of salicylic acid in *UGT1A6*2* homozygotes compared with *UGT1A6*1* homozygotes. Furthermore, overall exposure to salicylic acid was also significantly lower in *UGT1A6*2* homozygotes. However, in a large study of 264 men and 264 women, Navarro and colleagues failed to detect any significant effect of *UGT1A6* genotype on urinary excretion of aspirin or its associated metabolites, although significant effects of gender and ethnicity were observed (Navarro et al., 2011). These data are in keeping with a study in 284 diabetic patients treated with aspirin (Postula et al., 2013), where no association was detected between three *UGT1A6* polymorphisms (rs17863783, rs1105880, rs2070959) and aspirin non-response as determined by PFA-100, VerifyNow, serum TXB2 and 11dhTXB2. Similarly, in a study of 165 patients with stable cardiovascular disease, Jalil et al (Jalil et al., 2015) demonstrated no significant association between carriage of the *UGT1A6*2* and *UGT1A6*3* and the risk of developing aspirin induced gastritis.

Whilst *UGT1A6* polymorphisms may significantly affect the pharmacokinetics of aspirin and its associated metabolites, data from large patient studies suggest that these polymorphisms do not significantly alter aspirin's pharmacodynamic effect. Given that UGT1A6 is involved only in salicylic acid metabolism, it is unlikely that *UGT1A6* polymorphisms would significantly impact on aspirin's pharmacodynamic or clinical effect. Aspirin's anti-platelet effect is exerted only prior to its rapid deacetylation to salicylic acid, which is not dependent on UGT1A6. In the current analysis, a potential association was only observed for one *UGT1A6* polymorphism (rs2070959) and one platelet function test. As discussed earlier, this polymorphism was not in Hardy-Weinberg equilibrium and the genotype call-rate was 20% lower than all other SNPs included in the analysis. Consequently, this is unlikely to represent a true effect of the polymorphism on platelet reactivity.

This analysis also failed to detect an association between the *CYP2C9*2* and **3* polymorphisms and aspirin response. These findings are in keeping with data from Postula's

study (Postula et al., 2013) where no association was observed between carriage of the *CYP2C9*2* and **3* polymorphisms and platelet reactivity as measured by four different assays. However, in Jalil's study of 165 patients with stable cardiovascular disease, carriage of the *CYP2C9*3* allele was significantly associated with the risk of aspirin induced gastritis (OR 6.8; 95% CI 1.39 – 33.19, P=0.033) (Jalil et al., 2015). Like UGT1A6, CYP2C9 is involved in aspirin metabolism only after conversion of aspirin to the inactive salicylic acid; consequently, it is unlikely that *CYP2C9* polymorphisms would directly affect the anti-platelet activity of aspirin.

The current analysis also failed to detect any associations between a number of other polymorphisms and aspirin response. Polymorphisms in the *COX-1* gene have been suggested as potentially important in determining aspirin effect, given that COX-1 is the pharmacodynamic target for aspirin. However, data on this relationship have been conflicting, with some studies detecting a significant association between *COX-1* polymorphisms and aspirin response whilst other studies have not. In a study of 38 healthy volunteers, Halushka et al (Halushka et al., 2003) detected a significant association between the A-824G/C50T haplotype and formation of PGH2, the precursor molecule for TXA2. Heterozygotes demonstrated a significantly greater inhibition of PGH2 production to aspirin as compared to wild type homozygotes. Importantly, this study also demonstrated that the A-824G and C50T polymorphisms were in complete linkage disequilibrium, with the variant G allele of the A-824G polymorphism creating a potential AP2 transcription factor binding site which may lower *COX-1* expression. Similarly, in a study recruiting patients with coronary artery disease, the haplotype of five polymorphisms in the *COX-1* gene (A-824G, C22T, G128A, C644A and C714A) was significantly associated with arachidonic acid induced platelet aggregation and serum TXB2 levels (Maree et al., 2005). Furthermore, Ulehlova and colleagues (Ulehlova et al., 2014) demonstrated a significant association between the *COX-1* A-824G polymorphism and aspirin induced platelet inhibition in a cohort of 124 patients recruited after an acute MI. In this study, G allele carriers were significantly more likely to be identified as aspirin resistant by LTA or Multiplate (P=0.003) than wild-type A allele carriers. However, other studies have not detected a clear effect from *COX-1* polymorphisms on aspirin response and clinical outcome. In a large study of 859 stroke patients, Cao et al (Cao et al., 2014) did not demonstrate any significant association between the A-824G polymorphism and the risk of adverse cardiovascular outcomes, although a modest increase in risk was observed for one polymorphism, G1676A (HR 1.92; 95% CI 1.15 to 3.33, P=0.013). In addition, several other studies have demonstrated putative associations between other

*COX-1* alleles and clinical outcome (Lee et al., 2008, Yi et al., 2017b) whilst other studies have not (Hillarp et al., 2003, Lordkipanidze et al., 2011). Given these discordant data, it is likely that the effect of individual *COX-1* polymorphisms are small, which limits their utility for predicting aspirin response or clinical outcome. Importantly, individual *COX-1* polymorphisms exist as part of larger haplotypes and investigating those haplotypes against clinical or pharmacodynamic outcomes is likely to better reflect the effect of *COX-1* genotype on aspirin response.

The current analysis failed to detect an association between *PEAR1* genotype and aspirin induced platelet inhibition, although several studies have demonstrated associations between *PEAR1* genotype and aspirin response. In a study of 1486 healthy participants from at-risk families for cardiovascular disease, Herrera-Galeano et al (Herrera-Galeano et al., 2008) identified a number of polymorphisms in the *PEAR1* gene, with one SNP (rs2768759) being significantly associated with aspirin related platelet inhibition. Similarly, in a cohort of 965 patients with stable coronary artery disease, Wurtz and colleagues demonstrated a strong association between the *PEAR1* rs12041331 polymorphism and aspirin response determined by Multiplate. However, Peng et al failed to demonstrate any significant association between the rs12041331 polymorphism and platelet aggregation in 288 aspirin treated stroke patients. Nonetheless, a genome-wide association study from the Pharmacogenomics of Anti-Platelet Intervention (PAPI) cohort identified a strong association between anti-platelet response and the *PEAR1* rs1204133 polymorphism (Lewis et al., 2013), which was then replicated in 1227 patients with cardiovascular disease. In the patient studies, the rs12041331 polymorphism was significantly associated with clinical outcomes, with A allele carriers demonstrating a higher risk of adverse cardiovascular events compared to GG homozygotes. These findings are in keeping with other recent studies assessing platelet reactivity or clinical outcomes, which demonstrate a clear association between *PEAR1* genotype and higher platelet reactivity or adverse clinical outcomes (Backman et al., 2017, Yao et al., 2018). Furthermore, deep sequencing of the *PEAR1* locus in 1709 participants of the GeneSTAR genome-wide association study has also demonstrated a significant association between the *PEAR1* rs12041331 polymorphism and aspirin related platelet inhibition (Keramati et al., 2018). Taken together, these data suggest that *PEAR1* polymorphisms may be important modifiers of aspirin response, although the underlying mechanism of how the polymorphisms affect aspirin response remains unclear. Whilst a number of studies have demonstrated an association between *PEAR1* polymorphisms and platelet reactivity in patients on aspirin, the relationship with COX-1 sensitive assays, such as

serum thromboxane B2 or urinary 11dhTXB2, has been less clear. This suggests that the effect of the *PEAR1* polymorphism on platelet aggregation is unlikely to be COX-1 or aspirin specific. Moreover, *PEAR1* polymorphisms have also been associated with response to other anti-platelet drugs, such as clopidogrel, which is in keeping with recent data suggesting that PEAR1 has pluripotent effects on platelet aggregation via multiple pathways including GPIIb/IIIa activation and PI3K/Akt signalling (Backman et al., 2017, Keramati et al., 2018).

A recent-meta-analysis of 53 studies assessing the relationship between aspirin response and genetic polymorphisms (Yang et al., 2018a) by Yang and colleagues identified six genetic polymorphisms that may be associated with aspirin response. These included SNPs in *GPIb* (-5T/C), *GPIa* (807C/T), *COX-1* (-1676A/G), *COX-2* (-1195A/G) and *TBXA2R* (924T/C), with some SNPs being associated with platelet outcome only in a specific ethnicity or disease group. Notably, this meta-analysis failed to detect significant associations between a number of other SNPs in different genes and aspirin response, including platelet receptors and other surface glycoproteins. These data are in keeping with another meta-analysis of studies investigating polymorphisms in *COX-1*, *COX-2*, *GPIa* and *GPIb* (Weng et al., 2013), which demonstrated a strong association between the *GPIa* 807C/T and *COX-2* 765G/C polymorphisms and aspirin non-response. Similarly, other meta-analyses have also failed to detect associations between aspirin response and other polymorphisms, such as *GPIIIa* PIA1/A2 (Floyd and Ferro, 2014) or *COX-1*, *P2Y1* and *P2Y12* (Goodman et al., 2008).

Whilst a number of genetic variants have been associated with poor aspirin response, it remains unclear how patients demonstrated to be poorly responsive to aspirin should be treated. In a sub-analysis of the ASPECT study (Gurbel et al., 2007), Gurbel et al could not demonstrate a clear dose-response relationship at three different aspirin doses (81, 162 and 325mg) in COX-1 specific platelet function tests, although higher doses did reduce resistance and platelet reactivity when non-COX-1 dependent platelet function tests were used. Furthermore, a sub-study of the CHARISMA study failed to demonstrate any clinical benefit of aspirin doses greater than 100mg daily (Steinhubl et al., 2009). However, recent data have suggested that increasing aspirin dose in aspirin poor responders may improve aspirin sensitivity and potentially clinical outcomes. Mrdovic et al (Mrdovic et al., 2016) treated aspirin non-responders (N=190) with 300mg aspirin daily for 30 days, whilst aspirin sensitive patients remained on low dose aspirin (N=771). After 30 days, the clinical outcomes of MACE and bleeding were not significantly different between the two groups, suggesting that aspirin 300mg in poor responders was as effective as low dose aspirin in aspirin sensitive patients. Similarly, Gengo et al (Gengo et al., 2016) demonstrated that increasing the dose of aspirin

beyond 81mg daily in non-responders substantially improved platelet inhibition. Out of 100 patients deemed non-responders, 79 patients became aspirin sensitive following doses of 162mg or 325mg, although no clinical outcome or adverse event data were available for this study. Interestingly, a further study by Paikin et al (Paikin et al., 2015) demonstrated that a four-time daily dose of 81mg aspirin may be more effective than a single 325mg dose of aspirin in reducing platelet reactivity in aspirin non-responders.

Despite these positive data on higher doses of aspirin to reduce platelet reactivity in aspirin non-responders, there are no high quality, randomised, clinical trial data to support this in clinical practice. In addition, treatment of other causes of poor aspirin response, such as diabetes, interacting medication and poor adherence, should be considered first-line measures.

There are a number of limitations to the current analysis. Firstly, whilst patients were recruited from a prospective cohort of NSTEACS patients, patients were selected for this analysis on the basis of available platelet function test data and it has not been possible to control for other factors that may significantly affect platelet function such as diabetes, hyperlipidaemia and high body mass index. However, the incidence of these conditions in the current analysis is broadly similar to other pharmacogenetic studies investigating aspirin non-response. In addition, a statistically significant difference was observed in a number of co-morbidities between the PFA-100 and Multiplate groups, which is likely to represent a location effect. Most patients with Multiplate data were recruited from tertiary cardiothoracic units (Blackpool Victoria Hospital and Liverpool Heart and Chest Hospital) whereas the PFA-100 data were from patients mostly admitted to the Royal Liverpool University Hospital, with a more general and less-specialised cardiology unit.

Secondly, the number of patients included in the current analysis is relatively low. As described in the methods section, platelet function was assessed at only three of the sixteen UK hospital sites that recruited patients into the PhACS study. Furthermore, this analysis is from an interim analysis undertaken after approximately 900 of the 1470 patients had been enrolled. Consequently, this has limited the power of this analysis to detect associations between genotype and phenotype, particularly after correction for multiple testing.

Thirdly, this analysis was undertaken using a candidate gene approach based on an assessment of aspirin's pharmacokinetic and pharmacodynamic pathway. Consequently, polymorphisms in other potential candidate genes (such as platelet glycoproteins and receptors) were not assessed. However, several meta-analyses have failed to identify

polymorphisms in platelet receptors and platelet glycoproteins as significant modifiers of aspirin response; consequently, it is unlikely that any significant effect would have been observed had those polymorphisms been included in this analysis.

Fourthly, a number of SNPs chosen for this analysis could not be included due to difficulties encountered during genotyping (low call rate, assay failure) or from very low minor allelic frequencies. Consequently, this has limited the scope of the analysis given that a number of polymorphisms in aspirin's pharmacodynamic pathway could not be assessed.

Fifthly, the current analysis did not investigate the agreement between the two methods used to assess platelet reactivity. Whilst the PFA-100 system was used to identify non-responders to aspirin, the Multiplate platform assessed overall platelet reactivity and consequently it was not possible to directly assess correlation between the two platforms. A further limitation was the small number of patients that had platelet function data from both the PFA-100 and Multiplate systems. However, a potential strength of this analysis is the use of one COX-dependent platelet function test (Multiplate) and one COX-independent test (PFA-100), which offers a greater opportunity to assess genotype against the complex phenotype of platelet aggregation and response to anti-platelets.

Finally, this analysis reported a relatively high level of aspirin non-response. Whilst patients included in this analysis reported good adherence to aspirin, aspirin adherence was not assessed formally by measuring serum TXB2 or Ur11dhTXB2.

In conclusion, the current investigation failed to detect any significant associations between candidate genes in aspirin's pharmacokinetic and pharmacodynamic pathway and aspirin response as assessed by two different platelet function tests, although possible associations were detected for polymorphisms in *TBXA2R* and *UGT1A6* prior to correction for multiple testing. In addition, several studies have demonstrated potentially clinically relevant SNPs in the *PEAR1* gene which may be important for assessing the risk of aspirin non-response and poor clinical outcome. Similarly, two meta-analyses have also identified SNPs in the *GPIa* and *GPIb* genes that may also increase the risk of aspirin non-response. Further studies, utilising relevant, high risk, patient populations are clearly necessary to determine the relevance of these genetic variants. These studies should include robust clinical outcomes, platelet function testing and assessment of aspirin compliance in addition.

# Chapter 5 – The relationship between OxLDL-β2GPI levels, lipid profile, platelet function and clinical outcomes in patients with an acute coronary syndrome treated with aspirin

## 5.1: Introduction

Atherosclerosis is the fundamental process underlying most acute coronary syndromes. It leads to the formation of atheromatous plaques within arteries causing progressive vascular stenosis, haemodynamic insufficiency and consequent ischaemic symptoms. Rupture of atheromatous plaques in coronary arteries lead to rapid, platelet-rich, thrombus formation with acute ischaemia and infarction of distal myocardium.

Atherosclerosis is primarily driven by lipids, and in particular, low-density lipoprotein cholesterol (LDL) (Pirillo et al., 2013) which is deposited within the intima of the arterial wall. Subsequent oxidation of the deposited LDL leads to immune activation and inflammatory cell activation via several mechanisms (Hartley et al., 2019) including expression of vascular adhesion molecules and endothelial cell dysfunction. A defining feature of atherosclerosis is the production of foam cells within the arterial wall, which are derived from macrophages and are primarily responsible for cholesterol uptake into atherosclerotic plaques mediated via scavenger receptor class A (SR-A) and CD36 (Yu et al., 2013). Oxidised LDL, derived from lipid oxidation, is one of the principal factors that promote the formation of foam cells from macrophages (Peluso et al., 2012), increasing the rate of lipid disposition with consequent formation of lipid rich atherosclerotic plaques. In addition, macrophage activation induces the production of pro-inflammatory cytokines which attracts other immune cells, such as lymphocytes, into the plaque. The resulting pro-inflammatory state in the plaque increases production of various proteolytic enzymes and reactive oxygen species (ROS) which further increases immune activation and lipid oxidation. Ultimately, the extensive inflammatory cell infiltration, lipid peroxidation and enzyme mediated degradation leads to plaque instability and eventual rupture, culminating in an acute coronary syndrome.

Lipid peroxidation is likely to be the critical step in this process. OxLDL production stimulates the production of a wide range of mediators, some of which may be recognised by the immune system as ‘danger associated molecular patterns’ (DAMPs) (Hartley et al., 2019, Leibundgut et al., 2013). These include ‘oxidation-specific epitopes’ (OSEs) which may have varying effects: some, such as malondialdehyde-acetaldehyde (MDA)-LDL, increase the

uptake of OxLDL by macrophages, whilst others are recognised by the innate immune system and lead to further immune activation. Ultimately, their pleiotropic effect results in propagation of both atherosclerosis and immune activation and therefore OxLDL can be viewed as a primary regulator of atherosclerosis. In addition, OxLDL exerts its effect via the lectin-like oxidised LDL receptor-1 (LOX-1) (Pirillo et al., 2013) which is expressed on a number of different vascular and immune tissues, including endothelial cells, vascular smooth muscle and macrophages. Its expression is up-regulated by a number of factors, including inflammation, hypertension, hyperglycaemia, oxidative stress and shear stress (Pirillo et al., 2013). LOX-1 activation also has deleterious effects on vascular biology, inducing endothelial dysfunction as well as increasing overall oxidative stress (Jin and Cong, 2019), which forms a positive feedback loop with OxLDL. Endothelial cell dysfunction may itself accelerate the development of atherosclerosis due to immune dysregulation, platelet activation and alterations in vascular haemodynamics, all of which may increase the likelihood of plaque rupture (Jin and Cong, 2019, Pirillo et al., 2013). Finally, LOX-1 may also have effects on vascular smooth muscle cells causing both proliferation and apoptosis in experimental models (Pirillo et al., 2013).

Higher OxLDL and LOX-1 levels have been associated with clinical manifestations of cardiovascular disease and, in some studies, an increased risk of adverse cardiovascular events. Several studies have demonstrated an association between higher levels of OxLDL and increases in carotid artery intima-media thickness (cIMT) (Calmarza et al., 2014, Gao et al., 2018), with one study demonstrating significant increases in carotid plaque inflammation and consequent stroke risk (Markstad et al., 2019). Furthermore, in the CHANCE study, OxLDL level was strongly associated with the risk of recurrent stroke (HR 1.43; 95% CI 1.03 to 1.98) (Wang et al., 2018b), as was the ratio between OxLDL and HDL (HR 1.50; 95% CI 1.08 to 2.08) (Wang et al., 2018a). A meta-analysis of 12 studies also demonstrated a consistent association between OxLDL level and cardiovascular prognosis with a 79% increase in the risk of adverse cardiovascular events reported (Gao et al., 2018). However, this meta-analysis also highlighted that the number of high-quality studies investigating the relationship between OxLDL and clinical outcome is low, with individual studies including different patient types and utilising variable outcome measures.

Importantly, OxLDL also forms stable complexes with beta-2-glycoprotein I, a phospholipid binding protein, which may also be similarly pro-atherogenic. The OxLDL-β2GPI complex promotes macrophage differentiation to foam cells (Xu et al., 2014) via pathways specific to the complex itself and, consequently, the effects of the OxLDL-β2GPI complex is likely to be

additive to the known effects of OxLDL. In addition, the complex is pro-inflammatory and widely distributed throughout atherosclerotic plaques, further enhancing immune activation, inflammation, lipid deposition and plaque instability (Ames et al., 2018, Wang et al., 2018c).

Several clinical studies have demonstrated that the OxLDL-β2GPI complex is associated with more severe atherosclerosis and risk of adverse cardiovascular outcomes. In a large study of 500 patients with cardiovascular disease, Bliden et al (Bliden et al., 2016) demonstrated a strong association between severe atherosclerosis and higher OxLDL-β2GPI complex levels. Interestingly, there was no association between OxLDL levels and degree of atherosclerosis, suggesting that OxLDL-β2GPI levels may be a more sensitive marker of lipid oxidation and overall vascular inflammation. Similarly, other studies have demonstrated associations between OxLDL-β2GPI complex levels and carotid artery disease, stroke and diabetic microvascular disease (Berger et al., 2014, Yu et al., 2015, Zhang et al., 2018), although recent data on clinical outcomes in unstable cardiovascular disease is lacking, with a single study demonstrating a significant increase in the risk of adverse cardiovascular events in ACS patients with elevated OxLDL-β2GPI complex levels (Greco et al., 2010).

Lipid oxidation may also be associated with higher rates of platelet activation. Several studies have demonstrated that OxLDL may significantly increase platelet aggregation via a number of different mechanisms. These include activation of platelets via the scavenger receptor, CD36, or via other mechanisms such as ROS generation, phospholipase C activation, increased platelet-monocyte interactions or dysregulation of the PI3K/AKT/mTOR pathway (Berger et al., 2018, Hua et al., 2009, Wang et al., 2018d). As these mechanisms are not directly inhibited by anti-platelet drugs, such as aspirin, it is conceivable that they may be involved in the underlying causes of aspirin non-response. However, little data exists on the relationship between OxLDL and platelet reactivity in patients and therefore the impact of the interaction between platelet reactivity and OxLDL is unclear.

In summary, whilst OxLDL has a critical role in the development of atherosclerosis and subsequent acute coronary syndromes, there remains a paucity of clinical outcome data in relevant patient cohorts, such as unstable cardiovascular disease. In addition, whilst OxLDL has been demonstrated to increase platelet activation *in vitro*, it remains unclear whether there is an effect on platelet reactivity in patients and whether this is associated with a poorer response to anti-platelets such as aspirin. Finally, OxLDL exerts some of its pro-atherogenic action via the formation of OxLDL-β2GPI complexes, which, to date, have not

been robustly investigated in the context of acute coronary syndromes or via the assessment of platelet reactivity.

In order to address these questions, a case-control study investigating the relationship between OxLDL-β2GPI complex levels and clinical and platelet reactivity outcomes was conducted in a cohort of patients with non-ST elevation acute coronary syndromes (NSTEACS).

## 5.2: Methods

### 5.2.1: Patient Cohort

Patients were recruited from the prospective 'Pharmacogenetics of Acute Coronary Syndrome' (PhACS) study which has been described previously.

Briefly, subjects were included in this study if they were in hospital with a primary diagnosis of an acute coronary syndrome. Principal exclusions included ST elevation MI, other diagnoses likely to explain a positive troponin and being unwilling or unable to consent to the study.

All included subjects were followed up for a minimum of 12 months from recruitment with two physical visits at month 1 and month 12 and annual telephone follow up with a case note review. Follow up continued annually until the final patient recruited had completed 12 months of follow up.

Blood samples were taken at all physical visits. These included a sample for genotyping (baseline visit only), platelet function (all visits), RNA sample (all visits), serum sample (all visits) and urine samples (all visits). Platelet function was assessed using the PFA-100 system and the Multiplate platform.

The primary outcome measure for the PhACS study was a composite of cardiovascular mortality, non-fatal MI and stroke. Secondary outcomes included all-cause mortality, bleeding and development of left ventricular failure.

Cardiovascular mortality was defined using the PLATO trial (Wallentin et al., 2009) criteria of "cardiovascular deaths, cerebrovascular deaths and any other death for which there was not a clearly documented non-vascular cause". Non-fatal myocardial infarction was defined using the TRITON-TIMI 38 (Wiviott et al., 2007) criteria of "MI must be distinct from the index

event and is defined by symptoms suggestive of ischaemia/infarction, electrocardiographic data, cardiac biomarker or pathologic evidence of infarction dependent on the clinical situation using criteria adapted from the definition developed by the American College of Cardiology". Non-fatal stroke was defined using adapted criteria from the HORIZONS-AMI study (Mehran et al., 2008) of "an acute neurological event or deficit lasting for greater than 24 hours, as classified by a physician".

This study received ethical approval from the Liverpool Adult Research Ethics Committee and was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki.

**5.2.2: Patient Selection**

Patients were selected for this study on a case-control basis. Cases were defined as patients who had suffered an ischaemic event consistent with the primary outcome measure definition. Cases were matched 1:1 with other patients recruited to the PhACS study who did not suffer an ischaemic event. Subjects were matched for the following criteria:

- Gender
- Age (+/- 5 years)
- Body Mass Index (obese / not obese)
- Diabetes Status
- Follow up period for the control subject exceeds the case subject's time to first cardiovascular event

In addition, subjects had to be receiving aspirin at discharge form the index admission. Wherever possible, other cardiovascular risk factors were matched between the case and control subjects. Given that platelet function was only assessed at three sites in the PhACS study, cases and controls were selected only from those sites.

**5.2.3: OxLDL-β2GPI Assay**

The OxLDL-β2GPI complex was tested using the hsAtherOx Test Kit (Corgenix, Broomfield USA) in compliance with the manufacturer's instructions. Briefly, the assay is performed as an indirect enzyme-linked immunosorbent assay (ELISA). The subject's diluted serum samples, as well as calibrator and control samples, were incubated in microwells coated with a monoclonal antibody against human complexed β2GPI. Samples were washed to remove protein present within the serum, which was followed by addition of biotin conjugated anti-human apoB100 (LDL) monoclonal antibodies in order to form complexes with the bound

antigen. After a further washing step, horseradish peroxidase conjugated Streptavidin (HRP-SA) was added to the biotin-conjugated antibody-antigen complex. After washing, tetramethylbenzidine (TMB) / hydrogen peroxide was added and colour allowed to develop over 30 minutes. Optical density was then read using a 450 nm wavelength; with results being calculated against the calibration curves prepared using the kit's calibration samples. Results are reported as U/mL. Samples were analysed in the Wolfson Centre for Personalised Medicine by Dr Eunice Zhang, Dr Valentina Manzo and myself.

**5.2.4: Lipid profile analysis**

All lipid profile samples were analysed by the Clinical Biochemistry Department at the Royal Liverpool University Hospital for a standard profile of total cholesterol, high density lipoprotein (HDL) and triglycerides. Results are reported as mmol/L.

Briefly, the cholesterol and HDL assays were performed using an automated Roche/Hitachi cobas c analyser using the CHOL2 and HDLC4 assay kits (Roche, Mannheim, Germany) in line with the manufacturer's instructions and standard procedures. The CHOL2 assay utilises an enzymatic, colorimetric method where cholesterol esterase is used to generate free cholesterol. The free cholesterol is then oxidised to cholest-4-en-3-one and hydrogen peroxide via cholesterol oxidase and the hydrogen peroxide generated induces changes in the oxidative-coupling of phenol and 4-aminophenazone which forms a red quinone-imine dye, measured by an increase in absorption. Similarly, the HDLC4 assay is a homogenous enzymatic colorimetric test, using the differential sensitivity of LDL, VLDL and HDL to cholesterol esterase and cholesterol oxidase following addition of polyanions and detergent. HDL is then isolated via the actions of cholesterol esterase and cholesterol oxidase in the presence of oxygen, resulting in the generation of hydrogen peroxide. The hydrogen peroxide, in the presence of peroxidase, generates a dye from 4-amino-antipyrine which is then read photometrically. The triglyceride assay (Trig/GB) was performed using the automated Roche/Hitachi MODULAR P analyser (Roche, Mannheim, Germany). This assay measures free glycerol using a colorimetric method following enzymatic hydrolysis of triglycerides catalysed by glycerol kinase, glycerol-3-phosphate oxidase and lipase.

LDL values were derived and reported using the following formula:

$$\text{LDL cholesterol} = \text{Total cholesterol} - \text{HDL cholesterol} - \text{VLDL cholesterol}$$

VLDL cholesterol was estimated using the following formula:

$$\text{VLDL cholesterol} = \text{Triglycerides} / 2.19$$

### 5.2.5: Platelet function testing

Platelet function was assessed as described previously using the PFA-100 system and Multiplate platforms. PFA-100 data were only available for patients recruited from the Royal Liverpool University Hospital and Liverpool Heart and Chest Hospital whilst Multiplate data were available from Blackpool Victoria Hospital, Royal Liverpool University Hospital and Liverpool Heart and Chest Hospital. Both PFA-100 and Multiplate data were analysed as categorical variables (responder / non-responder). A cut off value of <193 seconds was used as the definition of non-response to aspirin for the PFA-100 system. For the Multiplate platform, a cut-off value of >39 units for the Multiplate ASPI test was used to define aspirin non-response, as advised by the manufacturer. Platelet function tests were performed at the local recruiting site by research nurses.

### 5.2.6: Statistical Analysis

Data were presented as mean and standard deviation for continuous data, and frequencies or percentages for categorical variables. For continuous variables, data were compared by either a t-test or ANOVA. Categorical data were compared using a Chi-squared test. Simple and logistic regression analyses were conducted to investigate the relationship between OxLDL-β2GPI levels, platelet function, lipid profile, clinical variables and case-control status. A result was considered statistically significant if the P value was less than 0.05. Data were analysed using SPSS version 24 (SPSS, Chicago, USA).

## 5.3: Results

### 5.3.1: Patient Characteristics

A total of 835 patients were recruited to the PhACS study from the Royal Liverpool University Hospital, Blackpool Victoria Hospital and Liverpool Heart and Chest Hospital. Out of those 835 patients, 155 patients (18.6%) had a further ischaemic event which was considered to be consistent with the primary outcome measure. Ninety-five of the 155 patients were successfully matched with a control subject. Measurement of OxLDL-β2GPI complex levels was unsuccessful in 19 of the case-control pairs, leaving a total 76 cases and 76 controls which were included in the final analysis. A summary of patient demographics, clinical risk factors and medications is provided in **Table 5.1**. No statistically significant differences in cardiac risk factors, previous cardiac history, symptom scores or concomitant medications between the case and control groups were observed. In the case group, the first ischaemic

event was myocardial infarction in 44 patients (57.9%), cardiovascular death in 27 patients (35.5%) and CVA in 5 patients (6.6%).

### 5.3.2: Platelet function data

Platelet function data were available for only a sub-set of patients as described previously. PFA-100 data were available for 68 patients in total, of which 28 were cases and 40 were controls. Similarly, only 56 patients had available data on the Multiplate ASPI test, of which 23 were cases and 33 were controls. The overall rate of non-response observed with the PFA-100 system across both cases and controls was 54.4% (n=37). However, the Multiplate system reported a substantially lower rate of aspirin non-response (8.9%, n=5) across both cases and controls. A summary of platelet function data is provided in **Table 5.2**.

### 5.3.3: Relationship between OxLDL –β2GPI complex levels and case-control status

The mean OxLDL-β2GPI level was 1.62 +/- 2.31 U/mL in cases and 2.65 +/- 4.13 in controls. Similarly, the median OxLDL-β2GPI level was 0.65 U/mL (IQR 0.38 - 1.49) in cases and 0.83 (0.45 - 2.28). Univariate analysis did not demonstrate a significant association between OxLDL-β2GPI and case-control status (P=0.071). However, following adjustment for clinical co-variates including age, gender, obesity, diabetes, hypertension, hyperlipidaemia and chronic kidney disease, a significant association between lower OxLDL-β2GPI complex levels and further cardiovascular events was observed (P=0.0341) (**Table 5.3**).

### 5.3.4: Relationship between OxLDL-β2GPI levels and lipid profiles

There was no significant association detected between LDL cholesterol and OxLDL-β2GPI levels (P=0.099) in univariate analyses. Similarly, no association was observed between OxLDL-β2GPI levels and total cholesterol (P=0.066) or triglycerides (P=0.711).

### 5.3.5: Relationship between OxLDL-β2GPI levels and platelet reactivity

No significant association was observed between PFA-100 defined aspirin non-response and OxLDL-β2GPI levels (P=0.847). Similarly, for the Multiplate ASPI test no significant association between OxLDL-β2GPI level and aspirin non-response status (P=0.533) or overall ASPItest AUC (P=0.273) was observed (**Table 5.4**).

### 5.3.6: Relationship between platelet reactivity and case-control status

For PFA-100 determined aspirin non-response, the aspirin non-response rate was numerically higher in control patients compared to cases although this was not statistically significant (60.0% versus 46.4% respectively, P=0.415). In addition, for aspirin non-response

defined using the Multiplate ASPI test, a higher rate of non-response was detected in the controls compared to the cases which was not statistically significant (12.1% vs 4.4%, P=0.336) (**Table 5.2**).

### 5.3.7: Relationship between lipid profile and case-control status

There were no significant differences observed in LDL, HDL and total cholesterol levels between case and control patients. Whilst mean total cholesterol values were marginally higher in cases compared to controls, this did not meet statistical significance (P=0.47). Conversely, mean HDL cholesterol levels were also numerically higher in cases compared to controls, but this did not meet statistical significance (1.06 +/- 0.49 vs 0.96 +/- 0.26 mmol/L, P=0.10). Notably, the case and control groups were well matched for statin usage (89.0% vs 91.8% respectively, P=0.57), with mean total cholesterol and LDL-cholesterol demonstrating good control of hyperlipidaemia across both groups (**Table 5.3**).

### 5.3.8: Relationship between lipid profile and platelet function

A significant association between HDL:cholesterol ratio level and aspirin non-response determined by the Multiplate ASPItest was observed, with aspirin non-responders having a higher HDL:cholesterol ratio compared to aspirin responders (5.18+/-1.99 vs 3.68 +/-1.23 mmol/L, P=0.017). This appeared to be primarily driven by a lower HDL cholesterol level in non-responders compared to responders, although the difference was not statistically significant (0.72+/-0.08 vs 1.02+/-1.02 mmol/L, P=0.059). No significant associations between Multiplate defined aspirin response and LDL or total cholesterol were observed. In addition, there was no significant difference in lipid profiles between aspirin responders or non-responders as determined by the PFA-100 system (**Table 5.4**).

| | | Cases | Controls | P-value |
|---|---|---|---|---|
| | **N** | 76 | 76 | 1.00 |
| **Males** | **N** | 56 | 56 | 1.00 |
| | **%** | 73.68 | 73.68 | |
| **Age** | **Mean** | 70.58 | 69.72 | 0.59 |
| | **SD** | 10.75 | 10.19 | |
| | **Median** | 71.96 | 70.03 | |
| | **Q1** | 63.51 | 63.13 | |
| | **Q3** | 79.60 | 77.73 | |
| | **IQR** | 16.08 | 14.61 | |
| **BMI** | **Mean** | 28.97 | 29.00 | 0.98 |
| | **SD** | 6.93 | 5.94 | |
| | **Median** | 27.69 | 28.40 | |
| | **Q1** | 24.10 | 25.45 | |
| | **Q3** | 33.34 | 32.10 | |
| | **IQR** | 9.24 | 6.65 | |
| **Hypertension** | **N** | 51 | 54 | 0.60 |
| | **%** | 67.11 | 71.05 | |
| **Hyperlipidaemia** | **N** | 49 | 46 | 0.62 |
| | **%** | 64.47 | 60.53 | |
| **PAD** | **N** | 7 | 2 | 0.09 |
| | **%** | 9.21 | 2.63 | |
| **CKD** | **N** | 12 | 8 | 0.34 |
| | **%** | 15.79 | 10.53 | |
| **DM** | **N** | 21 | 21 | 1.00 |
| | **%** | 27.63 | 27.63 | |
| **Prior MI** | **N** | 40 | 32 | 0.19 |
| | **%** | 52.63 | 42.11 | |
| **Prior PCI** | **N** | 18 | 12 | 0.22 |
| | **%** | 23.68 | 15.79 | |
| **Prior CABG** | **N** | 15 | 11 | 0.39 |
| | **%** | 19.74 | 14.47 | |
| **Current Smoker** | **N** | 15 | 10 | 0.27 |

| | | | | |
|---|---|---|---|---|
| | % | 19.74 | 13.16 | |
| **Previous Smoker** | **N** | 45 | 47 | 0.74 |
| | **%** | 59.21 | 61.84 | |
| **Non Smoker** | **N** | 16 | 18 | 0.70 |
| | **%** | 21.05 | 23.68 | |
| **CCS** | **Mean** | 1.32 | 1.11 | 0.12 |
| | **SD** | 0.91 | 0.83 | |
| | **Median** | 1 | 1 | |
| | **N** | 73 | 73 | |
| **Aspirin** | **N** | 73 | 73 | 1.00 |
| | **%** | 100.00 | 100.00 | |
| **Clopidogrel** | **N** | 66 | 66 | 1.00 |
| | **%** | 90.41 | 90.41 | |
| **Warfarin** | **N** | 2 | 1 | 0.56 |
| | **%** | 2.74 | 1.37 | |
| **LMWH** | **N** | 4 | 5 | 0.73 |
| | **%** | 5.48 | 6.85 | |
| **Beta-Blocker** | **N** | 56 | 64 | 0.08 |
| | **%** | 76.71 | 87.67 | |
| **CCB** | **N** | 17 | 16 | 0.84 |
| | **%** | 23.29 | 21.92 | |
| **Nitrate** | **N** | 45 | 40 | 0.40 |
| | **%** | 61.64 | 54.79 | |
| **Statin** | **N** | 65 | 67 | 0.57 |
| | **%** | 89.04 | 91.78 | |
| **ACE-I** | **N** | 56 | 53 | 0.57 |
| | **%** | 76.71 | 72.60 | |
| **ARB** | **N** | 5 | 7 | 0.55 |
| | **%** | 6.85 | 9.59 | |
| **PPI** | **N** | 39 | 32 | 0.25 |
| | **%** | 53.42 | 43.84 | |
| **Death** | **N** | 27 | NA | NA |
| | **%** | 35.5 | | |

| | | | | |
|---|---|---|---|---|
| **MI** | **N** | 44 | | |
| | **%** | 57.9 | | |
| **CVA** | **N** | 5 | | |
| | **%** | 6.6 | | |

**Table 5.1 – Characteristics of included patients**

| | | **Cases** | **Controls** | **P-value** |
|---|---|---|---|---|
| **PFA** | **Number with Data** | 28 | 40 | 0.415 |
| | **N Resistant** | 13 | 24 | |
| | **% Resistant** | 46.4 | 60.0 | |
| **Multiplate** | **Number with Data** | 23 | 33 | 0.336 |
| | **N Resistant** | 1 | 4 | |
| | **% Resistant** | 4.4 | 12.1 | |

**Table 5.2 – Relationship between case-control status and platelet function**

| | | Cases | Controls | P-value |
|---|---|---|---|---|
| | **N** | 76 | 76 | NA |
| **OxLDL-β2GPI (U/mL)** | **Mean** | 1.62 | 2.65 | 0.07 (Unadjusted) |
| | **SD** | 2.31 | 4.13 | |
| | **Median** | 0.65 | 0.83 | **0.03** (Adjusted) |
| | **IQR** | 0.37-1.49 | 0.45-2.28 | |
| **LDL (mmol/L)** | **Mean** | 2.03 | 2.07 | 0.79 |
| | **SD** | 0.96 | 0.81 | |
| **Cholesterol (mmol/L)** | **Mean** | 3.83 | 3.69 | 0.47 |
| | **SD** | 1.28 | 0.91 | |
| **HDL (mmol/L)** | **Mean** | 1.06 | 0.96 | 0.10 |
| | **SD** | 0.49 | 0.26 | |
| **HDL:Chol Ratio** | **Mean** | 4.20 | 4.11 | 0.76 |
| | **SD** | 2.33 | 1.57 | |

**Table 5.3 – Relationship between case-control status, OxLDL-β2GPI and lipid profile**

| | | PFA-100 | | | Multiplate | | |
|---|---|---|---|---|---|---|---|
| | | SENS | RESIST | P-value | SENS | RESIST | P-value |
| | N | 31 | 37 | NA | 51 | 5 | NA |
| | % | 45.6 | 54.4 | | 91.1 | 8.9 | |
| OxLDL-β2GPI (U/mL) | Mean | 3.12 | 3.33 | 0.847 | 4.18 | 2.31 | 0.141 |
| | SD | 4.26 | 4.40 | | 4.85 | 3.45 | |
| | Median | 1.21 | 1.28 | | 1.69 | 1.17 | |
| | IQR | 0.64-4.14 | 0.51-5.95 | | 0.48-6.67 | 0.37-4.83 | |
| LDL (mmol/L) | Mean | 2.05 | 2.10 | 0.82 | 1.91 | 2.36 | 0.26 |
| | SD | 1.11 | 0.78 | | 0.80 | 1.15 | |
| Cholesterol (mmol/L) | Mean | 3.75 | 3.86 | 0.68 | 3.52 | 3.70 | 0.69 |
| | SD | 1.23 | 1.13 | | 0.94 | 1.37 | |
| HDL (mmol/L) | Mean | 1.07 | 1.01 | 0.62 | 1.02 | 0.72 | 0.06 |
| | SD | 0.39 | 0.52 | | 0.34 | 0.08 | |
| HDL:Chol Ratio | Mean | 3.85 | 4.31 | 0.30 | 3.68 | 5.18 | **0.02** |
| | SD | 1.76 | 1.82 | | 1.23 | 1.99 | |

**Table 5.4 – Relationship between platelet function tests, OxLDL-β2GPI and lipid profile**

## 5.4: Discussion

In this analysis, a potential association was detected between OxLDL-β2GPI complex levels and case-control status, with controls having higher levels of OxLDL-β2GPI compared to patients who had recurrent cardiovascular events in the PhACS study. This suggests that the OxLDL-β2GPI complex may be protective in the context of atherosclerosis rather than being pro-atherogenic and pro-inflammatory.

However, our finding is out of keeping with the majority of the published clinical outcome data. In a study by Greco et al (Greco et al., 2010), 339 patients with acute coronary syndrome had OxLDL-β2GPI measured and were followed up for a median of two years. OxLDL-β2GPI levels were significantly higher in patients with more severe coronary artery disease as assessed by coronary angiography, and clinical outcomes were worse for patients in the highest quartiles of OxLDL-β2GPI level, with an overall threefold risk increase in the risk of adverse cardiovascular outcomes in patients in the highest quartiles of OxLDL-β2GPI levels (RR 3.53; 95% CI 1.20 to 10.38, P=0.026). These data are in keeping with others, with a study by Bliden et al (Bliden et al., 2016) also demonstrating a significant association between higher OxLDL-β2GPI levels and more severe coronary atherosclerosis in a cohort of 435 patients undergoing elective coronary angiography. Similarly, Ames et al (Ames et al., 2018), in a cross-sectional case-control study of 57 patients with cardiovascular disease and 90 healthy controls, higher OxLDL/β2GPI levels were significantly associated with myocardial infarction and venous thromboembolism. Furthermore, higher OxLDL/β2GPI levels have been associated with ischaemic stroke, particularly in the context of diabetes (Zhang et al., 2018).

However our findings are in keeping with recent data suggesting that β2GPI may prevent, in some cases, the binding of OxLDL to LOX-1, thereby reducing the pro-inflammatory effects of OxLDL. Chi et al (Chi et al., 2018) demonstrated a substantial reduction in the OxLDL mediated expression of tissue factor by murine macrophages following incubation with domain 5 (DV) of β2GPI. In addition, the expression of LOX-1 was also substantially reduced following incubation of DV- β2GPI, as was the binding of OxLDL to the LOX-1 receptor. These data are in keeping with earlier studies that demonstrated that OxLDL- β2GPI may have protective effects on the vasculature rather than being pro-inflammatory. Several older studies have demonstrated that binding of β2GPI to OxLDL prevented uptake of both OxLDL and cholesterol into macrophages (Hasunuma et al., 1997, Lin et al., 2001), in keeping with Chi's data.

Whilst other data suggest that the OxLDL-β2GPI complex does have pro-inflammatory effects, its effects may be mediated by anti-β2GPI antibodies bound to the OxLD-β2GPI complex rather than by the complex itself. In Toll-Like Receptor 4 (TLR-4)-competent and TLR4 mutant mice, Zhang et al (Zhang et al., 2014d) demonstrated that treatment with OxLDL-β2GPI /anti- β2GPI induced the formation of foam cells from peritoneal macrophages via expression of TLR-4 and activation of NFκB, with a subsequent increase in pro-inflammatory cytokines observed. However, administration of OxLDL-β2GPI alone did not significantly increase NFκB activation or expression of pro-inflammatory cytokines compared to media alone. Interestingly, administration of OxLDL alone significantly increased the level of NFκB activation compared to media alone, but the increase was significantly lower than that achieved by administration of OxLDL-β2GPI/anti-β2GPI. Similarly, the conversion of macrophages into foam cells was also lower following OxLDL-β2GPI administration compared to both OxLDL alone and OxLDL/ β2GPI/anti-β2GPI. In addition, OxLDL can bind to CRP, which may also increase its pro-inflammatory effects. In mice, Wang et al (Wang et al., 2016) described 5-fold increase in macrophage cholesterol content following administration of CRP-OxLDL, compared to 3- and 4-fold increases for OxLDL-β2GPI and CRP-OxLDL-β2GPI groups. Importantly, there was a significant difference in macrophage cholesterol content between the OxLDL alone and OxLDL-β2GPI groups, with the OxLDL-β2GPI group demonstrating significantly lower macrophage cholesterol content compared to OxLDL alone. However, both OxLDL, OxLDL-β2GPI and their CRP bound entities all increased macrophage expression of scavenger receptors, although this was less pronounced following OxLDL-β2GPI administration compared to all other groups. However, in vascular smooth muscle cells (SMCs), the effects of the OxLDL-β2GPI complex appear to be broadly similar to OxLDL- β2GPI /Anti-β2GPI and OxLDL alone. Wang et al (Wang et al., 2018c) demonstrated that lipid uptake in vascular SMCs was significantly increased by the administration of OxLDL, OxLDL-β2GPI and OxLDL-β2GPI/Anti-β2GPI, with no significant differences between any of the three. This effect appears to be mediated by TLR4, and leads to expression of pro-atherogenic molecules such as MMP-9 and MCP-1. Taken together, these data suggest that whilst the OxLDL-β2GPI complex has some pro-atherogenic effects, it appears to be less functional than OxLDL itself, and in some cases may blunt the pro-inflammatory effects of OxLDL which is partially in keeping with the findings of our study. However, our analysis did not measure OxLDL in addition to OxLDL-β2GPI levels. It is therefore not possible to determine whether the higher levels of OxLDL-β2GPI in the control patients were consistent

with a reduction in the pro-atherogenic effects of OxLDL, as has been observed in the *in vitro* and animal data above.

However, in the published clinical studies, OxLDL-β2GPI levels were generally associated with OxLDL levels (Yu et al., 2015, Zhang et al., 2018) with similar increases in the risks of adverse clinical outcomes. Nonetheless, it is not clear whether the associations observed in those clinical studies are mechanistically driven by OxLDL or the OxLDL- β2GPI complex although in Bliden et al's study, only OxLDL-β2GPI was associated with the presence of severe atherosclerotic disease with no association detected for OxLDL alone (Bliden et al., 2016). Given the lack of agreement between the pre-clinical and clinical study findings, as well as the paucity of clinical data on the relationship between OxLDL-β2GPI and outcome, it remains unclear whether OxLDL-β2GPI complexes are by themselves pro-atherogenic or whether the observed effect is mediated by OxLDL alone.

Importantly, OxLDL has been long identified as a marker of atherosclerosis and increased vascular risk. As discussed previously, one of the key effects of OxLDL is the transformation of macrophages into foam cells with consequent development of lipid rich atherosclerotic plaques. In addition, this transformation is associated with the generation of various danger associated molecular patterns (Leibundgut et al., 2013) that lead to inflammatory cell activation and secretion of multiple pro-inflammatory cytokines. Ultimately this leads to activation of the NLRP3 inflammasome via NFκB activation in macrophages, resulting in IL-1β secretion and further inflammatory cell recruitment (Grebe et al., 2018). OxLDL has been serially associated with activation of TLR4, but may also have additional effects via interactions with scavenger receptors such as CD36 (Grebe et al., 2018), which also increases activation of the NLRP3 inflammasome. OxLDL has also been associated with other pro-atherogenic effects including endothelial cell dysfunction, effects on vascular smooth muscle and increases in oxidative stress via generation of ROS (Chen et al., 2012a, Chang et al., 2015, Katouah et al., 2015, Watt et al., 2016). These in-vitro effects are mirrored by clinical studies, with OxLDL levels being associated with more severe atherosclerosis and increased risk of adverse cardiovascular events. In a meta-analysis of 12 studies which included over 12,000 patients, higher OxLDL levels conferred an almost twofold higher risk of adverse cardiovascular events (Gao et al., 2017). Importantly, this meta-analysis also identified that the effects of OxLDL on cardiovascular outcome are independent of LDL cholesterol levels, an important cardiovascular risk factor. However, the relationship between LDL levels and OxLDL is unclear, with some studies demonstrating (Aydin et al., 2015, Ogawa et al., 2015) a close relationship between OxLDL levels and LDL cholesterol, whilst others do not (Russo et

al., 2018). In the current analysis, we did not demonstrate a clear association between OxLDL-β2GPI levels and LDL cholesterol levels. However, given that OxLDL levels are likely to be driven by the pro-inflammatory milieu within atherosclerotic lesions, it is likely that the degree and severity of atherosclerosis is a more important determinant of OxLDL levels than LDL cholesterol in patients. This is in keeping with the published data where OxLDL levels are most closely associated with LDL in healthy volunteer or registry studies (Zuliani et al., 2013) where the burden of atherosclerosis is likely to be very low. We did not measure the levels of inflammatory markers or pro-inflammatory cytokines in this study, and it is consequently unclear whether the observed lack of association between OxLDL-β2GPI levels and LDL-cholesterol is due to the independent effects of inflammation on OxLDL production or not. However, it should be noted that the patients included in this study had an index diagnosis of ACS and are therefore more likely to have a substantial atherosclerotic burden with consequent higher levels of inflammation.

The current analysis also failed to demonstrate a significant association between platelet reactivity and OxLDL-β2GPI levels. The relationship between the OxLDL-β2GPI complex and platelet reactivity has not been previously investigated in the context of acute coronary syndromes and, consequently, the relevance of this finding is unclear. However, a number of published studies have reported an association between higher OxLDL levels and increased platelet reactivity. In a study by Carnevale et al (Carnevale et al., 2014), agonist stimulated platelets from healthy volunteers incubated with LDL demonstrated an increase in ROS and formation of OxLDL. OxLDL generation was greater in platelets sampled from patients with hypercholesterolaemia, whilst greater platelet aggregation was observed in LDL-incubated platelets. These data suggest that hyperlipidaemia significantly increases overall platelet reactivity, and platelets themselves may be able to generate OxLDL independently. These data are in keeping with a study by Chatterjee et al (Chatterjee et al., 2017) which demonstrated that LDL and OxLDL stimulate generation of ROS and mitochondrial superoxide generation in platelets. Furthermore, in a lipidomic analysis, the lipid profile in platelets is substantially altered in patients with coronary artery disease, with specific increases detected in oxidised lipid derivatives consistent with higher oxidative stress. Finally, LDL and OxLDL were demonstrated to significantly increase both GPIIbIIIa activation and platelet degranulation, thereby providing a clear mechanism by which hyperlipidaemia and OxLDL activates platelets. OxLDL and hyperlipidaemia induced ROS production is likely to be mediated via a phospholipase Cγ2 dependent pathway (Berger et al., 2018) whilst inhibition of ROS production in platelets reduces OxLDL related platelet

activation, mediated via increased activity of the PI3K/AKT/mTOR pathway (Wang et al., 2018d) and a reduction in consequent autophagy. Interestingly, OxLDL may also increase platelet-monocyte interactions. Badrnya et al (Badrnya et al., 2014) demonstrated that following stimulation with OxLDL, platelets and monocytes rapidly form platelet-monocyte aggregates, predominantly involving CD16+ monocytes. This interaction increased monocyte OxLDL uptake and conversion to foam cells, mediated via a CD36-OxLDL interaction. Furthermore, addition of aspirin or ticagrelor appeared to reduce OxLDL uptake although it did not abolish it entirely. In human umbilical vein endothelial cells, Chen et al (Chen et al., 2012a) demonstrated that aspirin also reduced OxLDL mediated ROS generation by downregulation of Nox4 and nitric oxide synthase expression as well as NFκB activation. In keeping with Badrnya et al's findings, aspirin also reduced the expression of MCP-1 with consequent lower monocyte attraction and potential for generation of pro-atherogenic foam cells. Taken together, these data suggest that OxLDL may be an important modifier of platelet reactivity, although it remains unclear whether the OxLDL-β2GPI complex would be similarly pro-aggregatory given that the OxLDL-β2GPI complex may decrease the pro-inflammatory effect of OxLDL itself.

Our study did detect a significant association between higher HDL:cholesterol ratios and an increased frequency of aspirin non-response when measured by the Multiplate platform. This observation appeared to be driven mostly by a reduction in HDL-cholesterol rather than an increase in total cholesterol, although no significant differences were demonstrated in HDL levels between non-responsive and responsive patients. These findings are in keeping with data suggesting that hyperlipidaemia may increase platelet reactivity and reduce effectiveness of anti-platelet drugs such as aspirin and clopidogrel. In a study of 48 patients with hypercholesterolaemia, Chan and colleagues (Chan et al., 2015) demonstrated that platelet reactivity was significantly higher in hypercholesterolaemic patients compared to healthy volunteers. Importantly, whilst high LDL cholesterol was associated with increased platelet reactivity, patients with both high LDL cholesterol and low HDL cholesterol levels had significantly higher platelet reactivity compared to patients with raised LDL cholesterol alone. With regard to anti-platelet drug response, Labuz-Roszak and colleagues (Labuz-Roszak et al., 2014) demonstrated that aspirin response, assessed by the Multiplate platform, was associated with lipid profile in a cohort of 96 patients with diabetes. A strong association was detected between poor aspirin response and higher LDL levels, although no association was demonstrated between total cholesterol or HDL levels and aspirin response in this study. Following a lipid challenge, Yassine et al demonstrated a significant increase in

urine11dhTXB2 levels in a cohort of 11 diabetic patients being treated with aspirin (Yassine et al., 2010) although there was no significant increase in VerifyNow measured platelet reactivity.

Given the emerging evidence of the adverse effects of OxLDL on platelet reactivity and clinical outcomes, an increasing focus has been placed on how high levels of OxLDL should be treated. Despite the effects of OxLDL on platelet reactivity, aspirin has been demonstrated to reduce the effects of OxLDL on vascular cells. In OxLDL stimulated macrophages (Hua et al., 2009), aspirin significantly reduced the expression of MMP-2 and MMP-9 which are important mediators associated with the risk of atherosclerotic plaque rupture. In addition, aspirin was also demonstrated to upregulate tissue inhibitors of metalloproteinases (TIMP) -1 and -2, further reducing the effects of MMP-2 and MMP-9. In endothelial cells exposed to Ox-LDL, aspirin also reduced the expression of COX-2 and ICAM-1 (Zhao et al., 2008), which may reduce the levels of oxidative stress and monocyte binding. In addition, aspirin was demonstrated to significantly reduce NFκB activation and p38 MAPK phosphorylation induced by OxLDL.

Statins, given their beneficial effect on lipids, also have beneficial effects on OxLDL. Statins have been demonstrated to have anti-inflammatory effects on endothelial cells. In an in-vitro study, Wang and colleagues (Wang et al., 2017) demonstrated that OxLDL activates the NLRP3 inflammasome in vascular endothelial cells, which is significantly reduced by the addition of simvastatin. The beneficial effects of statin therapy on OxLDL and inflammation has also been extensively demonstrated in clinical studies and randomised controlled trials. In a retrospective study of 600 patients undergoing angiography, Ogawa and colleagues (Ogawa et al., 2015) demonstrated that an observed association between OxLDL levels and smoking status was only observed in patients not being treated with statins. In patients on statins, no significant association was observed between smoking status and any component of the investigated lipid profile. In STEMI patients, Aydin et al (Aydin et al., 2015) randomised 120 statin naïve patients to receive either atorvastatin 80mg or rosuvastatin 20mg daily. After four weeks of treatment, both atorvastatin and rosuvastatin significantly reduced OxLDL levels by 60% from baseline values which was accompanied by a corresponding decrease in inflammatory markers (hs-CRP, IL-6 and TNF-R1). Similarly, in 153 hypercholesterolaemic patients, Moutzouri et al (Moutzouri et al., 2013) randomised patients to receive either simvastatin 40mg, rosuvastatin 10mg or simvastatin 10mg and ezetimibe 10mg daily. All three treatments significantly reduced OxLDL levels to a similar extent after a three-month treatment period, with corresponding reductions in other

markers of oxidative stress (8-Epi PGF2α) and inflammation (lipoprotein associated phospholipase A2). Finally, Altunkeser et al (Altunkeser et al., 2019) randomised 106 ACS patients to receive atorvastatin 80mg or rosuvastatin 40mg daily for four weeks. After four weeks, the levels of OxLDL were significant lower compared to baseline assessment, with no significant difference observed between the two treatment groups. However, this was at the expense of an increased PCSK9 level which is in keeping with the previously known effects of high-dose statin therapy. Importantly, statin therapy may also have beneficial effects on platelet reactivity and the response to anti-platelet drugs. In a study of 83 patients with stable coronary artery disease, Pesaro et al (Pesaro et al., 2012) demonstrated that statin therapy significantly reduced platelet reactivity in aspirin treated patients, which was mirrored by reductions in CRP, OxLDL and LDL cholesterol. These data are in keeping with two further studies which demonstrated a significant improvement in anti-platelet drug response following treatment with statins (Godino et al., 2017, Tacconelli et al., 2018). In this study, we did not investigate the potential effect of statin treatment on platelet reactivity or OxLDL-β2GPI levels, particularly in relation to statin potency or dose. However, there are no significant differences between statin treatment in the case and control groups, with similar numbers of patients in both groups receiving high potency or high dose statins.

However, OxLDL and OxLDL-β2GPI may not best represent the pathological effects of hyperlipidaemia or oxidative stress on atherosclerosis. Recent data suggest that other factors, such as lipid particle size, may have a critical effect on cardiovascular risk and development of acute coronary syndromes. Smaller HDL particle size may reduce the affinity of the HDL particles for the cholesterol scavenger receptor, thereby reducing overall cholesterol efflux and increasing cardiovascular risk (Parra et al., 2014). In a study of 284 patients, classified as low cardiovascular risk, smaller HDL particle size was significantly associated with greater cIMT and higher CRP values (Parra et al., 2014). Similarly, smaller HDL particle size was observed in patients with acute coronary syndromes as compared to non-ischaemic controls in a recent case-control study (de Miranda Teixeira et al., 2019). In addition, smaller LDL particle size has been demonstrated to increase cardiovascular risk through a higher propensity of smaller particles to be oxidised to OxLDL, with consequent effects on the development of atherosclerosis (Shiffman et al., 2017). In the large Malmo Prevention Project Study of 5764 participants (Shiffman et al., 2017), very small LDL particles were associated with a 23% increase in risk of cardiovascular events following adjustment for overall lipid profile (HR 1.23; 95% CI 1.06 – 1.43, P=0.007). Furthermore, LDL particle size was an independent risk factor for further cardiovascular events irrespective of overall

calculated cardiovascular risk, suggesting that measurement of LDL particle size may increase the sensitivity of existing risk scores. Additional observational studies have confirmed this finding, with the Hortega-Liposcale Follow-up study (Pichler et al., 2018) demonstrating a higher risk of incident cardiovascular events in patients with smaller LDL particles as well as an increased risk in patients who switch from larger to smaller LDL particles over time. Moreover, smaller LDL particles may adversely affect otherwise protective lipid components, such as OxHDL and large HDL particles, thereby increasing cardiovascular risk further (Sorokin et al., 2018). Whilst our study failed to detect a clear association between OxLDL-β2GPI levels, case-control status or other components of the lipid profile, we did not measure lipid particle size. Given the published data on the importance of lipid particle size and its effects on cardiovascular risk independent of overall LDL or HDL levels, further studies should assess particle size as a critical measure of the effect of overall lipid metabolism on atherosclerosis.

Oxidative stress, and the production of ROS, is a critical component in the development of atherosclerosis and production of OxLDL (Kattoor et al., 2017), whilst OxLDL production itself leads to further oxidative stress, expression of cell adhesion molecules and induction of pro-inflammatory cytokines (Lara-Guzman et al., 2018). Elevated markers of oxidative stress, such as LOX-1, total anti-oxidant capacity and reactive oxygen metabolites, are associated with acute cardiovascular events and are significantly lower in patients with stable disease or in healthy individuals (Lubrano et al., 2019). Furthermore, in young adults and patients with stable cardiovascular disease, elevated markers of oxidative stress are associated with the risk of new or recurrent cardiovascular events and correlate well with other, conventional, risk factors such as hypertension, smoking and hyperlipidaemia (RodrIguez et al., 2019). Reduction in oxidative stress by dietary modification, smoking cessation and physical exercise significantly reduces the risk of cardiovascular events in otherwise healthy individuals and cardiovascular disease patients (Marchio et al., 2019). Whilst OxLDL and oxidative stress are closely associated, it is important to note that oxidative stress has a pluripotent effect on the vasculature beyond its effects on lipid peroxidation. Consequently, future studies investigating the effect of OxLDL on cardiovascular events and platelet reactivity should assess markers of oxidative stress to better determine the overall effect of OxLDL on the vasculature and atherosclerotic disease.

The current analysis has a number of additional limitations. Firstly, whilst this analysis was conducted as a case-control study, it was not possible to match cases and controls for all cardiac risk factors and concomitant medications. However, no significant differences in

baseline demographics, concomitant medications or cardiac risk factors were detected when the case and control groups have been compared.

Secondly, the number of patients included in the current analysis is relatively low. Despite the number of available cases for matching, it was not possible to successfully match a significant proportion of cases to control subjects despite adopting a pragmatic approach to the matching criteria. In addition, a small number of cases and controls were not included in the final analysis due to unavailable OxLDL-β2GPI data. Whilst this analysis was not formally powered to detect an association between OxLDL-β2GPI level and case-control status, the power to detect an association has been reduced by the inability to match some cases to controls as well as unavailable ELISA data. A post-hoc power calculation demonstrates that the current analysis had only 47.5% power to detect a significant association between OxLDL-β2GPI and case-control status, and is lower for other reported outcomes such as LDL, total cholesterol and HDL.

Thirdly, platelet function data were not available for all patients included in this analysis and consequently the power to detect an association between platelet reactivity and OxLDL-β2GPI levels, lipid profiles or clinical outcome has been reduced. In addition, the rate of PFA-100 defined aspirin non-response was higher than most published data. Whilst all patients included in this analysis were reported to be treated with aspirin with good adherence, serum or urinary thromboxane levels were not measured to confirm this. However, Multiplate defined aspirin non-response was substantially lower and in keeping with previously published data.

In conclusion, the current analysis demonstrated that higher OxLDL-β2GPI levels are associated with a lower occurrence of cardiovascular events. In addition, we detected an association between a higher HDL:cholesterol ratio and aspirin non-response determined using the Multiplate platform but not the PFA-100 system. This analysis failed to detect any association between OxLDL-β2GPI level and aspirin response. However, several studies have identified potential mechanisms by which platelet reactivity may be affected by OxLDL, and further studies in patients using well validated and relevant methods for platelet function testing should be conducted. Furthermore, such studies should include assessment of inflammatory phenotype to investigate more closely the overall effect of inflammation on lipid peroxidation and platelet function. Finally, given that higher levels of OxLDL have been repeatedly associated with adverse cardiovascular outcomes, further studies should be performed to assess its potential use as a prognostic biomarker. In addition, these studies

should give consideration to how raised OxLDL levels should be treated, either via conventional lipid lowering therapies or mechanistic treatments.

# Chapter 6 – The relationship between *Helicobacter Pylori* serology, *CYP2C19* genotype and clinical outcomes in patients with an acute coronary syndrome treated with clopidogrel and proton-pump inhibitors

## 6.1: Introduction

The interaction between proton-pump inhibitors and clopidogrel has been cited as one of the most important causes of clopidogrel non-response. Clopidogrel is a pro-drug that requires a two-step process for metabolism into its active metabolite, with CYP2C19 being the primary enzyme responsible for its biotransformation (Gurbel et al., 2009). Similarly, most PPIs are metabolised by CYP2C19 with a consequent risk of interaction due to competition for CYP2C19 or CYP2C19 inhibition by the PPI. In addition, PPIs may also be responsible for other interactions within clopidogrel's pharmacokinetic pathway, such as a reduction in absorption, mediated by an increase in gastric pH (Scott et al., 2014). Lower rates of formation of clopidogrel's active metabolite and subsequent lower levels of clopidogrel induced platelet inhibition increase the risk of clopidogrel non-response and adverse cardiovascular events.

Several studies have investigated the potential interaction between PPIs and clopidogrel. In a study of 52 patients who had undergone PCI with drug eluting stent (DES) implantation, Arbel and colleagues (Arbel et al., 2013) demonstrated a clear association between omeprazole usage and clopidogrel related HTPR as measured by the VerifyNow system. Similarly, in a large, 59,000 patient, observational study, PPI usage in clopidogrel treated patients was associated with a 27% increase in the risk of thrombotic events (HR 1.27; 95% CI 1.12 to 1.45) (Kim et al., 2019), with CYP2C19-inhibiting PPIs increasing the risk further. Furthermore, several meta-analyses have demonstrated a clear association between PPI usage and elevated cardiovascular risk in patients being treated with clopidogrel. In a recent meta-analysis of 11 studies published between 2012 and 2016 (Bundhun et al., 2017), a 37% increase in the risk of MACE was observed in clopidogrel treated patients who were co-administered PPI. These data are in keeping with earlier meta-analyses which have also demonstrated similar increases in the level of risk from clopidogrel and PPI co-administration (Siller-Matula et al., 2010). Given these data, regulatory agencies worldwide have recommended against co-prescription of PPIs and clopidogrel which has resulted in a

significant decline in the use of PPIs in clopidogrel treated patients, with co-prescription dropping by 53% between 2006 and 2012 alone (Guerin et al., 2016).

However, the observed association between clopidogrel, PPIs and adverse cardiovascular outcomes has not been universally observed. No significant association between PPI use and adverse cardiovascular outcomes in clopidogrel treated patients was observed in post hoc analyses of three large cardiovascular outcome studies (TRANSLATE-ACS, TRILOGY-ACS and CREDO) (Dunn et al., 2013, Jackson et al., 2016, Nicolau et al., 2015), whilst pharmacodynamic studies have also failed to detect an association between PPI usage and clopidogrel induced platelet inhibition (Przespolewski et al., 2018). In addition, a recent meta-analysis from Demcsak and colleagues (Demcsak et al., 2018), which included 27 studies and over 150,000 recruited patients, did not demonstrate a significant association between PPI use and the risk of MACE in clopidogrel treated patients following a robust sub-group analysis (RR 0.99; 95% CI 0.76 to 1.28, P=0.93).

The underlying cause for these discordant data is unclear. In a subgroup analysis of the TRIUMPH study, Depta et al (Depta et al., 2015) demonstrated that ethnicity and *CYP2C19* genotype may have a significant influence on the risk of adverse cardiovascular outcome in patients co-prescribed clopidogrel and PPI. In this study, a significant association between PPI use in clopidogrel treated patients and outcome was detected for Caucasian patients but not for African-American patients. In addition, whilst an overall association between outcome and PPI use was observed in Caucasian patients (HR 1.62; 95% CI 1.19 to 2.19, P=0.002), this was only observed in patients carrying the *CYP2C19*17* allele (HR 2.05; 95% CI 1.26 to 3.33, P=0.003), whilst *CYP2C19* wild-type homozygotes did not demonstrate a significant association between PPI use and outcome (HR 1.51; 95% CI 0.90 to 2.54, P=0.12). Furthermore, a number of studies have highlighted that PPIs are associated with adverse effects which may increase the risk of adverse cardiovascular events independent of their putative interactions with clopidogrel. These include development of *Clostridium difficile* associated diarrhoea, osteoporosis and renal dysfunction. In addition, Pello-Lazaro et al (Pello Lazaro et al., 2017) demonstrated that PPI use in ACS patients increased the risk of heart failure and death, but not recurrent ischaemia, independently of clopidogrel usage in a cohort of ACS patients. However, these data have not been replicated in other studies (Fortuna et al., 2016) or in meta-analyses (Batchelor et al., 2018).

An alternative explanation for the clopidogrel-PPI interaction is to consider the indication for PPI treatment. In the context of cardiovascular disease, PPIs are often prescribed to prevent

gastrointestinal bleeding from aspirin administration (Pelliccia et al., 2015). However, they are also commonly prescribed for a variety of gastro-intestinal disorders including gastro-oesophageal reflux disease, peptic ulcer disease and non-ulcer related dyspepsia (Batchelor et al., 2018), some of which may be related to or caused by infection with *Helicobacter Pylori*.

*H. Pylori* is a spiral shaped, Gram negative, bacterium that colonises the gastric mucosa and is associated with the development of gastritis, peptic ulcer disease and gastric cancer (Jamkhande et al., 2016). Infection with *H. Pylori* is subclinical with no overt signs and symptoms and as such it is regarded as a chronic, asymptomatic, infection, diagnosed only at the point of development of gastrointestinal symptoms. In addition, *H. Pylori* secretes a number of virulence factors, such as cytotoxin gene A (CagA) which may generate a local and systemic inflammatory response (Kucukazman et al., 2015). This inflammatory response may also increase the level of vascular inflammation with consequent endothelial cell dysfunction, immune cell activation and increased platelet reactivity, culminating in a greater atherosclerotic burden and higher risk of adverse cardiovascular events.

Several studies have demonstrated an association between *H. Pylori* infection and cardiovascular disease. In a study of 204 patients with ACS, Tabata et al (Tabata et al., 2016c) demonstrated a significant association between positive *H. Pylori* serology and occurrence of ST-elevation MI. Similarly, in a further study by the same group, *H. Pylori* seropositivity was associated with worse clinical outcomes in ACS patients followed up for three years (Tabata et al., 2016b). These data are in keeping with a large meta-analysis by Liu and colleagues (Liu et al., 2014) of 26 studies and over 20,000 patients which demonstrated a twofold increase in risk of myocardial infarction in *H. Pylori* seropositive patients (OR 2.10; 95% CI 1.75 to 2.53, P=0.006). Importantly, several studies have also demonstrated that *H. Pylori* eradication may reduce markers of vascular dysfunction and improve clinical outcomes. Blum et al (Blum et al., 2011) demonstrated a significant improvement in endothelial dysfunction following eradication therapy with histopathologically confirmed *H. Pylori* infection, with other studies demonstrating similar reductions in inflammatory markers, such as sCD40L, post eradication therapy (Kebapcilar et al., 2009). Similarly, eradication of *H. Pylori* has been demonstrated to significantly reduce the risk of further adverse cardiovascular events in acute coronary syndromes (Elizalde et al., 2004).

Taken together, these data suggest that *H. Pylori* may be an important risk factor for cardiovascular disease. Given that a common reason for PPI prescription is non-specific gastrointestinal symptoms, it is possible that a number of PPI treated ACS patients have an

undiagnosed *H. Pylori* infection that may contribute to an increased risk of adverse cardiovascular events caused by *H. Pylori* induced vascular inflammation and endothelial dysfunction. As previously discussed, inflammation is an important contributor to anti-platelet treatment failure and it is therefore possible that part of the observed clopidogrel-PPI interaction is due to the pro-inflammatory effects of *H. Pylori* as opposed to a pharmacokinetic interaction at CYP2C19. In addition, *H. Pylori* infection rates are likely to vary widely between different study populations which may contribute to the lack of consistent association between adverse clinical outcomes and clopidogrel-PPI co-prescription.

In order to investigate this hypothesis, a nested case-control study investigating the association between *H. Pylori* infection and clinical outcome was performed in a cohort of patients with non-ST elevation acute coronary syndromes (NSTEACS) who were treated with both clopidogrel and a PPI.

## 6.2: Methods

### 6.2.1: Patient Cohort

Patients were recruited from the prospective 'Pharmacogenetics of Acute Coronary Syndrome' (PhACS) study which has been described previously. Briefly, subjects were included in this study if they had a primary diagnosis of an acute coronary syndrome. Specific exclusions included ST elevation MI and other diagnoses likely to be responsible for an elevated troponin.

Subjects were followed up for a minimum of 12 months from recruitment, with two physical visits at month 1 and month 12 for blood sampling and interview. Subjects were followed up annually thereafter with a telephone interview and review of their case notes. Blood sampling at physical visits included samples for genotyping (baseline visit only), platelet function, RNA and serum.

The primary outcome measure was a composite of cardiovascular mortality, non-fatal MI and non-fatal stroke, based on outcome definitions from the PLATO, TRITON-TIMI 38 and HORIZONS-AMI studies (Mehran et al., 2008, Wallentin et al., 2009, Wiviott et al., 2007). Secondary outcomes included all-cause mortality, bleeding and development of left ventricular failure.

The study received ethical approval from the Liverpool Adult Research Ethics Committee and was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki.

### 6.2.2: Patient Selection

Patients were selected for this study on a case-control basis. Cases were defined as patients who had suffered an ischaemic event consistent with the primary outcome measure definition. Cases were matched 1:1 with other patients recruited to the PhACS study who did not suffer an ischaemic event.

Subjects were matched for the following criteria:

- Gender
- Age (+/- 5 years)
- BMI (obese / not obese)
- On clopidogrel at discharge from the index admission
- On a PPI at discharge from the index admission
- Diabetes status
- Follow up period for the control subject exceeds the case subject's time to first cardiovascular event

Wherever possible, other cardiovascular risk factors were matched between the case and control subjects.

### 6.2.3: *H. Pylori* Serology

*H. Pylori* serology was measured by the Microbiology Department at the Royal Liverpool University Hospital. Anti-*H.Pylori* antibodies were measured using the INOVA Diagnostics (San Diego, CA, USA) QUANTA Lite H. Pylori IgG ELISA in accordance with the manufacturer's instructions and standard procedures. Results are given as Positive (>25 U/L), Equivocal (20-25 U/L) and Negative (<20 U/L).

### 6.2.4: *CYP2C19* Genotype

Raw genotype data were provided by Dr Vanessa Fontana, University of Liverpool, UK. Briefly, DNA samples from the PhACS study were genotyped using the Illumina HumanOmniExpressExome-8 v1.0 BeadChip array at Edinburgh Genomics. Samples and variants were excluded if the genotype call-rate was <95%, minor allele frequency <0.05 or the variant deviated from Hardy-Weinberg equilibrium. Imputation up to the 1000 Genomes Phase I reference panel was undertaken using IMPUTE2.

Data were reported separately for each genotype and were analysed using either an additive or dominant inheritance model. In addition, the data for the *CYP2C19*2* and **17* alleles were combined into a metaboliser status as defined by the genetic analyses of the PLATO, CURE and ACTIVE-A trials (Pare et al., 2010, Wallentin et al., 2010). Any carrier of a *17 allele was characterised as an ultra-rapid metaboliser (UM, unless they also carried a *2 allele, in which case they were defined as an indeterminate metaboliser(IndM)), *1 allele homozygotes were defined as extensive metabolisers (EM), *2 allele heterozygotes were characterised as intermediate metabolisers (IM, unless they also carried a *17 allele, in which case they were defined as indeterminate metabolisers (IndM)) and *2 allele homozygotes were defined as poor metabolisers (PM). Hardy-Weinberg equilibrium was assessed for both the *CYP2C19*17* and *CYP2C19*2* alleles.

**6.2.5: Statistical Analysis**

Data were presented as mean and standard deviation for continuous data and frequencies or percentages for categorical variables. For continuous variables, data were compared using either a t-test or ANOVA. Categorical data were compared using a Chi-squared test. Simple and logistic regression analyses were conducted to investigate the relationship between *H. Pylori* serology, *CYP2C19* genotype, clinical variables and case-control status. A result was considered statistically significant if the P value was less than 0.05. Data were analysed using SPSS version 24 (SPSS, Chicago, USA).

## 6.3: Results

### 6.3.1: Patient Characteristics

Out of 1470 patients recruited to the PhACS study, a total of 472 (32.1%) were discharged from the index admission receiving both clopidogrel and a PPI. 73 patients (15.5%) had a further ischaemic event, of which 69 were successfully matched with a control subject. Genetic or *H. Pylori* serology data were unavailable for two subjects, leaving a total of 67 cases and 67 controls which were included in the final analysis. A summary of patient demographics, clinical risk factors and medications is provided in **Table 6.1**. Control patients were noted to have a significantly lower incidence of prior coronary artery bypass grafting; otherwise there were no significant differences in cardiac risk factors, symptom scores or concomitant medications between the case and control groups. Importantly, both groups were well matched for PPIs which are CYP2C19 inhibitors (omeprazole and esomeprazole). In the case group, the first ischaemic event was myocardial infarction in 41 patients (61.2%), cardiovascular death in 20 patients (29.8%) and CVA in 6 patients (9.0%).

### 6.3.2: *H. Pylori* Serology

*H. Pylori* data were available for all subjects in the final analysis. In the 134 patients included in the current study, a total of 42 patients (31.3%) were positive for *H. Pylori* IgG and a further five (3.7%) had equivocal results (**Table 6.2**).

### 6.3.3: *CYP2C19* genotype

A total of 128 subjects had available *CYP2C19* genotype data. For the *CYP2C19*17* genotype, 46 patients were heterozygotes for the variant allele (35.9%) while seven patients were variant homozygotes (5.5%). Allelic frequencies were 0.77 for the *1 allele and 0.23 for the *17 allele. For the *CYP2C19*2* allele, 29 patients carried one copy of the variant allele (22.7%) while 2 patients (1.5%) carried two copies, i.e. were homozygotes. The allelic frequencies were 0.87 for the *1 allele and 0.13 for the *2 allele. No polymorphism deviated significantly from Hardy-Weinberg equilibrium. As discussed previously, the *CYP2C19*17* and *CYP2C19*2* genotypes were combined into a metaboliser status for each patient. A total of 48 patients (37.5%) were categorised as UMs, 49 as EMs (38.3%), 24 as IMs (18.8%), 2 as PMs (1.5%) and 5 (3.9%) were categorised as indeterminate metabolisers (IndMs) (**Tables 6.3, 6.4 and 6.5**).

| | | Cases | Controls | P-value |
|---|---|---|---|---|
| | **N** | 67 | 67 | 1.00 |
| **Males** | **N** | 46 | 46 | 1.00 |
| | **%** | 68.66 | 68.66 | |
| **Age** | **Mean** | 71.78 | 70.80 | 0.29 |
| | **SD** | 12.01 | 10.97 | |
| | **Median** | 73.64 | 73.59 | |
| | **Q1** | 64.48 | 64.32 | |
| | **Q3** | 81.30 | 77.82 | |
| | **IQR** | 16.82 | 13.49 | |
| **BMI** | **Mean** | 30.11 | 29.32 | 0.10 |
| | **SD** | 6.90 | 6.04 | |
| | **Median** | 28.41 | 27.74 | |
| | **Q1** | 25.44 | 25.60 | |
| | **Q3** | 33.18 | 31.66 | |
| | **IQR** | 7.74 | 6.06 | |
| **Hypertension** | **N** | 46 | 46 | 1.00 |
| | **%** | 68.66 | 68.66 | |
| **Hyperlipidaemia** | **N** | 43 | 41 | 0.72 |
| | **%** | 64.18 | 61.19 | |
| **PAD** | **N** | 4 | 7 | 0.35 |
| | **%** | 5.97 | 10.45 | |
| **CKD** | **N** | 12 | 6 | 0.13 |
| | **%** | 17.91 | 8.96 | |
| **DM** | **N** | 22 | 22 | 1.00 |
| | **%** | 32.84 | 32.84 | |
| **Prior MI** | **N** | 40 | 35 | 0.38 |
| | **%** | 59.70 | 52.24 | |
| **Prior PCI** | **N** | 18 | 14 | 0.42 |
| | **%** | 26.87 | 20.90 | |
| **Prior CABG** | **N** | 20 | 7 | **0.01** |
| | **%** | 29.85 | 10.45 | |
| **Current Smoker** | **N** | 12 | 16 | 0.40 |

| | | | | |
|---|---|---|---|---|
| | % | 17.91 | 23.88 | |
| **Previous Smoker** | **N** | 38 | 28 | 0.08 |
| | **%** | 56.72 | 41.79 | |
| **Non Smoker** | **N** | 17 | 22 | 0.34 |
| | **%** | 25.37 | 32.84 | |
| **CCS** | **Mean** | 1.38 | 1.30 | 0.50 |
| | **SD** | 1.09 | 1.10 | |
| | **Median** | 1 | 1 | |
| **Aspirin** | **N** | 58 | 63 | 0.14 |
| | **%** | 86.57 | 94.03 | |
| **Clopidogrel** | **N** | 67 | 67 | 1.00 |
| | **%** | 100.00 | 100.00 | |
| **Warfarin** | **N** | 2 | 0 | 0.15 |
| | **%** | 2.99 | 0.00 | |
| **LMWH** | **N** | 0 | 3 | 0.08 |
| | **%** | 0.00 | 4.48 | |
| **Beta-Blocker** | **N** | 53 | 54 | 0.83 |
| | **%** | 79.10 | 80.60 | |
| **CCB** | **N** | 19 | 15 | 0.43 |
| | **%** | 28.36 | 22.39 | |
| **Nitrate** | **N** | 49 | 46 | 0.57 |
| | **%** | 73.13 | 68.66 | |
| **Statin** | **N** | 62 | 64 | 0.47 |
| | **%** | 92.54 | 95.52 | |
| **ACE-I** | **N** | 54 | 50 | 0.41 |
| | **%** | 80.60 | 74.63 | |
| **ARB** | **N** | 6 | 11 | 0.19 |
| | **%** | 8.96 | 16.42 | |
| **PPI** | **N** | 67 | 67 | 1.00 |
| | **%** | 100.00 | 100.00 | |
| **Omeprazole** | **N** | 33 | 29 | 0.49 |
| | **%** | 49.25 | 43.28 | |
| **Esomeprazole** | **N** | 1 | 2 | 0.56 |

| | | | | |
|---|---|---|---|---|
| | **%** | 1.49 | 2.99 | |
| **Omep or Esomep** | **N** | 34 | 31 | 0.60 |
| | **%** | 50.75 | 46.27 | |
| **Death** | **N** | 20 | NA | NA |
| | **%** | 29.9 | | |
| **MI** | **N** | 41 | | |
| | **%** | 61.2 | | |
| **CVA** | **N** | 6 | | |
| | **%** | 9.0 | | |

**Table 6.1 – Characteristics of the included patients**

| | | **All** | **Case** | **Control** |
|---|---|---|---|---|
| | **Total n** | 134 | 67 | 67 |
| ***H. Pylori* Positive** | **n** | 42 | 20 | 22 |
| | **%** | 31.3 | 29.9 | 32.8 |
| ***H. Pylori* Negative** | **n** | 87 | 45 | 42 |
| | **%** | 64.9 | 67.2 | 62.7 |
| ***H. Pylori* Equivocal** | **n** | 5 | 2 | 3 |
| | **%** | 3.7 | 3.0 | 4.5 |

**Table 6.2 – *H. Pylori* serology results**

| | | | Genotype frequency | | | | | | | | | | | Allelic frequency | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *1*1 | | *1*17 | | *17*17 | | P-Value | P-value (Regr) | Any *17 | | P-value (Dom) | *1 | | *17 | |
| SNP | Group | Total | n | % | n | % | n | % | | | n | % | | n | Freq | n | Freq |
| CYP2C19*17 | All patients | 128 | 75 | 58.6 | 46 | 35.9 | 7 | 5.5 | NA | NA | 53 | 41.4 | NA | 196 | 0.77 | 60 | 0.23 |
| | Case Patients | 64 | 34 | 53.1 | 26 | 40.6 | 4 | 6.3 | 0.454 | 0.322 | 30 | 46.9 | 0.209 | 94 | 0.73 | 34 | 0.27 |
| | Control Patients | 64 | 41 | 64.1 | 20 | 31.3 | 3 | 4.7 | | | 23 | 35.9 | | 102 | 0.80 | 26 | 0.20 |
| | *H. Pylori* Positive | 39 | 24 | 61.5 | 13 | 33.3 | 2 | 5.1 | 0.970 | NA | 15 | 38.5 | 0.832 | 61 | 0.78 | 17 | 0.22 |
| | *H. Pylori* Negative | 84 | 50 | 59.5 | 29 | 34.5 | 5 | 6.0 | | | 34 | 40.5 | | 129 | 0.77 | 39 | 0.23 |

**Table 6.3 – Relationship between the CYP2C19*17 polymorphism, case-control status and *H.Pylori* serology results** (Regr – regression model, Dom – dominant inheritance model)

| | | | Genotype frequency | | | | | | | | | | | Allelic frequency | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *1*1 | | *1*2 | | *2*2 | | P-value | P-value (Regr) | Any *2 | | P-value (Dom) | *1 | | *2 | |
| SNP | Group | Total | n | % | n | % | n | % | | | n | % | | n | Freq | n | Freq |
| CYP2C19*2 | **All patients** | 128 | 97 | 75.8 | 29 | 22.7 | 2 | 1.6 | NA | NA | 31 | 24.2 | NA | 223 | 0.87 | 33 | 0.13 |
| | **Case Patients** | 64 | 54 | 84.4 | 10 | 15.6 | 0 | 0.0 | **0.049** | **0.014** | 10 | 15.6 | **0.023** | 118 | 0.92 | 10 | 0.08 |
| | **Control Patients** | 64 | 43 | 67.2 | 19 | 29.7 | 2 | 3.1 | | | 21 | 32.8 | | 105 | 0.82 | 23 | 0.18 |
| | ***H. Pylori* Positive** | 39 | 30 | 76.9 | 8 | 20.5 | 1 | 2.6 | 0.752 | 0.313 | 9 | 23.1 | 0.711 | 68 | 0.87 | 10 | 0.13 |
| | ***H. Pylori* Negative** | 84 | 62 | 73.8 | 21 | 25.0 | 1 | 1.2 | | | 22 | 26.2 | | 145 | 0.86 | 23 | 0.14 |

**Table 6.4 – Relationship between the CYP2C19*2 polymorphism, case-control status and *H.Pylori* serology results** (Regr – regression model, Dom – dominant inheritance model)

| | | | Phenotype frequency | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ultra-rapid | | Extensive | | Intermediate | | Poor | | Indeterm. | | P-value | P-value (Regr) |
| SNP | Group | Total | n | % | n | % | n | % | n | % | n | % | | |
| Metaboliser | All patients | 128 | 48 | 37.5 | 49 | 38.3 | 24 | 18.8 | 2 | 1.6 | 5 | 3.9 | NA | NA |
| | Case Patients | 64 | 26 | 40.6 | 28 | 43.8 | 6 | 9.4 | 0 | 0.0 | 4 | 6.3 | **0.025** | **0.008** |
| | Control Patients | 64 | 22 | 34.4 | 21 | 32.8 | 18 | 28.1 | 2 | 3.1 | 1 | 1.6 | | |
| | *H. Pylori* Positive | 39 | 14 | 35.9 | 16 | 41.0 | 7 | 17.9 | 1 | 2.6 | 1 | 2.6 | 0.944 | 0.570 |
| | *H. Pylori* Negative | 84 | 30 | 35.7 | 32 | 38.1 | 17 | 20.2 | 1 | 1.2 | 4 | 4.8 | | |

**Table 6.5 – Relationship between the CYP2C19 metaboliser status, case-control status and *H.Pylori* serology results** (Regr – regression model)

### 6.3.4: Relationship between *H.Pylori* serology and case-control status

There was no significant association detected between case-control status and positive *H. Pylori* serology. A total of 20 patients (29.9%) in the case group tested positive for *H. Pylori* antibodies compared to 22 (32.8%) in the control group (P=0.819). After including a number of clinical co-variates (Age, gender, obesity, diabetes status, hypertension and chronic kidney disease) in a regression analysis, the overall P-value for the association between positive *H. Pylori* serology and case-control status was 0.88. In addition, no other included clinical co-variates were significantly associated with case-control status (**Table 6.2**).

### 6.3.5: Relationship between *CYP2C19* genotype and case-control status

For *CYP2C19*17*, no significant association was detected between genotype frequency and case-control status. For case patients, the genotype frequencies for the *1*1, *1*17 and *17*17 genotypes were 53.1%, 40.6% and 6.3% respectively, in comparison to 64.1%, 31.3% and 4.7% in the control arm (P=0.454 for an additive model, P=0.209 for a dominant model). After including clinical co-variates (age, gender, obesity, diabetes, hyperlipidaemia and CKD), the overall association between *CYP2C19*17* genotype and case-control status remained non-significant (P=0.322). Furthermore, no associations between clinical co-variates and *CYP2C19*17* genotype were detected (**Tables 6.3 and 6.6**).

However, for *CYP2C19*2*, a significant association between genotype frequency and case-control status was demonstrated. For case patients, the genotype frequencies for the *1*1, *1*2 and *2*2 genotypes were 84.4%, 15.6% and 0% respectively, compared to 67.2%, 29.7% and 3.1% in the control subjects (P=0.049 for an additive model, P=0.023 for a dominant model). Following inclusion of clinical factors in a regression model (age, gender, obesity, diabetes, hyperlipidaemia and CKD), the overall association between *CYP2C19*2* genotype and case-control status remained significant (P=0.014). In a sub-group analysis, a significant association between *CYP2C19*2* genotype and case-control status was only detected in patients who were *H. Pylori* negative, with *H. Pylori* positive patients demonstrating no significant association between genotype and case-control status (P=0.614). In *H. Pylori* negative case patients, 86% had a *1*1 genotype, 14% had a *1*2 genotype and 0% had the *2*2 genotype. In comparison, the *H. Pylori* negative control patients reported genotype frequencies of 61.0% for *1*1, 36.6% for *1*2 and 2.4% for *2*2 (P=0.028 for an additive model, P=0.009 for a dominant model) (**Table 6.4 and 6.7**).

In addition, a significant association was demonstrated between combined metaboliser status and case-control status, with control patients having a significantly higher number of intermediate and poor metabolisers compared to the case patients. For the case patients, the metaboliser frequencies for UMs, EMs, IMs, PMs and IndMs were 40.6%, 43.8%, 9.4%, 0% and 6.3% respectively, in comparison to 34.4%, 32.8%, 28.1%, 3.1% and 1.6% in the control patients (P=0.025). Following inclusion of clinical factors in a regression analysis, the association remained significant for intermediate metabolisers only (P=0.016). As expected, a significant association between metaboliser status and case-control status was only detected in cases and control patients that tested negative for *H. Pylori* (P=0.617 for *H. Pylori* positive patients, P=0.022 for *H. Pylori* negative patients) (**Table 6.5 and 6.8**).

### 6.3.6: Relationship between *H. Pylori* serology and CYP2C19 genotype

For *CYP2C19*17*, no significant association was detected between genotype and *H. Pylori* serology. In the *H. Pylori* positive patients, the genotype frequencies for *1*1, *1*17 and *17*17 were 61.5%, 33.3% and 5.1% respectively, which compared to 59.5%, 34.5% and 6.0% in *H. Pylori* negative patients (P=0.970 for an additive model, P=0.832 for a dominant model) (**Table 6.3 and 6.6**).

Similarly, for *CYP2C19*2* no significant association was detected between *H. Pylori* status and genotype. Genotype frequencies were 76.9%, 20.5% and 2.6% for the *1*1, *1*2 and *2*2 genotypes in *H. Pylori* positive patients, which compared to 73.8%, 25.0% and 1.2% respectively in *H. Pylori* negative patients (P=0.752 for an additive model, P= 0.711 for a dominant model) (**Table 6.4 and 6.7**).

Given the detected association between the *CYP2C19*2* polymorphism and case-control status in *H. Pylori* negative patients, the association between genotype and *H. Pylori* status was assessed in the individual case and control groups. In *H. Pylori* positive case patients, the genotype frequencies for *1*1, *1*2 and *2*2 were 78.9%, 21.1% and 0% respectively, in comparison to 86.0%, 14.0% and 0% in *H. Pylori* negative case patients (P=0.484 for both additive and dominant models). In the control patients, the genotype frequencies were 75.0%, 20.0% and 5.0% for the *1*1, *1*2 and *2*2 genotypes respectively in *H. Pylori* positive controls, which compared to 61.0%, 36.6% and 2.4% respectively in the *H. Pylori* negative controls (P=0.394 for an additive model, P=0.279 for a dominant model). With inclusion of clinical variables (age, gender, obesity, diabetes, hypertension, hyperlipidaemia and CKD), there remained no significant association between *CYP2C19*2* genotype and *H. Pylori* status overall (P=0.313) (**Table 6.4 and 6.7**).

Finally, no association was detected between a combined metaboliser status and *H. Pylori* serology. The metaboliser frequencies for UMs, EMs, IMs, PMs and IndMs in *H. Pylori* positive patients was 35.9%, 41.0%, 17.9%, 2.6% and 2.6% respectively, which compared to 35.7%, 38.1%, 20.2%, 1.2% and 4.8% in the *H. Pylori* negative patients (P=0.944). When analysed within the individual case and control groups, there remained no association between metaboliser status and *H. Pylori* status with inclusion of clinical variables having no effect (P=0.570) (**Table 6.5 and 6.8**).

| | | | Genotype frequency | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *1*1 | | *1*17 | | *17*17 | | P-value (A) | P-value (B) | Any *17 | | P-value (A) | P-value (B) |
| SNP | Group | Total | n | % | n | % | n | % | | | n | % | | |
| CYP2C19*17 | Case, HP Positive | 19 | 12 | 63.2 | 7 | 36.8 | 0 | 0.0 | 0.358 (HP Pos) | 0.364 (Case) | 7 | 36.8 | 0.839 (HP Pos) | 0.425 (Case) |
| | Cont, HP Positive | 20 | 12 | 60.0 | 6 | 30.0 | 2 | 10.0 | | | 8 | 40.0 | | |
| | Case, HP Negative | 43 | 22 | 51.2 | 17 | 39.5 | 4 | 9.3 | 0.218 (HP Neg) | 0.426 (Cont) | 21 | 48.8 | 0.132 (HP Neg) | 0.522 (Cont) |
| | Cont, HP Negative | 41 | 28 | 68.3 | 12 | 29.3 | 1 | 2.4 | | | 13 | 31.7 | | |

**A = Comparison between Cases & Controls with same HP serology status**
**B = Comparison between HP positives and negatives with same case-control status**

**Table 6.6 – CYP2C19*17 polymorphism sub-group analysis**

| | | | Genotype frequency | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *1*1 | | *1*2 | | *2*2 | | P-value (A) | P-value (B) | Any *2 | | P-value (A) | P-value (B) |
| SNP | Group | Total | n | % | n | % | n | % | | | n | % | | |
| CYP2C19*2 | Case, HP Positive | 19 | 15 | 78.9 | 4 | 21.1 | 0 | 0.0 | 0.614 (HP Pos) | 0.484 (Case) | 4 | 21.1 | 0.770 (HP Pos) | 0.484 (Case) |
| | Cont, HP Positive | 20 | 15 | 75.0 | 4 | 20.0 | 1 | 5.0 | | | 5 | 25.0 | | |
| | Case, HP Negative | 43 | 37 | 86.0 | 6 | 14.0 | 0 | 0.0 | **0.028** (HP Neg) | 0.394 (Cont) | 6 | 14.0 | **0.009** (HP Neg) | 0.279 (Cont) |
| | Cont, HP Negative | 41 | 25 | 61.0 | 15 | 36.6 | 1 | 2.4 | | | 16 | 39.0 | | |

**A = Comparison between Cases & Controls with same HP serology status**
**B = Comparison between HP positives and negatives with same case-control status**

**Table 6.7 - CYP2C19*2 polymorphism sub-group analysis**

| | | | Phenotype frequency | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ultra-rapid | | Extensive | | Intermediate | | Poor | | Indeterm. | | P-value (A) | P-value (B) |
| SNP | Group | Total | n | % | n | % | n | % | n | % | n | % | | |
| Metaboliser | Case, HP Positive | 19 | 6 | 31.6 | 9 | 47.4 | 3 | 15.8 | 0 | 0.0 | 1 | 5.3 | 0.617 (HP Pos) | 0.302 (Case) |
| | Cont, HP Positive | 20 | 8 | 40.0 | 7 | 35.0 | 4 | 20.0 | 1 | 5.0 | 0 | 0.0 | | |
| | Case, HP Negative | 43 | 18 | 41.9 | 19 | 44.2 | 3 | 7.0 | 0 | 0.0 | 3 | 7.0 | **0.022** (HP Neg) | 0.702 (Cont) |
| | Cont, HP Negative | 41 | 12 | 29.3 | 13 | 31.7 | 14 | 34.1 | 1 | 2.4 | 1 | 2.4 | | |

**A = Comparison between Cases & Controls with same HP serology status**
**B = Comparison between HP positives and negatives with same case-control status**

**Table 6.8 - CYP2C19 metaboliser phenotype sub-group analysis**

## 6.4: Discussion

In this study, we did not detect a significant association between positive *H. Pylori* serology and the risk of further cardiovascular events in the context of acute coronary syndromes. Whilst these findings are inconsistent with most *in vitro* and clinical biomarker studies that suggest a mechanistic link between *H. Pylori* infection and increased cardiovascular risk, our findings are in keeping with larger clinical outcome studies that do not clearly demonstrate an association between *H. Pylori* infection and poor cardiovascular outcomes.

Several studies have demonstrated potential molecular pathways by which *H. Pylori* may increase the degree and severity of atherosclerosis. Li and colleagues (Li et al., 2017b) demonstrated that *H. Pylori* CagA positive strains induced greater expression of cytokines compared to CagA negative strains, via activation of the c-Met-PI3K/Akt-mTOR signalling pathway. In addition, *H. Pylori* has been demonstrated to induce NLRP3 expression (Pachathundikandi and Backert, 2018) in THP-1 monocytes via specific microRNA upregulation. Analysis of other *H. Pylori* associated miRNAs and mRNA interactions by Yang and colleagues (Yang et al., 2018b) has also identified a number of genes and proteins that are significantly altered by *H. Pylori* infection and may increase the risk of cardiovascular disease, inflammation and cancer.

*H. Pylori* infection has also been associated with the development and acceleration of atherosclerosis. In mice infected with *H. Pylori* and fed with a high-fat diet, atherosclerotic burden is significantly increased compared to non- *H. Pylori* infected animals (Ayada et al., 2009). Furthermore, eradication of *H. Pylori* or immunisation with *Hp*-HSP60 reduced the burden of atherosclerosis and reduced the elevated Th1 mediated immune response observed on *H. Pylori* infection. However, unlike other chronic bacterial infections such as *Chlamydia pneumoniae*, *H. Pylori* specific Th1-cells have not been demonstrated in atherosclerotic plaques from *H. Pylori* infected patients (Benagiano et al., 2003), although other studies have demonstrated that *H. Pylori* can be cultured from human atherosclerotic plaques (Izadi et al., 2012).

An alternative mechanism by which *H. Pylori* could increase the risk of adverse cardiovascular events is by altering platelet reactivity. In healthy volunteers, *H. Pylori* has been demonstrated to increase platelet reactivity by binding vWF with consequent interaction with the platelet GPIb receptor which, importantly, also requires the presence of anti-*H. Pylori* IgG (Byrne et al., 2003). In addition, *H. Pylori* urease has also been demonstrated to activate platelets in rabbits via a lipo-oxygenase mediated pathway (Wassermann et al.,

2010). Scopel-Guerra and colleagues (Scopel-Guerra et al., 2017) also demonstrated that *H. Pylori* urease related platelet activation induces production of pro-inflammatory mediators such as IL-1β and CD14, which may further increase the pro-inflammatory state induced by *H. Pylori* infection. However, in this current study, we could not assess platelet reactivity as an outcome as only a small proportion of the included patients were recruited from sites which could perform platelet function tests.

In human studies, *H. Pylori* infection has been associated with a number of different biomarkers associated with cardiovascular disease. In a study of 185 patients with coronary artery disease and 80 healthy controls, Badran and colleagues (Badran and Mahfouz, 2007) demonstrated a significant association between *H. Pylori* CagA IgG positive serology and raised inflammatory markers, whilst CagA positive patients had a fourfold increase in the risk of atrial fibrillation (OR 3.59; 95% CI 1.87-6.94, P<0.001). Similarly, in a cohort of 159 patients with coronary artery disease, Huang et al (Huang et al., 2011) observed a clear association between *H. Pylori* infection and higher levels of total cholesterol, LDL-cholesterol, OxLDL and hsCRP, with CagA expressing *H. Pylori* strains demonstrating the largest increases in lipid and inflammatory markers compared to the CagA negative strains. In addition, *H Pylori* infection has been demonstrated to induce endothelial dysfunction which was also associated with higher levels of inflammatory and cell-adhesion markers (Oshima et al., 2005). Taken together, these data suggest that *H. Pylori* infection is pro-inflammatory, with direct effects on the vascular endothelium which may consequently increase the risk of vascular disease. Furthermore, several studies have reported associations between *H. Pylori* infection and the presence of conventional vascular risk factors, such as hyperlipidaemia (Kim et al., 2016) and hypertension (Wan et al., 2018). However, several other studies have failed to replicate the association between *H. Pylori* infection and the presence of vascular risk factors (Kim et al., 2016, Lu et al., 2014), which is in keeping with the data from our study where no such association was detected.

Despite clear associations between *H. Pylori* infection and a number of different inflammatory and vascular biomarkers, the association between clinical outcome and *H. Pylori* is less clear, with some studies reporting positive associations and others not. For example, in patients with diabetes mellitus, Hamed et al demonstrated a significant association between *H. Pylori* infection and risks of micro and macrovascular diabetic complications, which was also associated with higher levels of TNF-α and IL-6 in *H. Pylori* positive patients (Hamed et al., 2008). Furthermore, in a large, retrospective, cross-sectional study of 17,322 patients with *H. Pylori* and 69,328 matched controls, Huang and colleagues

(Huang et al., 2014) demonstrated a 50% increase in risk of ischaemic stroke (HR 1.52; 95%CI 1.40-1.65).

However, most studies have failed to replicate an association between *H. Pylori* and clinical outcomes. In a prospective nested case-control study of 29,876 subjects without overt cardiovascular disease, Ikeda and colleagues (Ikeda et al., 2013) could not detect an association between positive *H. Pylori* IgG serology and adverse cardiovascular outcomes, such as stroke and MI, over an eight year follow up period. Similarly, in data from the 9895 patient NHANES III study (Chen et al., 2013), *H. Pylori* status was not associated with the risk of all-cause or stroke mortality with an inverse association reported between *H. Pylori* Cag A positive patients and stroke related mortality (HR 0.45; 95% CI 0.27-0.76). Finally, in a nested case-control study of similar design to our study, Lin et al (Lin et al., 2015) also failed to detect any association between positive *H. Pylori* serology and the risk of cardiovascular mortality in a large cohort of otherwise healthy subjects. Whilst a meta-analysis of 26 case-control studies (Liu et al., 2015) demonstrated a strong association between positive *H. Pylori* serology and risk of myocardial infarction, it should be noted that most included studies were cross-sectional and did not include prospective data. In the included prospective studies, the association between *H. Pylori* serology and outcome was weaker and often not significant. It is therefore unclear whether the overall association reported by Liu's meta-analysis represents a true effect of *H. Pylori* or whether it has been confounded by study design and retrospective data capture. Furthermore, in a meta-analysis of ten prospective cohort and case-control studies, Yu et al did not demonstrate any significant association between positive *H. Pylori* serology and risk of stroke (Yu et al., 2014).

Taken together, these data suggest that the impact of positive *H. Pylori* serology on clinical outcomes is minimal despite its well characterised effects on a cellular and biomarker level. Indeed, the lack of association with clinical outcomes is entirely consistent with the findings from our study, which failed to detect a significant association between *H. Pylori* serology and case-control status. Importantly, however, our study did not measure any inflammatory biomarkers and therefore we cannot determine whether higher levels of inflammatory markers or pro-inflammatory cytokines were associated with either case-control or *H. Pylori* status, as has been described in previous studies. In addition, a significant limitation of our study (and most of the published data) is the use of *H. Pylori* antibody status alone as a marker of active *H. Pylori* infection. Stool tests for *H. Pylori* antigen and the urea breath test better represent active infection in comparison to the serum *H. Pylori* antibody, which may be a marker of previous, rather than active, exposure (Braden, 2012). However, stool tests

and the urea breath test are more complex and costly to perform than the standard serology test and consequently most studies have investigated only *H. Pylori* antibodies. In addition, a significant proportion of studies investigating the association between *H. Pylori* infection and clinical outcome have been conducted in mostly asymptomatic patients without overt cardiovascular disease or significant cardiovascular risk factors. It is therefore unclear whether *H. Pylori* infection may be an important additive factor in the context of acute coronary syndrome given its potential pro-inflammatory and pro-atherogenic effects demonstrated *in vitro* and *in vivo*.

Our study also demonstrated a significant association between *CYP2C19*2* genotype and case-control status, with control patients having significantly higher allelic frequencies for the variant, loss-of-function, *2 allele. In addition, this effect was only observed in *H. Pylori* negative patients and not in patients with positive *H. Pylori* serology. Whilst the *CYP2C19*2* allele has been determined as a risk factor for adverse cardiovascular events in patients treated with clopidogrel (Mega et al., 2010b), our study observed a higher frequency of the *2 allele in the control group rather than the case group. This suggests that the observed association in our study is not mediated by non-response to clopidogrel but perhaps by an effect on PPI metabolism, which is also catalysed by the CYP2C19 isoenzyme.

Several studies have suggested that *CYP2C19* polymorphisms may affect the efficacy of PPIs. In a study of 120 Japanese, *H. Pylori* negative, healthy volunteers Sugimoto and colleagues (Sugimoto et al., 2014) demonstrated that intra-gastric pH values were significantly higher in subjects with *CYP2C19*2* and *CYP2C19*3* genotypes compared to wild-type subjects administered either omeprazole, lansoprazole or rabeprazole. Similarly, Deshpande and colleagues demonstrated a significant association between *CYP2C19* genotype and pharmacokinetics of esomeprazole. Poor metabolisers were noted to have significantly higher exposure and maximum concentrations of esomeprazole in comparison to ultra-rapid metabolisers and extensive metabolisers (Deshpande et al., 2016).

The relationship between *CYP2C19* polymorphisms and clinical effectiveness of PPIs has been well studied, particularly in the context of *H. Pylori* eradication. PPIs form a cornerstone of eradication therapy by potentiating the effects of the co-prescribed antibiotics in addition to their own intrinsic anti- *H. Pylori* activity (Kuo et al., 2014). In a study of 200 *H. Pylori* positive patients, Ormeci and colleagues (Ormeci et al., 2016) demonstrated a significantly higher rate of *H. Pylori* eradication failure in patients with a wild-type *CYP2C19* genotype in comparison to carriers of the variant *CYP2C19*2* and **3* alleles. Similarly, Hong et al (Hong

et al., 2016) also demonstrated a significant association between *CYP2C19* genotype and the rate of *H. Pylori* eradication failure in a cohort of 374 patients with duodenal ulcer disease, with an eradication rate of 80.6% in EMs compared to 90.0% in IMs and PMs (OR 4.65; 95% CI 0.257 – 0.843, P = 0.005). However, Chang and colleagues (Chang et al., 2018) failed to demonstrate any significant effect of *CYP2C19* genotype on the rate of *H. Pylori* eradication therapy failure in a cohort of 190 patients with chronic gastritis. Importantly, the effect of *CYP2C19* genotype may be dependent on the type of PPI prescribed, with PPIs that are dependent on *CYP2C19* for their metabolism (e.g. omeprazole) being most closely associated with *CYP2C19* genotype. In a study of 160 *H. Pylori* positive dyspeptic patients, Lin et al (Lin et al., 2017) demonstrated a significant association between the *CYP2C19* extensive metaboliser patients and *H. Pylori* eradication therapy failure in omeprazole treated patients but not in patients treated with rabeprazole, a PPI that is not significantly metabolised by CYP2C19. Of note, in our study, nearly half of the patients included were treated with either omeprazole or esomeprazole, with the remainder being treated with lansoprazole, which is also largely CYP2C19 metabolised.

Whilst *CYP2C19* genotype has been demonstrated to affect the success rate of *H. Pylori* eradication and PPI efficacy, it is not clear how this may relate to the observed higher numbers of *CYP2C19*2* allele carriers in *H. Pylori* negative controls in our study. Furthermore, we did not detect any association between *H. Pylori* serology status and *CYP2C19* genotype, irrespective of whether this was analysed as cases and controls combined or individually within the case and control groups. This suggests that any effect of *CYP2C19* polymorphisms on PPI efficacy is not related to carriage of *H. Pylori* in this study, which is broadly in keeping with the limited published data investigating the effects of PPIs on the gastric microbiota (Parsons et al., 2017). However, given that the association between case-control status and *CYP2C19*2* genotype was not observed in *H. Pylori* positive cases and controls; it is conceivable that there remains an undetected interaction between *H. Pylori* status, case-control status and *CYP2C19* genotype although the underlying cause remains unclear. It should also be noted that the *H. Pylori* seropositive rate in this case-control population is relatively low in comparison to other published data (circa 30% in this study compared to 60-70% in the majority of the published data), and it is likely that the actual number of included patients with active infection is much lower given the limitations of the *H. Pylori* antibody test. It is possible that the lower prevalence of *H. Pylori* seropositivity is related to the criteria used to identify cases and controls for this study, given that all participants were treated with a PPI. However, whilst PPIs have been recognised to

have intrinsic anti-*H. Pylori* activity, little data have been published investigating whether long term PPI treatment lowers the risk of *H. Pylori* infection. Several studies have demonstrated that any increase in gastric pH alters the gastric microbiome, with either species restriction or expansion depending on the underlying cause for the pH alteration (Parsons et al., 2017). PPIs have been associated with significant species expansion in the gastric flora (Paroni Sterbini et al., 2016), potentiating the growth of a number of bacterial species that are usually inhibited by the unfavourable gastric environment, although the effect on *H. Pylori* growth remains largely unknown. However, the alteration in the gastric microbiome may induce local and systemic inflammation, which may potentially affect the risk of cardiovascular disease. Nonetheless, the lower *H. Pylori* prevalence in our study, the effect of PPIs on the gastric microbiome and the potential effect of the *CYP2C19*2* polymorphism on PPI efficacy do not appear to explain our finding of a significantly higher number of *CYP2C19*2* genotypes in H*. Pylori* negative controls compared to *H. Pylori* negative case patients. Furthermore, our finding does not appear to be explained by the effect of the *CYP2C19*2* polymorphism on clopidogrel metabolism, given that the frequency of the variant allele is higher in patients who did not have further cardiovascular events, which is not in keeping with the known effect of the *CYP2C19*2* on clopidogrel's pharmacokinetics and pharmacodynamics.

There are several other limitations to our study. As discussed previously, we used the serum *H. Pylori* antibody to define *H. Pylori* status which, whilst in keeping with most of the published literature, does not differentiate between currently and previously infected individuals. In addition, we did not record whether subjects had previously received eradication therapy and it is therefore likely that a proportion of *H. Pylori* positive patients in this study may not have had current infection. This represents a significant weakness given that the underlying hypothesis relies on active infection increasing vascular inflammation and consequent cardiovascular risk. Furthermore, we did not assess for *H. Pylori* associated virulence factors, such as CagA, which have been better associated with inflammation and vascular dysfunction in previous studies.

The current study is also limited by the small number of included patients which is partially a consequence of a relatively low proportion of patients being co-prescribed a PPI and clopidogrel following recent regulatory advice regarding the potential clopidogrel and PPI interaction. Consequently, our power to detect associations between *H. Pylori* and case-control status is reduced.

In conclusion, this study failed to detect a significant association between *H. Pylori* infection and the risk of adverse cardiovascular events or *CYP2C19* genotype. However, we did detect a significant association between *CYP2C19*2* genotype and case-control status, with higher numbers of patients with the *CYP2C19*2* allele in the control arm compared to the patients with further cardiovascular events. In addition, this association was detected only in *H. Pylori* negative patients, with no significant differences in *CYP2C19* genotype between the *H. Pylori* positive cases and controls. The mechanism underlying this observation remains unclear and requires replication within the whole PhACS cohort to determine its significance.

Furthermore, given the published data demonstrating associations between *H. Pylori* infection, vascular inflammation, platelet reactivity and endothelial dysfunction, further studies investigating the association between *H. Pylori* and the risk of adverse cardiovascular outcomes should be performed, particularly in the context of unstable cardiovascular disease and PCI. These studies should include robust measures of *H. Pylori* virulence factors, inflammatory biomarkers and platelet reactivity.

# Chapter 7 – Final Discussion

Cardiovascular disease remains one of the greatest health challenges worldwide. Over the last 25 years, outcomes have improved significantly by focussing on better management of both acute and chronic disease manifestations. In particular, the evidence-based usage of anti-platelet agents, both aspirin and the ADP receptor blockers, have significantly improved outcomes by inhibiting platelet aggregation which, ultimately, is fundamental to the underlying pathology of acute coronary syndromes.

However, response to anti-platelet agents is variable. Sub-optimal response to anti-platelet agents is common, with several studies demonstrating a significantly worse prognosis in patients deemed non-responsive to anti-platelet drugs. A meta-analysis from Krasopoulos et al (Krasopoulos et al., 2008) of almost 3000 patients and 20 studies identified that 28% of patients were categorised as aspirin resistant, with a fourfold increase in adverse cardiovascular events and a six-fold increase in the risk of death. Similarly for clopidogrel, a meta-analysis of 25 studies and 3688 patients by Snoep et al (Snoep et al., 2007b) reported a mean clopidogrel non-response rate of 21% and an eight-fold higher risk of adverse cardiovascular outcomes in clopidogrel non-responsive patients. Furthermore, the newer anti-platelet agents, such as prasugrel and ticagrelor, are also reported to have variable responses. For prasugrel, several studies have reported high on-treatment platelet reactivity (HTPR) in prasugrel treated patients which is associated with poorer outcomes (Sato et al., 2017, Bonello et al., 2011). Similarly, HTPR in ticagrelor treated patients has also been reported, although this may be significantly less prevalent than clopidogrel or prasugrel related HTPR (Lemesle et al., 2015). These data suggest that non-response to anti-platelet drugs is relatively common, with clear effects on cardiovascular outcomes.

The rationale for this thesis was to investigate some of the most important causes for this observed non-response. In particular, this thesis has focussed on the impact of genetic polymorphisms, inflammation and cardiac risk factors on anti-platelet drug response and clinical outcomes in patients with unstable cardiovascular disease. In addition, we have also investigated two potential strategies for personalisation of anti-platelet therapy: genetic markers and pharmacodynamic response via platelet function tests.

## 7.1: Genetic Factors

In chapters 2 & 3, we demonstrated a clear association between carriage of the *CYP2C19*2* polymorphism and poor clinical or pharmacodynamic outcomes in clopidogrel treated patients using large meta-analyses of published data. For the pharmacodynamic meta-analysis, we included 165 studies and performed 25 meta-analyses investigating four methods of testing platelet function and seven genetic polymorphisms. In the clinical meta-analysis, a total of 81 studies were included with four genetic polymorphisms and eight clinical outcome measures investigated. Together, these data demonstrate the critical importance of *CYP2C19* loss-of-function polymorphisms on clopidogrel response, with higher platelet reactivity and worse clinical outcomes consistently demonstrated in carriers of the *CYP2C19*2* or **3* alleles. This finding is in keeping with a meta-analysis by Mega and colleagues (Mega et al., 2010b) demonstrating a 55% increase in the risk of adverse cardiovascular events in clopidogrel treated patients carrying the variant *CYP2C19* alleles. In addition, in the clinical meta-analysis, we also demonstrated clear association between carriage of the *CYP2C19*2* or **3* allele and a range of clinical outcome measures, despite only including studies where survival analysis data were provided or extractable. This finding emphasises the results of Mega's meta-analysis (Mega et al., 2010b) but is not in keeping with the data from Bauer's and Holmes' meta-analysis (Bauer et al., 2011, Holmes et al., 2011), where no consistent association between CYP2C19 variant alleles and clinical outcomes was detected, aside from a weak association with stent thrombosis in both studies and myocardial infarction in Holmes' analysis only. Whilst both Holmes' and Bauer's meta-analyses undertook extensive meta-regression and control of confounding variables, it remains unclear whether their results truly represent the effect of the *CYP2C19* genotype on clinical outcome. For example, both meta-analyses rely on published data rather than pooled data used in Mega's meta-analysis, with consequent limitations in reporting (use of odds ratio or relative risk) which may not best represent the time to event analyses reported in many of the included studies. In addition, both meta-analyses include stable and unstable populations together for analysis which may dilute any relationship between *CYP2C19* genotype and clinical outcome.

Importantly, we also failed to detect any association between pharmacodynamic or clinical outcomes and other genetic polymorphisms that had variably been demonstrated to be associated with clopidogrel response. This includes polymorphisms involved in clopidogrel absorption (*ABCB1* C3435T) and clopidogrel metabolism (*CYP3A5*3*, *PON1* Q192R). However, a significant association was observed between *ABCB1* 3435T allele carriers and

platelet reactivity when VASP was used for pharmacodynamic assessment in our pharmacodynamic meta-analysis, but this was not detected when LTA or VerifyNow was used for the assessment of platelet function. The relevance of this finding is unclear, and no association between *ABCB1* C3435T genotype and clinical outcome was detected in the clinical meta-analyses. However, it should be noted that several studies have demonstrated an association between carriage of the T allele and clopidogrel related HTPR (Harmsze et al., 2010a) and adverse clinical outcomes (Mega et al., 2010a) which, in tandem with our findings, suggest that *ABCB1* polymorphisms may have a small but significant role in determining clopidogrel response. Similarly, whilst we did not detect any association between the CYP3A5*3 polymorphism and clinical or pharmacodynamic outcome, the *CYP3A5*3* polymorphism may have functional relevance in certain circumstances such as co-administration of CYP3A4 metabolised or inhibiting drugs (e.g. amlodipine) (Park et al., 2012).

Given the impact of genetic variants on clopidogrel pharmacodynamics and clinical outcome, we sought to investigate the effect of genetic polymorphisms on the pharmacodynamic response to aspirin, using a cohort of patients with acute coronary syndrome. A number of previous meta-analyses have failed to demonstrate a clear effect of several polymorphisms in the *COX-1* or platelet glycoprotein genes on the response to aspirin, despite evidence that the response to aspirin may be a heritable trait (Faraday et al., 2007). Furthermore, a number of novel polymorphisms were identified in the GeneSTAR genome-wide association study (Mathias et al., 2010) but the detected associations between aspirin response and genotype were highly platelet function test specific. However, newly identified variants in the *PEAR-1* gene were demonstrated to be associated with aspirin response (Keramati et al., 2018), which is in keeping with other genome-wide association studies (Lewis et al., 2013). Consequently, in chapter 4, we undertook a review of aspirin's pharmacokinetic and pharmacodynamic pathway using published literature and the Platelet Aggregation Inhibitor Pathway on the PharmGKB website (www.pharmgkb.org/pathway/PA154444041/overview) and selected a total of 16 polymorphisms in ten genes for genotyping, although only 8 polymorphisms in five genes were successfully genotyped. Whilst significant associations between the *UGT1A6* rs2070959 and *TBXA2R* rs4523 polymorphisms and aspirin response were detected, statistical significance was lost following correction for multiple testing. In addition, it should be noted that there was very poor agreement between the two platelet function tests utilised for this study (Multiplate and PFA-100) which is in keeping with data from the GeneSTAR study and other published data demonstrating associations between

genotype and only certain platelet function tests, with poor agreement across other tests utilised in the study. Nonetheless, our finding that the *TBXA2R* rs4523 polymorphism may be associated with aspirin response has biological plausibility, given that *TBXA2R* is expressed on several tissues, including platelets, leucocytes and atherosclerotic plaques. *TBXA2R* polymorphisms have been demonstrated to increase the sensitivity of the thromboxane receptor to TXA2 (Wang et al., 2013), with elevated levels of TXA2 being generated via over-expressed, aspirin-insensitive COX-2 on atherosclerotic plaques. Consequently, this may increase arachidonic acid induced platelet aggregation via a partially aspirin-independent pathway, with a consequent increase in adverse cardiovascular outcomes. We also observed a potential association between *UGT1A6* polymorphisms and aspirin response, although this was compromised by a relatively low genotype call rate and the polymorphism not being in Hardy-Weinberg equilibrium. Furthermore, *UGT1A6* polymorphisms are unlikely to affect aspirin related platelet inhibition, given that *UGT1A6* is involved in aspirin metabolism only after aspirin's conversion to inactive salicylic acid which has no anti-platelet effect (Kuehl et al., 2006). Taken together with a small sample size, it is likely that the association with UGT1A6 is a false-positive signal. Importantly, we did not demonstrate any association between *COX-1*, *PEAR-1* and *CYP2C9* polymorphisms and aspirin response, which is in keeping with several published studies and meta-analyses (Goodman et al., 2008, Weng et al., 2013).

### 7.2: Platelet Function Testing

As discussed previously, we noted a lack of agreement between aspirin response defined by the Multiplate platform and the PFA-100 system used in Chapter 4. In addition, in our pharmacodynamic meta-analysis, we observed an association between *ABCB1* C3435T polymorphism and platelet aggregation for the VASP assay but not LTA or VerifyNow. Furthermore, in Chapter 5, we demonstrated a clear association between lipid profiles and platelet reactivity for the Multiplate platform but not the PFA-100 system. These findings are in keeping with data from other studies which have demonstrated poor correlation between individual platelet function tests, particularly for arachidonic acid induced platelet activation (Gremmel et al., 2015). Moderate agreement and good reliability over time has been demonstrated between LTA, VASP, Mutiplate and VerifyNow for measuring ADP induced platelet aggregation but, for arachidonic acid induced platelet aggregation, only Multiplate demonstrated moderate reliability over time (Karon et al., 2014). Importantly, whilst the PFA-100 system demonstrates a significant association with clinical outcome, it is generally poorly correlated with other assays and is regarded as not being aspirin specific, with

significant interferences from other variables such as vWF, haematocrit and platelet count (Fitzgerald and Pirmohamed, 2011, Kovacs et al., 2014). In addition, several other clinical variables have been demonstrated to affect the results of specific platelet function tests such as haematocrit and VerifyNow (Kim et al., 2017b) as well as platelet count and Multiplate (Choi and Kim, 2018). Furthermore, platelet function tests have been demonstrated to be inconsistent over time, with several studies demonstrating poor reproducibility in both healthy volunteers and aspirin treated patients across a range of different assays (Miller et al., 2014, Muir et al., 2009).

These data reflect the complex nature of platelet reactivity, with significant effects from clinical and non-clinical factors. Whilst platelet function tests can be used to monitor anti-platelets in clinical practice, recent data suggest that they are used relatively infrequently in the context of ACS despite their potential utility in identifying patients with high or low on-treatment platelet reactivity who may be at risk of adverse clinical outcomes (Wang et al., 2015). Several reasons may explain this finding. Firstly, it is not clear what platelet function test are most specific to the anti-platelet agent being tested. Whilst some tests, for example thromboxane B2 or its metabolites, are thought to be specific to aspirin, it is likely that there are other mechanisms by which anti-platelet drugs modulate platelet reactivity, which may not be related to the primary mechanism of action of the anti-platelet drug. Importantly, each platelet function test investigates anti-platelet response in different ways, which may explain the lack of consensus and high variability in studies where these assays are directly compared. Assays that are often considered gold-standard and with the lowest inter-assay and intra-assay variability (e.g. LTA) are complex to perform and cannot be used as a rapid, point-of-care test. Cut-off values to define HTPR are often variable and inconsistent for individual assays, with published data often using multiple definitions, making it difficult to compare individual studies. Finally, European Society of Cardiology guidelines (and others) suggest universal use of highly potent P2Y12 inhibitors in combination with aspirin; these drugs are subject to lower rates of HTPR in comparison to clopidogrel and, consequently, platelet function testing is unlikely to change management for patients. However, stratification of anti-platelet therapy may have significant benefits, even with the now routine use of ticagrelor. Whilst ticagrelor has been demonstrated to be a more potent anti-platelet agent than clopidogrel, its use is associated with a significantly higher risk of bleeding as well as a number of unique adverse effects, such as dyspnoea, which are a consequence of its effect on adenosine metabolism. In addition, the observed variability in platelet function tests suggest that platelet reactivity is more complex than a simple drug-receptor

interaction and that other clinical and biological factors modulate platelet function. Whilst platelet function tests are potentially sensitive to these additional factors, their utility for stratification may be limited by the lack of data demonstrating which assay best represents overall platelet reactivity and, specifically, which assay best represents the action of a particular drug. Further studies investigating the relationship between clinical outcomes and platelet function tests are clearly necessary, with a focus on development of clear cut-off values for non-response and identification of a 'gold standard' assay that best reflects drug response in patients.

### 7.3: Inflammation and other clinical factors

One of the additional biological factors demonstrated to significantly affect platelet reactivity and clinical outcomes is vascular inflammation, with inflammation being a critical component of the development of atherosclerosis. Several studies have demonstrated that higher levels of inflammation are associated with the severity of atherosclerosis and risk of acute coronary syndromes (Ertem et al., 2017, Odeberg et al., 2016). Data from large clinical cohorts such as CLARITY-TIMI 28 (O'Donoghue et al., 2016) and CREDO (Dosh et al., 2009) have also demonstrated a significant association between higher levels of inflammation and poor outcome following myocardial infarction or PCI. Importantly, treatment with anti-inflammatory agents may significantly reduce the risk of further cardiovascular events. In the CANTOS study (Ridker et al., 2017), treatment with canakinumab, a monoclonal antibody against IL-1β, significantly reduced adverse cardiovascular events in a cohort of 10,061 patients who had suffered a previous myocardial infarction and had an hsCRP>2mg/L (HR 0.85; 95% CI 0.74-0.98, P=0.021 for the 150mg canakinumab dose). In addition, anti-platelet agents may also significantly lower inflammatory markers (Hajsadeghi et al., 2016b), with the more potent agents, such as prasugrel or ticagrelor, reducing inflammatory markers to a greater extent than clopidogrel (Hajsadeghi et al., 2016a, Wei et al., 2017b). Similarly, higher levels of inflammatory markers may be associated with HTPR although the data are not consistent, with some studies demonstrating a clear association with certain inflammatory markers and others not (Muller et al., 2010, Osmancik et al., 2012).

Vascular inflammation may be induced by lipid oxidation. Lipid oxidation and the production of oxidised LDL (OxLDL) is a critical step in the production of foam cells and the development of atherosclerotic plaques (Yu et al., 2013). OxLDL induces a pro-inflammatory state within the atherosclerotic plaque, which leads to subsequent plaque instability and rupture with consequent development of an acute coronary syndrome (Hartley et al., 2019). In addition,

OxLDL forms stable complexes with β2GPI which may better represent the effect of OxLDL *in vivo*, although data are conflicting on whether the complex is pro- or anti-atherogenic. In Chapter 5, we investigated the effect of OxLDL-β2GPI complex levels on clinical outcome and platelet reactivity in a cohort of patients with ACS. Our findings suggested that higher levels of OxLDL-β2GPI complexes were associated with fewer adverse cardiovascular events and were unrelated to either LDL levels or platelet reactivity. However, our data were limited by the lack of contemporaneous measurement of OxLDL and inflammatory biomarkers, which makes it difficult to determine whether OxLDL-β2GPI complexes moderate or enhance the effect of OxLDL in our study, or whether OxLDL-β2GPI complexes are pro-inflammatory or anti-inflammatory. However, there are few published data investigating the effect of OxLDL-β2GPI in unstable cardiovascular disease although in stable cardiovascular disease, higher OxLDL-β2GPI levels have been associated with more severe atherosclerosis and the occurrence of stroke or diabetic microvascular complications (Berger et al., 2014, Bliden et al., 2016, Yu et al., 2015). Whilst the majority of *in vitro* data suggest that OxLDL-β2GPI complexes are pro-inflammatory, recent data have demonstrated that OxLDL-β2GPI complexes may reduce binding of OxLDL to the LOX-1 receptor, thereby reducing the pro-inflammatory effects of OxLDL (Chi et al., 2018).

We also demonstrated that levels of OxLDL-β2GPI were independent of LDL and total cholesterol levels. This is in agreement with several studies demonstrating poor correlation between LDL and OxLDL levels (Gao et al., 2017, Russo et al., 2018) and is likely to reflect the importance of overall atherosclerotic and inflammatory burden in determining OxLDL levels as opposed to being dependent on overall LDL levels.

We also failed to demonstrate any significant association between the levels of OxLDL-β2GPI levels and platelet reactivity as measured by both the PFA-100 system and Multiplate platform. Whilst there are few published data investigating OxLDL-β2GPI levels and platelet reactivity, *in vitro* data suggest that OxLDL may modify platelet function by increasing platelet activation via several potential mechanisms. These include generation of reactive oxygen species (Berger et al., 2018), increased platelet-monocyte interactions (Badrnya et al., 2014) and overall effects of a pro-inflammatory milieu on platelet reactivity (Wang et al., 2018d). In addition, some of these mechanisms may be common to the effects of hyperlipidaemia on platelet function and it was notable that we observed an association between higher levels of aspirin related platelet reactivity in patients and raised HDL:cholesterol ratios in our study, which was driven predominantly by lower HDL levels in aspirin non-responsive patients as opposed to raised cholesterol. These findings are in

keeping with other clinical studies that have demonstrated a significant relationship between hyperlipidaemia and higher levels of platelet reactivity (Labuz-Roszak et al., 2014). Indeed, in a study by Chan and colleagues (Chan et al., 2015), platelet reactivity was significantly higher in hypercholesterolaemic patients in comparison to healthy volunteers. Interestingly, patients in this study with low HDL cholesterol levels had significantly higher platelet reactivity in comparison to patients with normal HDL cholesterol irrespective of the levels of LDL cholesterol, which is in keeping with our observations.

Finally, we investigated (Chapter 6) the potential mechanism underlying the observed interaction between clopidogrel and PPIs. Several studies have demonstrated that clopidogrel is less effective when co-administered with PPIs, with a consequent increase in the risk of adverse cardiovascular events (Kim et al., 2019). As previously discussed, clopidogrel is a pro-drug that requires metabolism to its active metabolite which is primarily catalysed by CYP2C19. CYP2C19 is also the primary CYP450 enzyme responsible for the metabolism of PPIs and, consequently, there is a risk of interaction between both drugs. However, post-hoc analysis of data from large clinical outcomes trials such as TRANSLATE-ACS (Dunn et al., 2013) and others have failed to demonstrate a clear association between poor clinical outcomes and co-administration of clopidogrel and PPIs, which suggests that alternative mechanisms may underlie the putative pharmacokinetic interaction. In particular, PPIs are often prescribed for a variety of non-specific gastrointestinal conditions, such as dyspepsia, which may be associated with *H. Pylori* infection. *H. Pylori* infection has been associated with an increased risk of adverse cardiovascular events (Tabata et al., 2016c) which may be caused by an increase in vascular inflammation (Kucukazman et al., 2015). Give the previously discussed importance of the relationship between inflammation and cardiovascular risk, the observed interaction between clopidogrel and PPIs may be related to *H. Pylori* infection and consequent vascular inflammation rather than a pharmacokinetic interaction at CYP2C19.

We conducted a case-control study in patients with unstable cardiovascular disease who were co-prescribed clopidogrel and a PPI. We failed to demonstrate any significant association between positive *H. Pylori* serology and clinical outcome which is inconsistent with *in vitro* and biomarker data but in keeping with larger clinical outcome studies. *In vitro* studies clearly demonstrate that *H. Pylori* infection increases vascular inflammation and the risk of adverse cardiovascular outcomes by inducing inflammatory responses within atherosclerotic plaques (Li et al., 2017b), increasing endothelial cell dysfunction (Oshima et al., 2005) and, potentially, activating platelets (Scopel-Guerra et al., 2017). However, large,

prospective clinical outcome studies have failed to demonstrate a clear increase in the risk of adverse cardiovascular events from *H. Pylori* infection although retrospective studies have tended to demonstrate associations between *H. Pylori* infection and adverse clinical outcomes (Huang et al., 2014). In our cohort of unstable cardiovascular disease patients, we did not detect any association between *H. Pylori* infection and clinical outcome and it is notable that few published studies have investigated *H. Pylori* infection in the context of acute coronary syndromes and MI. However, like most published data, our study is limited by the use of *H. Pylori* serology as the measure of *H. Pylori* infection which may reflect previous infection rather than current, active infection. Furthermore, we did not assess any inflammatory biomarkers in the study which prevents assessment of whether inflammatory markers or pro-inflammatory cytokines were associated with *H. Pylori* serology or clinical outcome. Finally, we could not assess any putative effect of *H. Pylori* infection on platelet function given the low numbers of included patients who had available platelet function data.

Interestingly, we detected a significant association between *CYP2C19*2* genotype and case-control status, with higher rates of the loss-of-function, *2, allele carriage in patients who did not suffer additional cardiovascular events. In addition, this association was observed in *H. Pylori* negative but not in *H. Pylori* positive patients. The mechanism underlying this finding remains unclear. Whilst PPIs are sensitive to *CYP2C19* polymorphisms, with greater efficacy observed in patients with loss-of-function genotypes (Sugimoto et al., 2014), it is not clear how that may interact with *H. Pylori* infection given that the relationship between *H. Pylori* infection and long-term PPI use is poorly understood despite the well characterised changes in the gastric flora in patients prescribed PPIs long term (Parsons et al., 2017). In addition, the observed association between carriage of the *CYP2C19*2* allele and a lower risk of further cardiovascular events is not in keeping with the known effects of the allele on clopidogrel metabolism and efficacy.

### 7.4: Optimisation of anti-platelet therapy

In summary, this thesis has identified several important points in relation to anti-platelet response. Firstly, *CYP2C19* genotype is clearly associated with platelet function and clinical outcomes in clopidogrel treated patients. Secondly, whilst a number of genetic variants may alter aspirin response, it remains unclear whether any single polymorphism or panel of polymorphisms could represent aspirin response for the purposes of stratification. Thirdly, although platelet function tests directly measure anti-platelet response, their intrinsic inter-

assay variability limits their use as a potential biomarker for stratification. Finally, vascular inflammation and other cardiovascular risk factors may have significant impact on anti-platelet drug response which may necessitate specific treatments to reduce the risk of adverse cardiovascular outcomes.

On this basis, it appears that genetic stratification is the most appropriate method for personalisation of anti-platelet therapy. Small-scale adoption of *CYP2C19* guided dosing of clopidogrel has recently been reported with positive results (Cavallari et al., 2018, Lee et al., 2018).

Whilst large-scale, randomised clinical trials have clearly demonstrated that, in terms of efficacy and pharmacodynamic outcomes, the newer anti-platelets agents are superior to clopidogrel, there remains a clear case for personalisation of anti-platelet therapy. Clopidogrel is associated with a lower risk of bleeding in comparison to the newer, more potent, agents and does not share the adverse effects of dyspnoea associated with the use of ticagrelor. In addition, the mechanism by which clopidogrel is less efficacious is well known, easily testable for and, if detected *a priori*, potentially modifiable by using alternative ADP receptor antagonists. Furthermore, other clinical and biochemical factors, such as inflammation, hyperlipidaemia and drug interactions, are potentially easily monitorable and treatable. Treatment of such risk factors (e.g. with statins or with drugs to improve glycaemic control) could also substantially improve the efficacy of anti-platelet drugs in parallel with stratification of anti-platelet therapy. However, for aspirin, it remains unclear whether personalisation of therapy is currently possible given the lack of clear biomarkers for aspirin response, the absence of alternative agents and inconsistent data on the effects of an increased aspirin dose to overcome non-response (Dominiak et al., 2013, Mrdovic et al., 2016, Xian et al., 2015). Consequently, the discussion on stratification will focus primarily on clopidogrel and the other ADP receptor antagonists.

Crucially, the observed superiority of prasugrel and ticagrelor over clopidogrel may be due to poor clopidogrel response in patients carrying the *CYP2C19* loss-of-function alleles. In both TRITON-TIMI 38 (Mega et al., 2010a) and PLATO (Wallentin et al., 2010), the observed benefits of the newer anti-platelet agents were largely observed only in clopidogrel treated patients who had variant *CYP2C19* loss-of-function genotypes and not in those with wild-type genotypes. As a result, genotyping for *CYP2C19* alleles could be an effective strategy for personalisation of ADP receptor antagonists, with wild type genotypes being treated with

clopidogrel whilst those patients with variant, loss-of-function genotypes being treated with either ticagrelor or prasugrel.

Genotype guided therapy has been investigated in clinical trials with positive results. Pharmacodynamic outcome studies have demonstrated that patients treated on the basis of genotype achieve platelet inhibition in the therapeutic range more frequently than patients treated with universal clopidogrel or ticagrelor (Lee et al., 2016, Malhotra et al., 2015). Similarly, in clinical outcome studies, genotype guided dosing has been demonstrated to reduce the risk of adverse cardiovascular outcomes in clopidogrel treated patients. In a study by Shen et al (Shen et al., 2016), 628 post-PCI patients were randomised to receive 'routine' therapy with clopidogrel or 'individual' therapy on the basis of *CYP2C19* genotype. In the 'individual group', extensive metabolisers were treated with clopidogrel whilst intermediate metabolisers were treated with high-dose clopidogrel (150mg/day) and poor metabolisers were treated with ticagrelor. A composite outcome of death, myocardial infarction or target vessel revascularisation was assessed at 1, 6 and 12 months. A significant reduction in the composite endpoint was observed in the 'individual' group when compared to the 'routine' group and, importantly, within the 'individual' group, no significant differences in outcome were detected between the extensive, intermediate and poor metaboliser patients. Larger scale studies in PCI patients have also been positive, with two recent studies demonstrating no significant differences in adverse cardiovascular events between clopidogrel treated patients with wild type *CYP2C19* genotypes and ticagrelor or prasugrel treated patients with LOF *CYP2C19* genotypes (Cavallari et al., 2018, Lee et al., 2018). These emerging data suggest that genotype guided anti-platelet dosing is safe and efficacious, with further randomised trials now necessary to demonstrate its clinical effectiveness.

Platelet function test guided therapy has also been extensively assessed in clinical trials, with mixed results although this may be a consequence of the patient population included in the individual studies. Large clinical outcome studies such as GRAVITAS (Price et al., 2011), TRIGGER-PCI (Trenk et al., 2012) and ARCTIC (Collet et al., 2012) all failed to demonstrate any significant improvement in clinical outcomes following stratification of anti-platelet therapy on the basis of VerifyNow assessed platelet reactivity. However, all three studies were conducted largely in patients with stable coronary artery disease and it is therefore likely that failure to detect any benefit from stratification was largely due to the intrinsic low event rate in this group of patients. Studies in patients with acute coronary syndromes have demonstrated more positive results (Dridi et al., 2014) but, unlike the GRAVITAS, TRIGGER-PCI and ARCTIC trials, did not compare patients with clopidogrel related HTPR who received

stratified treatment against patients who had normal responses to clopidogrel. Instead, stratified therapy was compared only against patients with clopidogrel related HTPR who continued to receive standard doses of clopidogrel. It, therefore, remains unclear whether the observed benefit of pharmacodynamic stratification in ACS is merely a consequence of comparing stratified patients against high risk patients with HTPR who remained on treatment with clopidogrel.

Cost-effectiveness analyses have demonstrated that both genotype and platelet function guided stratification are likely to be cost-effective (Coleman and Limone, 2013, Jiang and You, 2017, Wang et al., 2018e). However, the incremental cost-effectiveness ratio (ICER) for genotype guided dosing is at least fourfold lower for genotype guided dosing (USD 2,560 to 10,153 per quality-adjusted life year) in comparison to platelet function guided dosing (USD 40,100 to 49,143 per QALY).

Taking all these data together, genotype guided stratification appears to be the most clinically and cost-effective method for personalisation of ADP receptor antagonist therapy. In addition, whilst genotype is not necessarily sensitive to additional clinical and biochemical factors that may affect anti-platelet response, it is the single most important determinant of clopidogrel non-response and can be easily and reliably measured in clinical practice. Consequently, further, adequately powered clinical trials in patients with unstable cardiovascular disease are required to determine its effectiveness.

Finally, attention should be given to the other clinical and biochemical factors that influence response to both the ADP receptor antagonists and aspirin. These include inflammation, hyperlipidaemia and diabetes, all of which have been demonstrated to increase platelet reactivity and lower the response to anti-platelets. Several studies have demonstrated the effectiveness of treating inflammation with anti-inflammatory agents, with the recent CANTOS study (Ridker et al., 2017) demonstrating significant reductions in the occurrence of adverse cardiovascular events in patients with previous MI treated with canakinumab, a monoclonal anti-IL1β antibody. However, it is likely that more specific therapies can be developed for the treatment of vascular inflammation as further data emerges on atherosclerosis specific inflammasomes. For example, recent focus has been placed on the development of specific therapies against OxLDL, with several pre-clinical and clinical trials ongoing at the present time (Hartley et al., 2019). Furthermore, better management of hyperlipidaemia and diabetes may significantly improve the response to anti-platelets in those conditions (Schuette et al., 2015). In particular, statins have been demonstrated to

have pluripotent effects by reducing LDL cholesterol and inflammation whilst also improving endothelial cell function and aspirin induced platelet reactivity (Pesaro et al., 2012, Wang et al., 2017). Consequently, stratification of ADP receptor antagonists should also be accompanied by robust management of other clinical and biochemical factors to ensure full optimisation and maximisation of anti-platelet therapy.

## 7.5: Conclusion

In conclusion, this thesis has investigated a number of genetic, clinical and biochemical factors that may affect the response to anti-platelet drugs. We have demonstrated, through robust meta-analysis of the published data, that *CYP2C19* polymorphisms are critical to identifying patients at risk of poor response to clopidogrel. At current, two randomised trials are being conducted (Popular Genetics and TAILOR-PCI) comparing genotype-guided anti-platelet therapy to universal therapy with either prasugrel and ticagrelor, with both trials expected to report within the next 12 to 18 months (Klein et al., 2019). In addition, we have identified potential polymorphisms that merit further investigation for their role in determining response to aspirin. Furthermore, we have also detected potential interactions between lipid oxidation and clinical outcomes in patients with unstable cardiovascular disease. Finally, we did not detect any significant association between *H. Pylori* infection and clinical outcomes in patients with ACS. Taken together, these data suggest that stratification of anti-platelet therapy on the basis of genotype may be possible for clopidogrel but not aspirin. In addition, modification of various risk factors, such as hyperlipidaemia, may also be important in reducing non-response to anti-platelet drugs.

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