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Personalised Medicine in New Zealand

A new approach to healthcare delivery

Dr. Patrick Alton Gladding, BHB, MBChB

A thesis submitted in fulfillment of the requirements for the degree of Doctorate of Philosophy in Medicine, The University of Auckland, 2011
Personalised Medicine in New Zealand

A New Approach to Healthcare Delivery

Personalised Medicine is expected to improve efficiency of healthcare delivery and reduce costs. Pharmacogenomics fits within this framework and seeks to identify individuals who are most likely to benefit from targeted interventions. The aim of this thesis was to focus on the application of personalised medicine in cardiovascular medicine, particularly as it applies to the use of antiplatelet medications, percutaneous coronary intervention, hereditary cardiac disease and population genomics.

A number of clinical trials and observational studies were undertaken. This included studies of drug-drug and drug-herb interactions in Chapters Two and Three, and pharmacogenetic studies of aspirin and clopidogrel in Chapters Four to Seven. Chapter Eight summarises these findings and discusses their relevance to Maori and Pacific Peoples in New Zealand. Biorepositories are discussed in Chapter Nine and their applied clinical use in Chapter Ten. Metabolomics and proteomics were applied in Chapter Eleven to elucidate mechanisms in coronary intervention. Chapters Twelve and Thirteen include a longitudinal study of a 9p21.3 SNP, associated with coronary disease and a simulation of warfarin dose requirement using pharmacogenetics. Chapter Fourteen discusses the barriers to the adoption of genomics and what future policy requirements are needed to overcome these impediments. Results from this thesis are as follows:

- Concomitant medications, such as the nonsteroidal drugs (ibuprofen and indomethacin) block the antiplatelet effect of aspirin
- The antiplatelet response to aspirin does not appear to be influenced by genetics
- Variants within the CYP2C19 gene (e.g. *2 allele) explain some of the response to clopidogrel
- Non-response to clopidogrel can be overcome with higher doses of the drug, however this may not be achieved with current maintenance doses (75-150mg daily)
- Integrating metabolomics and proteomics has significant value in elucidating novel mechanisms behind disease and identifying biomarkers
- DNA in a biorepository, linked to electronic medical records systems, has value in evaluating population genomics and in providing a clinical service

The response to antiplatelet drugs is complex and not always influenced by genetics. Clopidogrel treatment, however, can be individualised using pharmacogenomics. Genomic medicine has the potential to bridge population health and individualised clinical practice. The use of genomic medicine may be advantageous in addressing ethnic disparities and may be cost effective when applied on a population basis. Ethical issues and government policy need to be addressed.
Dedication

To Jo and Amelia, Oliver and Benjamin

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Preface

Pharmacogenetics has long held the promise of individualising pharmacological therapy using genetic biomarkers. Targeting treatments using pharmacogenetics is intended to maximise efficiency by preventing adverse events and targeting more expensive treatments to those who have the greatest need. With ever-growing healthcare costs, new pharmaceuticals providing only a modest incremental benefit over current therapies, and the world-wide economic downturn, a crisis is being realised. There has never been a greater need to ration treatment to those who have the most to gain from it. (1)

Cardiovascular medicine is well positioned to benefit from rapid advances in the field of pharmacogenetics. Elucidation of the genetic markers predicting response to clopidogrel, the second most-prescribed drug in the world, is of particular importance as a reduced antiplatelet effect with clopidogrel is associated with adverse clinical outcomes including cardiovascular death, myocardial infarction (MI), stroke and additional healthcare costs. Clopidogrel is attractive for pharmacogenetic study as it is a pro-drug that requires conversion to an active derivative, catalyzed by cytochrome P450 (CYPs). These CYPs are influenced by genetic variation.

This thesis can be considered in three parts, linked by the common theme of personalised medicine and targeted resource allocation.

Part I - Chapters One to Eight focuses on antiplatelet medication and pharmacogenetics
Part II - Chapters Nine to Eleven cover the storage of biological material for clinical purposes and biomarker research
Part III – Chapters Twelve to Fourteen explore population genomics and informatics

Part I
Chapter One reviews the current literature in the field of antiplatelet treatment and discusses the definition and incidence of non-response to treatment. Chapter Two describes a clinical trial aimed at demonstrating interactions between six commonly used non-steroidal anti-inflammatory drugs and aspirin. Chapter Three similarly describes a crossover trial aimed at showing whether herbal medicines interact with aspirin. Chapter Four explores the pharmacogenetics of aspirin. Chapters Five and
Six describe a randomised clinical trial investigating the pharmacodynamics and pharmacogenetics of clopidogrel, in patients undergoing percutaneous coronary intervention (PCI). Chapter Seven is a nonrandomised study that attempts to validate the findings of the previous two chapters. Chapter Eight places the context of these findings in the New Zealand population and argues the cost-benefits of adopting a personalised approach to antiplatelet prescribing.

**Part II**
Chapter Nine begins the second part of the thesis, and describes the methods and requirements for establishing biorepositories, an essential part of genomic research. Chapter Ten demonstrates the value of a biorepository in clinical practice, in the context of a hereditary cardiac disorder. Chapter Eleven describes an observational study of patients undergoing percutaneous coronary intervention and uses metabolomics, proteomics and functional network analysis to demonstrate disease mechanisms and potential biomarkers.

**Part III**
Chapter Twelve, Thirteen and Appendix A are population genomic studies that demonstrate the value of a biorepository in New Zealand. Chapter Twelve shows it is possible to link genomic data to electronic medical databases and Chapter Thirteen and Appendix A shows that a biorepository can be used for modeling and simulation studies. Chapter Fourteen discusses the barriers to the practical adoption of clopidogrel pharmacogenomics and Chapter Fifteen summarises this work and expands on the unresolved issues that remain for personalised and genomic medicine.

This thesis has taken a translational approach to clinical science, and is different from experimental benchtop work, which often applies exquisite focus in doctoral work. Clinical medicine spans many fields to encompass the entire individual, and a broader scope needed to be taken. To cover the breadth required in this work an interdisciplinary approach was made. Therefore much of this work was dependent upon teamwork and collaboration, particularly in the aspect of analytical work in the laboratory. Whereas in the past it may have been necessary for one individual to be expert at many steps in a process it is now more efficient to be the expert in one step and ensure the flow of information and discussion remains open. Hence in this thesis, at times, laboratory testing has been outsourced or performed by collaborators with expertise in a unique field.
Acknowledgements

Nothing can be done alone and incredible synergies are made when working in a team. Traditionally teams used to be geographically close, with face-to-face contact. As a result of the internet many interactions are now performed remotely. With the opening up of core laboratories within universities and the entrepreneurialism of individuals opening contract service laboratories it has now become possible for almost anyone to perform scientific research more cost-effectively. One does not need to purchase expensive equipment and spend time becoming expert in its use. Experts can now be contracted to work on high-end systems anywhere on the planet. Managing global virtual teams and maintaining good relationships however is a challenge. Openness, willingness to share, general enthusiasm and passion for a subject, along with an appreciation for the work and expertise of others, is critically important. Maintaining direction, focus and engagement can be difficult and virtual collaborations can be depersonalising. However, in saying that, during the course of this thesis I met many wonderful people who often went out of their way to provide assistance and support, even from afar. Many of these people had extraordinary skill but also humility, and without their assistance much of the technical work in this thesis could not have been completed.

The list to thank is long and, I apologise in advance if any are missed out. Thanks to Azru Gunes, Marja-Liisa Dahl, Irene Zeng, Helen Farrell, Mildred Lee, Barbara Semb, Charlene Nell, Gayl Humphrey, Jackie Crawford, Jon Skinner, Choy Hor Yee, Ralph Stewart, Harvey White, Mia Jullig, Martin Middleditch, Gordana Prijic, Andrew Shelling, Laura Panattoni, Silas Vilas-Boas, Katie Smart, Tom Marwick, Jim Thomas, Shafkat Anwar, Kazuaki Negishi, Jake Orville, Jim Mervis, Joerg Kistler, Margot Bethall, Dietrich Ruehlmann, Bill Ferenczy, Brian Taylor, Folarin Erogbogbo, Mark Swihart, Zoraida Aguilar, Will Leizerowicz, Nik Kasabov, Michelle Jamieson, Jagir Hussan, Peter Hunter, John Mackay. A particular thanks to Mark Webster and Ralph Stewart my co-supervisors and Robert Doughty my main supervisor, who collectively have helped contain my enthusiasm for this subject and produce, hopefully, clarity.

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Chapter 1: Antiplatelet drug non-response

This chapter provides an overview of the pharmacology and variability of response to commonly used antiplatelet medication. Individual medications are discussed as well as the definition of non-response and methods of measuring response. The clinical importance of how a patient responds to these medications is significant, as they are prescribed to a large proportion of the population. Lack of response, also known as non-response, to these medications is likely to have societal costs. These include increased cardiovascular events from ineffective treatment and treatment injury due to adverse side effects. This Chapter focuses principally on non-responders.

Introduction

The concept of antiplatelet drug “resistance” or non-response has received increasing attention over recent years. The inter-individual variable response to medicines is understood to be a significant cause of morbidity and wasted healthcare expenditure. Non-response to aspirin has been discussed as far back as 1993,(1) and has been recognised in all antiplatelet drugs, including clopidogrel and glycoprotein IIbIIIa inhibitors.(2, 3)

The evidence for the use of antiplatelet drugs in the treatment and prevention of acute coronary syndromes is extensive. A meta-analysis found that aspirin reduced the incidence of nonfatal myocardial infarction (MI), nonfatal stroke, and vascular death by 22 percent in those with vascular disease.(4) As an alternative single agent clopidogrel has a similar benefit,(5) and dual antiplatelet therapy is about 20 percent more effective than aspirin alone. Clopidogrel is an established adjuvant to aspirin in patients undergoing percutaneous coronary intervention (PCI) with stenting,(6) and in those presenting with acute non-ST segment elevation and ST elevation acute coronary syndromes.(7, 8) Individuals not responding to antiplatelet drugs are more likely to have recurrent vascular events, including an increased risk of stent thrombosis.(9, 10)

Point-of-care platelet function technology has made evaluation of the response to antiplatelet easier to perform,(11) but the field remains constrained by differences between testing methods and a lack of outcome and intervention studies. Definitions
of non-response are variable and include arbitrary thresholds set by some analysers.\(^{(12)}\)

**Antiplatelet agents**

Platelet activation plays a pivotal role in the pathogenesis of late stage and probably early stage atherothrombotic disease. Platelets are activated by a number of physiological agonists including thromboxane, adenosine diphosphate (ADP), thrombin, serotonin and collagen. Shear stress, a physical property of blood flow, also plays an important role. Platelets are capable of inducing their own aggregation, predominantly via thrombin generation, leading to an amplification reaction. Despite the wide range of platelet agonists, only four pathways are targeted by drugs in common use (Figure 1).

**Figure 1. Agonists to platelet activation and antiplatelet agents**

Drug classes include thromboxane pathway inhibitors such as aspirin and non-steroidal anti-inflammatory drugs, P2Y12 receptor antagonists such as ticlopidine and clopidogrel, phosphodiesterase inhibitors such as dipyridamole and cilostazol, and glycoprotein IIb/IIIa inhibitors including abciximab, eptifibatide and tirofiban.\(^{(13)}\)
A protease-activated receptor-1 (PAR-1, thrombin receptor) antagonist have been evaluated in Phase III trials (TRACER, TRA2P) and may be clinically available in the near future.(14) Thrombin is the most potent agonist of platelet activation and despite the current blockade of antiplatelet pathways with aspirin and clopidogrel persistent thrombin generation poses a considerable continued stimulus for platelets. Blocking the platelet PAR-1 receptor leaves other thrombin-mediated haemostatic functions intact, so theoretically bleeding events may not be increased.(15)

The glycoprotein IIb/IIIa inhibitors are currently considered to be the most potent antiplatelet agents as they inhibit the final common pathway of platelet aggregation. Platelets however have multiple functions, which include interacting with vessels and influencing haemodynamics. So although glycoprotein IIb/IIIa inhibitors are very effective at inhibiting platelets they do not mitigate platelet derived release of vasoactive substances.(16) Clinical trials suggest that combination therapy of glycoprotein IIb/IIIa inhibitors and P2Y12 receptor antagonists may reduce periprocedural myonecrosis and improve long-term ischemic outcomes, compared with either drug alone.(17, 18)

**Definitions**

Clinically-significant antiplatelet drug failure can be defined as a recurrent vascular atherothrombotic event occurring despite drug adherence. Given the multiple pathways of platelet activation, it is difficult to be certain that a drug has “failed”. For instance reinfarction after aspirin administration for an acute coronary syndrome may be due to ADP-mediated thrombus propagation rather than ongoing thromboxane-induced platelet aggregation.(19) Antiplatelet “resistance” or non-response might be best defined as residual post treatment activity (measured by a platelet function analyser) in a target pathway.(20)

An alternative terminology to non-responder or resistance is “high on treatment platelet reactivity” or HOTPR.(21) The term resistance has fallen out of favour due to the connotation that it is related to adherence to treatment. Non-response to a drug can be predicted prior to drug administration and relates to patient response to a treatment agent. High on treatment platelet reactivity however is a more encompassing term that may reflect not only the individual response to a drug but also the clinical state of the patient.
**Platelet function analysers**

Traditional platelet function testing using LTA is complicated to perform, requiring skilled and experienced phlebotomy and laboratory staff working under carefully controlled conditions. Laboratory-based LTA is accepted by most as the “gold standard”, but is labour intensive, operator-dependent and expensive. This has restricted its clinical use. These limitations have led investigators to use surrogates for platelet function, including biochemical markers such as serum or urine thromboxane B2 for aspirin activity and vasodilator-stimulated phosphoprotein (VASP) for P2Y12 receptor inhibition (Table 1).

Platelet function assays can be classified according to the method of analysis. Biochemical assays utilise either an ELISA assay (thromboxane) or flow cytometry (VASP). Non-biochemical platelet function analysers typically use light transmittance or electrical impedance to measure platelet aggregation directly, either in isolated platelets or in whole blood. The platelet agonist may differ between assays, making inter-assay comparisons difficult. Few analysers incorporate shear stress as a non-biochemical means of platelet stimulation.

<table>
<thead>
<tr>
<th>Platelet Function analyser</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Clinical Outcome Studies</th>
<th>Monitors ASA and Clopidogrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time*</td>
<td>Widely available, in vivo</td>
<td>Highly variable, non-specific activation, patient scarring</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>PFA-100*</td>
<td>Rapid, whole blood, hypothesised to mimic small vessel, uses shear stress</td>
<td>Requires pipetting of blood, inter-instrument variability, non-continuous output, dependent on vWF levels</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>VerifyNow*</td>
<td>Closed system, rapid,</td>
<td>Older model influenced by</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Method</td>
<td>Description</td>
<td>Advantages</td>
<td>Platelet Mapping</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td>Thromboelastogram (TEG)*</td>
<td>Small footprint, has advantages for cardiac anaesthetists</td>
<td>Requires pipetting of blood (operator dependent results), difficult to interpret output variables, minimal clinical studies</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Light transmittance aggregometry</td>
<td>Historic gold standard</td>
<td>Operator dependent, requires preparation of plasma and pipetting, costly, time consuming</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Multiplate analyser</td>
<td>Uses impedance method, compares favourably to other methods</td>
<td>Operator training dependent, requires pipetting of blood, not point-of-care</td>
<td>Yes</td>
<td></td>
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<tr>
<td>Urinary 11-dehydro-thromboxane B2</td>
<td>Specific to COX-1 activity</td>
<td>Not specific to platelet COX-1, dependent on renal function and urinary concentration</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>VASP</td>
<td>Specific to P2Y12 activity</td>
<td>Expensive, requires flow cytometer, technical experience required</td>
<td>Yes</td>
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</tbody>
</table>

*PFA = Platelet function analyser, COX = Cyclo-oxygenase, VASP = Vasodilator*
Point-of-care platelet function devices have simplified testing and in addition provide a rapid result available at the bedside or in the cardiac catheterisation laboratory. The three most widely-evaluated point-of-care devices are Dade-Behring’s PFA-100™, which measures platelet function under high shear stress by drawing blood through a small aperture and measuring the “closure time” of that aperture by a platelet plug; Accumetric’s VerifyNow™ assay which uses a light-based, whole blood aggregometry system; and the Thromboelastograph (TEG™), which measures clot tensile strength and has been most widely evaluated and used in patients undergoing cardiac surgery.

The PFA-100 has been used clinically in the diagnosis of platelet function disorders. Its cartridges have collagen and either arachidonic acid or ADP as the agonist. Whilst the PFA-100 has been tested against both aspirin response and clopidogrel its use has primarily been validated against aspirin response. The units of the assay are measured in seconds to closure time of a small aperture with clinically validated values which denote antiplatelet non-response. Closure times of ≥178 seconds, are generally considered to denote non-response to aspirin.

The VerifyNow device has three pathway-specific cartridges that test for the effects of aspirin, clopidogrel and GpIIbIIIa inhibitors, all validated against light transmittance aggregometry. The VerifyNow device has been used extensively in clinical trials and is an attractive option due to its closed, point-of-care system. The units of measure are either as a percentage of platelet inhibition or as a proprietary unit (Aspirin response unit, ARU or platelet response unit, PRU, for clopidogrel). ARU ≥550 denotes aspirin non-response and a PRU of ≥235 platelet reactivity units (PRU) denotes clopidogrel non-response.

Cutoff values that define resistance are still widely debated. The ASPECT study had seven different definitions for aspirin resistance, spanning five forms of platelet function testing. The cutoff value definitions for the different instruments varied widely, for example light transmittance aggregometry (LTA), ranging from ≥ 20 percent aggregation using an agonist of arachidonic acid to ≥ 70 percent using adenosine diphosphate (ADP). Clinical outcome studies have provided some
guidance with cutoff values for each individual platelet analyser, which are usually derived using receiver operator curves.

A receiver operator curve in a study of 380 patients undergoing PCI with platelet testing using the VerifyNow analyser showed an optimal cut-off for the combined endpoint was a post-treatment reactivity ≥235 platelet reactivity units (PRU) [area under the curve 0.71 (95% confidence interval 0.5-0.9), P = 0.03]. Patients with PRU values greater than the cut-off value had significantly higher rates of stent thrombosis and CV death.(39). Receiver operator curves have also been used to define LTA and PFA-100 cutoff points for testing.(41) Whilst it is valuable to have cutoff values for defining non-response or HOTPR it is important to realise that not all patients will be classified correctly on these values hence the sensitivity and specificity of such a cutpoint should be noted. For instance for defining non-response a PRU ≥235 has a sensitivity of 78% and specificity of 68%, with a negative predictive value of 99%.(39). Therefore although this cutoff value can be used to roughly discriminate responder status a minority of patients will be misclassified.

**Incidence**

The definition of antiplatelet drug response using these devices is somewhat arbitrary. There are seven different thresholds defining an aspirin response with the PFA-100 reported in the literature.(28-34) The incidence of non-response to aspirin varies with the platelet function test used and the threshold chosen to determine response. Reported incidence rates range between 9.5 and 33 percent.(31, 42, 43) There is also a weak correlation between different testing methods.(40) One study found no association between the PFA-100, using epinephrine as the agonist, and light transmittance aggregometry in patients taking low dose aspirin; 9.5 percent of individuals were considered non-responders using the PFA-100, compared with 5.5 percent non-responders and 23.8 percent semi-responders by light transmittance aggregometry.(31) Similarly a high heterogeneity of results from multiple platelet analysers has been demonstrated in patients both on aspirin and not on aspirin.(44)

Although population response to medication often fits a normal distribution it is uncertain whether the same is true of aspirin responsiveness. The aspirin response assessed with the VerifyNow device may follow a bimodal distribution with a value of 550 Aspirin Resistance Units (ARU) separating responders from non-responders.(45)
A bimodal response has not been found with other platelet function analysers, and this finding needs to be replicated.

Clopidogrel response appears to follow a normal distribution. (46) Varying definitions of non-response have been proposed, including a change in platelet response from baseline, and an absolute threshold. (46, 47) As there is no consensus definition the incidence of non-response to clopidogrel is uncertain.

Some argue that antiplatelet resistance does not exist and cite aspirin as a particular case example. A study of 700 patients found that residual arachidonic acid-induced platelet aggregation (resistance) was due to ADP-dependent rather than cyclooxygenase pathways. (48) The authors concluded that “aspirin resistance” is a misnomer and either a limitation of platelet analysers due to pathway nonspecificity or due to non-compliance. (48) Undetected nonadherence may have led to an overestimate of the rate of aspirin non-response. (49) This hypothesis is supported by the ASPECT study, a comprehensive assessment of a range of platelet function analysers, which concluded that aspirin non-response is rare, overcalled by some analysers like the PFA-100, and may be related to non COX-1 pathways such as those mediated by ADP. (40)

**Aetiology**

The cause of antiplatelet drug resistance is multifactorial. Some possible causes for aspirin non-response are outlined in Table 2. Risk factors include increasing age, smoking, and gender; (29) many are related to drug pharmacokinetics and pharmacodynamics. Diabetes is a particular problem as it is associated with generalised heightened platelet reactivity. The presence of diabetes increases platelet receptor expression, reduces platelet derived formation of nitrous oxide and increases sensitivity to ADP. (50) Despite treatment of diabetic patients with dual antiplatelet therapy there is still an increased rate of cardiovascular events, particularly in those who have measurably higher platelet reactivity. (51)

**Pharmacokinetics**

Pharmacokinetics includes the bioavailability, i.e. absorption and the first pass effect, volume of distribution, and clearance of a drug. The first pass effect on aspirin is considerable and the drug is rapidly cleared by carboxylesterases 1 and 2 in the
liver.(52) The antiplatelet effect of aspirin occurs predominantly in the portal circulation.(53) Although aspirin inhibits both COX-I and COX-II, the systemic COX-II effect is minimal below doses of 1,200mg.(54, 55) Platelets are anucleate and have no ability to regenerate COX-I and therefore aspirin-mediated platelet inhibition is permanent for the life of the platelet. However platelets are continually formed and COX-I sources, such as nucleated cells, can contribute prostaglandin precursors, leading to a recovery of platelet activity within twenty-four hours.(56)

Clopidogrel is a prodrug that requires hepatic biotransformation by cytochrome P450 3A4, 1A2, 2B6 and 2C19 to an active metabolite which irreversibly antagonizes the ADP receptor.(57-59) The active metabolite has an extremely short half-life.(60-62) Drugs co-administered with clopidogrel that inhibit the metabolism of CYP3A4 will, at least in theory, diminish the antiplatelet effect of clopidogrel. Although both atorvastatin and erythromycin inhibit CYP3A4 and reduce the ex vivo antiplatelet response to clopidogrel,(63) this interaction may not be clinically significant as post-hoc analysis of the CREDO study found no increase in vascular events in those on atorvastatin and clopidogrel.(64)
<table>
<thead>
<tr>
<th>Origin</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient Factors</strong></td>
<td>• Nonadherence to treatment</td>
</tr>
<tr>
<td></td>
<td>• Smoking</td>
</tr>
<tr>
<td></td>
<td>• Increasing Age</td>
</tr>
<tr>
<td></td>
<td>• Gender</td>
</tr>
<tr>
<td></td>
<td>• Co-morbidities e.g. diabetes</td>
</tr>
<tr>
<td></td>
<td>• Altered binding site</td>
</tr>
<tr>
<td></td>
<td>e.g. COX-1 polymorphisms</td>
</tr>
<tr>
<td></td>
<td>• Reduced absorption</td>
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<tr>
<td></td>
<td>e.g. p-glycoprotein polymorphisms</td>
</tr>
<tr>
<td></td>
<td>• Reduced CYP3A4 biotransformation</td>
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<tr>
<td></td>
<td>e.g. CYP3A4 polymorphisms</td>
</tr>
<tr>
<td></td>
<td>• Increased volume of distribution</td>
</tr>
<tr>
<td></td>
<td>e.g. increased body mass</td>
</tr>
<tr>
<td><strong>Drug Factors</strong></td>
<td><strong>Delivery vehicle</strong></td>
</tr>
<tr>
<td></td>
<td>• Reduced absorption</td>
</tr>
<tr>
<td></td>
<td>e.g. enteric coating</td>
</tr>
<tr>
<td><strong>Drug-drug Interactions</strong></td>
<td><strong>Decreased metabolism</strong></td>
</tr>
<tr>
<td></td>
<td>• Competitive inhibition of enzymes</td>
</tr>
<tr>
<td></td>
<td>e.g. reduced biotransformation of clopidogrel</td>
</tr>
<tr>
<td></td>
<td><strong>Steric hindrance</strong></td>
</tr>
<tr>
<td></td>
<td>• NSAIDs and aspirin</td>
</tr>
<tr>
<td><strong>Indirect mechanisms</strong></td>
<td><strong>• Overactive alternative pathways</strong></td>
</tr>
<tr>
<td></td>
<td><strong>• Rapid platelet turnover</strong></td>
</tr>
</tbody>
</table>
The ISAR-CHOICE study identified intestinal absorption as another factor limiting clopidogrel efficacy. (65) The intestinal P-glycoprotein efflux transporter, which has recently been implicated as an important pathway in clopidogrel absorption, is involved in a number of well recognised drug interactions. (66)

**Pharmacodynamics and pharmacogenomics**

Co-administered medications can alter the pharmacodynamics of antiplatelet agents. Concurrent use of some non-steroidal anti-inflammatory drugs including ibuprofen, naproxen, tiaprofenic acid and indomethacin may block the antiplatelet effect of aspirin by steric hindrance at the COX-1 receptor site. (67-70)

The interaction of a drug with its target site is also a point at which “resistance” may occur, an effect influenced more by an individual’s response rather than by the properties of the drug. Since receptors are coded by genes, pharmacogenetic testing is a potential method for estimating drug response. Using a candidate gene approach, studies have implicated a number of polymorphisms associated with aspirin resistance including a haplotype within the cyclo-oxygenase I enzyme, (71) and within the ADP receptor P2Y1. (72) As with most genetic analysis to date, these results need validating in other cohorts. (73) A recent longitudinal study of the CATHGEN cohort (CATHeterization GENetics, n=3,449) assessed multiple SNPs associated with aspirin resistance and showed no association between these SNPs and clinical events over 3.5 years. (74) Non-hypothesis driven genome wide association studies, in larger cohorts, may reveal polymorphisms in other genes of interest.

The site of action of clopidogrel, the P2Y12 receptor gene was the original target for pharmacogenetic studies. (75) A polymorphism of the P2Y12 gene, denoted haplotype H2, has been associated with increased platelet responsiveness to ADP, but does not appear to influence the clinical response to clopidogrel. (76) Further attention has focused on the metabolic activation of clopidogrel and possible genetic influences. Sequence variations within the hepatic CYP2C19 gene (CYP2C19*2, CYP2C19*3) have shown to associate with those who do not respond to the drug. (62, 77, 78). These variants although accounting for only 12% of the variability in response to clopidogrel are associated with a two-fold risk of cardiovascular death and three-fold risk of stent thrombosis in those taking clopidogrel. (79) This finding is supported by the result of three meta-analyses and has led to a black box warning
from the FDA on the drug.(79-81) A common polymorphism of the p-glycoprotein gene (ABCB1 aka MDR1 C3435T genotype) has been shown to influence the intestinal absorption of clopidogrel.(82) In addition this polymorphism has also been implicated with clinical non-response to clopidogrel.(83, 84)

It is unlikely that a single gene will be found that accounts for all aspirin or clopidogrel non-responders. The cause of antiplatelet drug resistance is multifactorial and influenced by both environmental and genetic factors. An advantage of using pharmacogenetics as opposed to platelet testing to individualise antiplatelet treatment is the availability of other relevant data within the genome. An example includes the 9p21.3 variant discussed in Chapter 12 has been associated with premature atherosclerosis, myocardial infarction and has also been associated with platelet reactivity.(85) Integrating this SNP into a decision pathway regarding antiplatelet treatment would take therapy beyond pharmacogenetics into preventative genomics. Studies have shown incremental value of combining multiple gene variants for drug metabolism and clinical factors to predict outcomes for individual patients.(84)

Translation of pharmacogenetics into clinical practice will require a number of factors to coincide. These include an increased availability of genotyping technology, lower cost of genotyping and studies to support testing, with clinical outcome data. A number of rapid low cost genotyping platforms are becoming available with the current FDA approved Verigene analyser being one example.(86) Other point-of-care genotyping platforms such as the SPARTAN Rx(87) and the handheld semiconductor minisequencing system from DNA electronics will improve the feasibility of integrating pharmacogenomics into clinical practice.

**Clinical importance**

1. **Aspirin non-response**

Stable coronary artery disease

Antiplatelet drug non-response appears to be clinically relevant in patients with stable coronary disease. An early study of aspirin non-response in 326 patients with stable cardiovascular disease found that 5.2 percent were resistant to aspirin, as measured by the PFA-100. At two years of follow up non-responders had a four-fold increase in
the incidence of death, MI or stroke.\(^{(10)}\) The PROSPECTAR study of patients with stable coronary disease, also using the PFA-100, reported a higher prevalence of aspirin non-responders (22 percent). The non-responder group had a higher rate of major adverse cardiac events over 21 months, although the difference between groups was not statistically significant.\(^{(88)}\)

The HOPE trial, of stable patients with coronary disease, used a high urinary 11-dehydro thromboxane B2 as a marker of aspirin nonresponse. After adjustment for baseline differences, the odds for major adverse cardiovascular events were increased with each increasing quartile of 11-dehydro thromboxane B2. Those in the highest quartile had a twofold higher risk of MI and a three and a half fold higher risk of cardiovascular death than those in the lowest quartile.\(^{(23)}\) A limitation of this study was that it was a post-hoc analysis and there was no rigorous assessment of drug non-compliance. However this finding has been reinforced in a substudy of the CHARISMA trial, a trial of stable patients with coronary disease. Urinary 11-dehydro thromboxane B2 in the highest quartile was associated with an increased risk for stroke, MI or cardiovascular death.\(^{(89)}\)

A study using the VerifyNow device found that aspirin non-response was present in 27 percent of the study population, and associated with a threefold increase in the risk of composite cardiovascular endpoint at one year.\(^{(90)}\) A number of meta-analyses have confirmed an association between platelet testing and clinical outcomes. In a meta-analysis of 6,450 patients the prevalence of aspirin non-responders was significantly higher in populations with vascular events.\(^{(91)}\)

Percutaneous coronary intervention

Aspirin non-response also appears important in patients undergoing elective percutaneous coronary intervention (PCI). Breet et al assessed aspirin response in 951 patients undergoing elective PCI and showed a higher event rate in those with high on treatment platelet reactivity. Furthermore in comparing a number of platelet testing platforms the VerifyNow platelet testing assay had the highest predictive ability for events.\(^{(41)}\)

Acute coronary syndromes

A number of studies have evaluated platelet function in patients presenting with an ACS. In a study of 151 patients with ACS undergoing PCI, all of whom received a
300mg clopidogrel loading dose, 19 percent were non-responders to aspirin, as assessed using the VerifyNow aspirin assay. The incidence of CK-MB or troponin I elevation post-PCI was significantly higher in aspirin-resistant than aspirin-sensitive patients.(92) Another study using the PFA-100 in 146 patients undergoing primary PCI for ST elevation myocardial infarction found a similar result.(93) More patients with major adverse cardiovascular events had aspirin non-response (39 percent versus 23 percent with a normal response, p <0.05).

In 216 patients with STEMI, enhanced platelet function under high shear stress, assessed with the PFA-100, was an independent predictor of elevated biomarkers for cardiac necrosis.(94) The STRATEGY study investigated this further in 70 STEMI patients undergoing PCI.(95) Platelet function assessed prior to intervention, using an PFA-100 ADP agonist, predicted the response to GP IIb/IIIa inhibition and long-term outcome. At one year, patients with an abnormal PFA-100 ADP time showed an adjusted five to 11-fold increase in the risk of death, reinfarction, and target vessel revascularization.(95) Further support comes from another trial in 153 patients using the PFA-100, finding that the closure time, measured after PCI, was independently associated with a higher rate of death or MI.(96)

In contrast, another study investigated aspirin non-response and long term outcome in 187 individuals with suspected acute MI.(97). Although no association was seen between aspirin non-response and adverse outcomes, only 26 percent of patients actually had an acute coronary syndrome. It is of interest that the platelet response in those with MI differed dramatically over time; platelets displayed hyper-aggregation at the time of presentation compared with later time points. Hence aspirin non-response at the time of MI may reflect the acute clinical state, rather than an individual difference in drug response.(97)

Indirect methods of measuring platelet activity also predict outcomes in patients with acute coronary syndromes. In one study partial inhibition of TXA₂ by ASA was found in 34 percent of patients and was associated with significant increases in serum troponin T, creatine kinase and creatine kinase-MB, compared with patients in whom TXA₂ production was blocked.(98)
2. Clopidogrel non-response

Several studies have investigated the importance of platelet reactivity after clopidogrel dosing. These studies are mostly in the context of coronary intervention with platelet function measured either before or after PCI (Table 3).

A key issue in clopidogrel non-response is the timing and dosing of the drug, particularly in those undergoing PCI and not pre-treated with clopidogrel. Two trials have shown that a 600mg loading dose, compared with a standard 300mg dose, is associated with more rapid and complete platelet inhibition and with reduced post-PCI myonecrosis.(99, 100) The CURRENT/OASIS 7 has addressed this in a 25,000 patient study of subjects with both STEMI and NSTEMI, many undergoing PCI.(101) Although increasing the dose of a drug for all appears to be a logical step to reduce the prevalence of non-responders this approach ignores the concerns of over treating hyper-responders. The double edged sword of antiplatelet treatment is thrombosis risk versus bleeding risk, with bleeding risk potentially limiting the approach of “one-size-fits all” medicine in the future.

An alternative strategy to a dose increase for all-comers is the use of a metric for non-response such as platelet function. Several studies have evaluated the Multiplate analyser and shown that high post treatment platelet reactivity correlates with poor clinical outcomes.(102-104) Emerging evidence suggests that those outcomes can be modified by changing treatment in non-responders.(105)

Table 3. Clopidogrel response studies in PCI

<table>
<thead>
<tr>
<th>Reference</th>
<th>No</th>
<th>Patient population</th>
<th>Platelet analyser</th>
<th>Prevalence of Clopidogrel non-response</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gurbel et al.(106)</td>
<td>192</td>
<td>81% with stable CAD</td>
<td>-</td>
<td>-</td>
<td>Higher post treatment ADP induced LTA and clot strength by TEG in patients with CV events</td>
</tr>
<tr>
<td>Gurbel et al.(107)</td>
<td>120</td>
<td>Case (SAT) vs control</td>
<td>-</td>
<td>-</td>
<td>Patients with subacute stent thrombosis (SAT) had</td>
</tr>
<tr>
<td>Study</td>
<td>n</td>
<td>CAD Status</td>
<td>Platelet Reactivity Measure</td>
<td>Platelet Reactivity</td>
<td>Results</td>
</tr>
<tr>
<td>-------</td>
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</tr>
<tr>
<td>Geisler et al. (108)</td>
<td>379</td>
<td>54% with stable CAD</td>
<td>Platelet inhibition &lt; 30% (20umol/L ADP LTA)</td>
<td>5.8%</td>
<td>Higher mean platelet reactivity than those without SAT</td>
</tr>
<tr>
<td>Lev et al. (9)</td>
<td>150</td>
<td>Elective PCI</td>
<td>Baseline minus post-treatment aggregation &lt; or = 10 in response to 5 and 20 umol/l ADP (LTA)</td>
<td>24%</td>
<td>Higher creatine kinase levels after coronary stenting in non-responsive patients</td>
</tr>
<tr>
<td>Cuisset et al. (99)</td>
<td>292</td>
<td>ACS patients RCT (300mg vs 600mg clopidogrel)</td>
<td>10 umol/L ADP (LTA) induced platelet aggregation &gt;70%</td>
<td>15% in 600mg group, 25% in 300mg group</td>
<td>Lower CV event rate in 600mg group, at 30 days</td>
</tr>
<tr>
<td>Hochholzer et al. (109)</td>
<td>802</td>
<td>Elective PCI</td>
<td>Quartiles of LTA platelet response to 5umol/L ADP</td>
<td>-</td>
<td>Platelet aggregation before elective stenting in patients pre-treated with clopidogrel correlates with early CV outcomes</td>
</tr>
<tr>
<td>Bliden et al. (110)</td>
<td>100</td>
<td>Elective PCI</td>
<td>-</td>
<td>-</td>
<td>High platelet reactivity measured by LTA and TEG is associated with increased CV events</td>
</tr>
<tr>
<td>Buonamici et al. (111)</td>
<td>804</td>
<td>34% with stable CAD</td>
<td>10 umol/L ADP ≥70% (LTA)</td>
<td>13%</td>
<td>HR 3.08 for stent thrombosis (acute, subacute or late) in non-responders</td>
</tr>
<tr>
<td>Geisler et al. (112)</td>
<td>1,019</td>
<td>50% with ACS</td>
<td>LTA</td>
<td>-</td>
<td>Increased ST at 3 months</td>
</tr>
<tr>
<td>Siller-Matula et al. (113)</td>
<td>416</td>
<td>9% undergoing elective PCI</td>
<td>Multiplate analyser</td>
<td>-</td>
<td>Increased ST at 6 months</td>
</tr>
</tbody>
</table>
Management of antiplatelet drug non-response

Due to a lack of randomised controlled clinical trials there is only emerging evidence on how to manage patients with antiplatelet non-response. Empiric strategies include increasing the dose of the antiplatelet agent, adding a second or third antiplatelet drug or switching to a more potent agent.

There is some evidence that aspirin response may be dose dependent. (30) Meta-analysis of the randomised clinical trials indicates that, across the study populations, the most effective aspirin dose with the fewest adverse consequences is 75-150mg once daily. (4, 114) However it is possible that in these large trials a small cohort of patients might have benefited from a higher aspirin dosage. The response to aspirin may decline over time due to tachyphylaxis meaning that response and treatment do not remain static. (115)

Once the problems related to measurement and definitions are overcome, clinical trials will be needed to develop and validate algorithms guiding optimal antiplatelet treatment. The Gauging Responsiveness with A VerifyNow assay—Impact on Thrombosis And Safety (GRAVITAS) study has addressed the issue of whether an increase in the clopidogrel maintenance dose reduced events in clopidogrel non-responders. (116) The trial did not show a benefit in individualising treatment, however the patients selected had mostly stable coronary disease (84% without troponin elevation), were low-risk and criticism has been drawn to the use of platelet function analysis and choice of clopidogrel dose. (117)

Newer drugs may overcome the limitations of current antiplatelet drugs. Prasugrel is a third generation thienopyridine that is not as dependent as clopidogrel on biotransformation to an active metabolite. In preclinical studies it was shown to have greater potency and achieve more rapid platelet inhibition than clopidogrel, when given orally. (118) The Joint Utilization of Medications to Block Platelets Optimally-Thrombolysis In Myocardial Infarction 26 (JUMBO-TIMI) trial found prasugrel to have a comparable safety profile to clopidogrel. (119) However the TRial to assess Improvement in Therapeutic Outcomes by optimizing platelet InhibitioN with
prasugrel Thrombolysis In Myocardial Infarction 38 (TRITON-TIMI 38) trial had a number of issues. Firstly, prasugrel was found to reduce ischemic events in an acute coronary syndrome population undergoing PCI, but at the cost of increased major bleeding.(15) Those assigned to clopidogrel received only a 300mg loading dose immediately prior to or during PCI; whereas 600mg is now more commonly-used.(100) Although this raised the question of dose equivalence, platelet function analysis in Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation—Thrombolysis in Myocardial Infarction 44 Trial (PRINCIPLE-TIMI 44) has shown that the dose of prasugrel used in TRITON leads to greater platelet inhibition than clopidogrel at the higher loading and maintenance doses.(120) Subgroup analysis of TRITON suggested prasugrel may have the greatest benefit over clopidogrel in the highest risk patients, such those with diabetes.

In the PLATelet Inhibition and Patient Outcomes (PLATO) study the reversible P2Y12 inhibitor ticagrelor was compared to clopidogrel in ACS patients undergoing PCI. Ticagrelor was more effective in reducing a composite endpoint than clopidogrel and represents an alternative to clopidogrel therapy. Treatment with ticagrelor may be a preferable strategy in patients who carry either the CYP2C19*2 or ABCB1 functional variants(121). In the PLATO study ticagrelor caused a significantly increased risk of non-CABG bleeding. Therefore exposure to the drug logically should be limited to those who with the most to gain from treatment. In addition ticagrelor has two significant drawbacks. Firstly it is a twice daily tablet with potential compliance issues. Secondly the medication is known to cause dyspnoea in a minority of patients. This is likely to indirectly increase the cost of use through unnecessary investigations.(122) An intravenous P2Y12 ADP receptor antagonist elinogrel has passed pilot studies and may in the future provide yet another alternative treatment in the acute setting.(123)

Vorapazar, a PAR-1, thrombin receptor antagonist, has been used in a triple antiplatelet regimen in patients with ACS in the TRA2P study. A reduction in ischaemic events was seen in patients receiving the drug compared to dual antiplatelet therapy. However despite original claims that this drug would not increase bleeding in those receiving triple therapy, a significant increase in intracranial haemorrhage was seen in the vorapaxar arm of the trial.(124)

Novel anticoagulants used in ACS are also worth considering in the context of antiplatelet drug non-response. Although not direct platelet inhibitors their impact on
factor Xa and thrombin inhibition have an influence on the clotting and platelet activation cascade. Rivaroxaban, a novel anti-Xa inhibitor, has been shown to be effective in reducing cardiovascular events in patients with ACS in both the ATLAS I and ATLAS II trials.(125, 126). Although the addition of rivaroxaban to dual antiplatelet therapy was superior to dual antiplatelet therapy alone it would seem logical that greater efficacy might be seen in non-responders to antiplatelet treatment. Prescreening trial participants in novel triple antiplatelet or anticoagulant trials for genetic non-responders has been proposed as a method for reducing the cost of megatrials.(127)

Alternatively, a future approach may be individualised antiplatelet therapy based on platelet function testing or pharmacogenetic profiling. This may be preferable to the above approaches of adding on treatments, which usually involves costly patented medications. Providing platelet or genetic testing is low cost and clinically feasible individualising treatment would rationalise the use of traditional medicine, and cost-effectively target novel treatments.(128) Emerging evidence is pointing to the possibility of personalised gene-based dosing of clopidogrel.(87, 129) Implementing personalised medicine in a large clinical trial would be complex given the multiple trial arms required, especially if an n=1 approach is taken. Therefore its application may preferably be applied in a registry, or real-world setting with historic controls.(130)

**Conclusion**

There is surprisingly limited published information on optimal dosages and combinations of antiplatelet agents for those with documented antiplatelet non-response, the definition of non-response also remains unclear. Whether clinical outcomes can be improved by platelet function testing and individualising treatment is uncertain. Although using higher doses of clopidogrel and glycoprotein IIb/IIIa inhibitors in non-responders seems logical it remains unproven whether these treatment strategies will further reduce thrombotic events, without increasing the risk of bleeding. Chapters Five to Seven, in this thesis, attempt to address this issue with clopidogrel therapy. Identifying those with a suboptimal response to antiplatelet drugs may influence other aspects of management such as the choice of a drug eluting or bare metal stent for percutaneous revascularisation.

Adjusting antiplatelet drug therapy on the basis of individual response is an appealing proposition but is not presently evidence based. There is a need for antiplatelet drugs
with rapid onset of effect and with a predictable degree of platelet inhibition in the population, as well as reversible agents for patients going to surgery. However whether we can afford these medicines will be a point of contention in the future. The issues of cost are discussed in Chapter Eight and Appendix A. An individualised approach to treatment theoretically may reduce the adverse consequences of antiplatelet therapy, allow more cost-effective use of expensive medication, and improve patient outcomes.
Chapter 2: The antiplatelet effect of six non-steroidal anti-inflammatory drugs and their pharmacodynamic interaction with aspirin in healthy volunteers

Chapter One reviewed the concept of drug “resistance” or non-response to antiplatelet medication and explored the possible causes. Chapter Two describes a clinical trial with the aim of identifying whether drug-drug interactions exist between aspirin and commonly used nonsteroidal anti-inflammatory drugs (NSAIDs). This has significant clinical implications, as both aspirin and NSAIDs are taken by a large proportion of the population. In addition many NSAIDs are now also available without a prescription (over-the-counter) and are widely available to patients with coronary disease, who are taking aspirin for secondary prevention.

Introduction

Patients with cardiovascular disease taking aspirin and some non-steroidal anti-inflammatory drugs (NSAIDs) appear to have increased vascular events. A recent meta-analysis of randomised trials found that COX-2 specific NSAIDs, ibuprofen and diclofenac, but not naproxen, were associated with an increased risk for vascular events, particularly myocardial infarction. (131) Similar findings have been reported in observational and case control studies of nonspecific NSAIDs. (132) One potential mechanism for this is a pharmacodynamic interaction between NSAIDs and aspirin through steric hindrance at the active site of COX-1. This prevents irreversible platelet inhibition by blocking the drug from the enzyme receptor. This has been suggested in some, (67, 68) but not all, (133) studies evaluating ibuprofen, and might also occur with indomethacin (69) and naproxen. (70) Various in vitro and ex vivo tests have been used but there are no standardised comparisons between NSAIDs.

Methods

The study protocol was approved by the Northern Regional Ethics Committee of New Zealand; all subjects gave written, informed consent. The haematocrit, platelet count, creatinine and von Willebrand factor were measured at baseline. Exclusion criteria included a history of cardiovascular disease, bleeding, gastrointestinal ulceration, allergy to aspirin or NSAID, renal impairment, anaemia, or thrombocytopenia.
Subjects abstained from medications or herbal supplements that might affect platelet function during the study.

24 healthy volunteers, mostly nurses, were randomly allocated into one of two groups of 12 in a series cross-over study. Six NSAIDs were evaluated in a double blind fashion; those in each group took three NSAIDs and placebo, in random order. The NSAIDs, per dose, were naproxen 550 mg (Synflex®, Roche), ibuprofen 400 mg (Brufen®, Knoll) celecoxib 200mg (Celebrex®, Pharmacia), indomethacin 25mg (Rheumacin 25®, Pacific), tiaprofenic acid 300mg (Surgam® SA, Aventis), sulindac 200 mg (Daclin®, Pacific), aspirin 300 mg (Solprin®, Reckitt Benckiser) and placebo (Pacific Pharmaceuticals). All were standard preparations, apart from slow-release tiaprofenic acid.

On Day one blood was taken for platelet function testing (Platelet Function Analyser-100® [PFA-100], Dade Behring Ltd, Illinois). Each individual was then given two doses of NSAID, 12 hours apart. The PFA-100 was measured on Day Two, 12 hours after the second NSAID dose. Subjects then received a final dose of NSAID and two hours later 300mg soluble aspirin. The PFA-100 was repeated on Day Three, 24 hours after the last NSAID dose (Figure 2). The cycle was repeated after a 12 day washout.
Figure 2. Study outline of aspirin, non-steroidal anti-inflammatory study

Ex-vivo platelet function was measured using the high shear PFA-100 epinephrine (CEPI) cartridge at the same time of the day, at rest, in all subjects. “Aspirin response” was defined as a closure time greater than 178 seconds, which was the weighted average threshold from seven studies in which it was evaluated (range greater than 142 to 196 seconds). (28-34) Aspirin non-response was defined as an increase of less than 20 seconds in the PFA-100 closure time after 300 mg aspirin, compared with baseline, and partial response was defined as an increase of greater than 20 seconds but less than 178 seconds after aspirin. Because the PFA-100 has an upper threshold of 300 seconds the data were not normally distributed. The Wilcoxon Rank test was used to compare treatment groups. For group means, values greater than 300 seconds were counted as 300 seconds. Based upon previous studies, it was calculated that a sample size of 18 (nine in each group) would afford a power greater than 90 percent to detect a difference between groups of greater than 20 percent in PFA-100 measurements, with a type I error rate of <0.05. (28-34) The study population was increased to 24 subjects to adjust for an estimated 25 percent aspirin non-response rate.
Results

The study subjects were aged 23–67 years (mean 38 years); 14 were female. Two were replaced at the start; one developed ataxia with the first NSAID dose (indomethacin) and the other had poor venous access. There were two aspirin non-responders and four partial-responders; they had higher levels of von Willebrand factor (vWF) than other subjects (188+/−77 versus 90+/−42, p=0.01). No subjects had depressed vWF levels.

The effect of each NSAID on the PFA-100 closure time is depicted in Table 4 and Figure 3. Naproxen and slow-release tiaprofenic acid significantly prolonged the closure time at the end of a 12 hour dosing interval. The closure time 24 hours after a NSAID dose followed two hours later by aspirin was significantly reduced by ibuprofen, indomethacin, tiaprofenic acid and naproxen, compared with placebo (Table and Figure 4). Neither sulindac nor celecoxib significantly reduced the closure time nor reduced the antiplatelet effect of aspirin. Comparing those NSAIDs which inhibited aspirin, the Day three closure time was lower with ibuprofen than with naproxen (p= 0.004).
Figure 3. PFA closure time on day one (baseline) and day two (12 hours post-NSAID dose).

Closed symbols are aspirin responders and open symbols aspirin poor-responders.
Figure 4. PFA closure time on day three, 24 hours after the last NSAID dose and 22 hours after aspirin 300 mg.

Closed symbols are aspirin responders and open symbols aspirin poor-responders.
Table 4. PFA-100 closure time - seconds (95% confidence intervals)

<table>
<thead>
<tr>
<th>Time</th>
<th>Placebo</th>
<th>Naproxen</th>
<th>Ibuprofen</th>
<th>Celecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>133±37</td>
<td>131±33</td>
<td>125±32</td>
<td>123±22</td>
</tr>
<tr>
<td>Day 2</td>
<td>126±20</td>
<td>252±73</td>
<td>131±35</td>
<td>126±35</td>
</tr>
<tr>
<td>Day 3</td>
<td>254±84</td>
<td>204±77</td>
<td>133±28</td>
<td>258±64</td>
</tr>
<tr>
<td>Day 2 vs Day 1 difference</td>
<td>(-27,12)</td>
<td>(81,162)</td>
<td>(-2,13)</td>
<td>(-19,27)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.42</td>
<td>&lt;0.0001</td>
<td>0.12</td>
<td>0.82</td>
</tr>
<tr>
<td>Day 3, NSAID vs placebo difference</td>
<td>-</td>
<td>(-94,-1)</td>
<td>(-153,-73)</td>
<td>(-38,69)</td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>0.04</td>
<td>0.001</td>
<td>&gt;0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Placebo</th>
<th>Indomethacin</th>
<th>Tiaprofenic acid</th>
<th>Sulindac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>126±31</td>
<td>118±29</td>
<td>120±35</td>
<td>127±44</td>
</tr>
<tr>
<td>Day 2</td>
<td>128±63</td>
<td>168±72</td>
<td>225±85</td>
<td>142±59</td>
</tr>
<tr>
<td>Day 3</td>
<td>250±79</td>
<td>164±53</td>
<td>173±61</td>
<td>234±73</td>
</tr>
<tr>
<td>Day 2 vs Day 1 difference</td>
<td>(-45,47)</td>
<td>(-4,102)</td>
<td>(67,144)</td>
<td>(-4,38)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.13</td>
<td>0.07</td>
<td>&lt;0.001</td>
<td>0.10</td>
</tr>
<tr>
<td>Day 3, NSAID vs placebo difference</td>
<td>-</td>
<td>(-129,-29)</td>
<td>(-125,-20)</td>
<td>(-49,7)</td>
</tr>
<tr>
<td>P-value</td>
<td>-</td>
<td>&lt;0.001</td>
<td>0.007</td>
<td>0.38</td>
</tr>
</tbody>
</table>

**Discussion**

The non-selective NSAIDs ibuprofen, indomethacin, tiaprofenic acid and naproxen all antagonise the antiplatelet effect of aspirin, as assessed by a high-shear ex-vivo platelet function analyser. This may, in part, explain findings from the primary
prevention US Physicians’ Health Study; those taking NSAIDs more than 60 days per year in addition to aspirin had a 2.8 fold increased risk of myocardial infarction.(134) Many are at risk for this pharmacodynamic interaction. In a Danish study of patients followed after a first myocardial infarction, 36 percent filled at least one prescription for an NSAID and more took low-dose ibuprofen, which is available over-the-counter in Denmark.(135)

Naproxen and slow release tiaprofenic acid may be cardioprotective if taken regularly, but hazardous if taken intermittently with aspirin. Our findings are consistent with those of Capone and colleagues who demonstrated that aspirin, naproxen twice daily, or both drugs given together reduced thromboxane B₂ levels at 24 hours to a similar extent.(136)

Ibuprofen and indomethacin do not have an antiplatelet effect at the end of a 12 hour dosing interval however submaximal doses were given in this study. These two drugs may be particularly hazardous when given with aspirin. The finding that ibuprofen inhibited aspirin to a greater extent than did naproxen is consistent with limited trial data. This occurs even with lower doses of ibuprofen, as used in this study. Subset analysis of patients taking aspirin in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET) study of NSAIDs for osteoarthritis found those on ibuprofen had more cardiovascular events than those on naproxen.(137)

Sulindac and celecoxib had no significant effect on the closure time, and did not reduce the prolongation with aspirin. Of the NSAIDs evaluated, it may be the drug of choice for patients requiring both aspirin and a NSAID; the safety and efficacy of the combination requires evaluation in appropriate clinical trials. Despite in vitro data to the contrary,(138) celecoxib, the least COX-2 specific coxib, did not block the effect of aspirin on the closure time, a finding consistent with a study of celecoxib co-administered with aspirin.(139)

How long does the gap between NSAID and aspirin administration need to be, to avoid this pharmacodynamic interaction? Ibuprofen, despite a short 2.2 hour half-life, inhibited an aspirin dose taken 12 hours later.(68) Similarly, coadministration of naproxen with aspirin antagonised the inhibition of thromboxane B₂ at one hour, despite naproxen alone having no measurable effect on COX-1 at that time.(70)
Conclusion

Taken together, it appears that NSAIDs may antagonise aspirin at blood levels considerably lower than those inhibiting platelet COX-1. Other interactions between aspirin and concomitant medications may also be important. This is explored further in the next chapter.
Chapter 3. The antiplatelet effect of commonly-used herbal medications and their interaction with aspirin: a randomised, placebo-controlled crossover trial

Chapter Two examined the interaction between aspirin and commonly used nonsteroidal anti-inflammatory medication. Herbal medications are frequently used by the general population, including those with cardiovascular disease taking aspirin. This chapter describes a randomised, placebo-controlled, crossover trial in 12 healthy volunteers to assess whether garlic, ginger, ginkgo, turmeric or ginseng exhibit an antiplatelet effect or interact with aspirin. The trial methodology used in this study is similar to that used in Chapter Two.

Introduction

Herbal medicinal products are regularly taken by at least 20 percent of the population. (140) Their use is higher in those aged 40-64 years, women, and those in higher socio-economic groups. Only one third discuss use of these medications with their physician. A number of commonly-taken herbal medications are thought to have cardiovascular benefits, including an antiplatelet effect. (140)

Aspirin is widely taken for the primary and secondary prevention of vascular disease. Aspirin irreversibly acetylates platelet cyclo-oxygenase 1 (COX-1). The interaction of aspirin with the COX-1 binding site may be blocked by other non-steroidal anti-inflammatory drugs, leading to only temporary impairment of platelet function. In Chapter Two it was shown that ibuprofen, indomethacin, tiaprofenic acid, and naproxen all inhibit the antiplatelet effect of aspirin, as determined by various measures of platelet function, including high shear, point-of-care platelet function analysers. (67-70, 138, 141) A reversible binding of herbal medication constituents with COX-1 has the potential to block the irreversible antiplatelet effect of aspirin.

Herbal medications are not well regulated or subject to the same scrutiny as prescription drugs, with little rigorous data on possible antithrombotic and antiplatelet benefits. This study assessed the ex-vivo antiplatelet effect of commonly-used herbal medications, and their potential to interact with aspirin.
**Methods**

**Design overview**

Platelet function was assessed using the point-of-care Platelet Function Analyser-100® [PFA-100] epinephrine (CEPI) cartridge (Dade Behring Ltd, Illinois, USA). This assay measures the time to closure of an aperture through which blood is entrained. Time to closure of greater than 300 secs is not measured. After a baseline PFA-100 test, each study subject took a herbal medication (or placebo) twice daily for four days. The PFA-100 was repeated on day five, 12 hours after the last herbal dose. Each subject then received a final dose of herbal supplement and two hours later 300 mg soluble aspirin. The PFA-100 was repeated on day six, 24 hours after the last herbal medication dose and 22 hours after aspirin. The cycle was repeated with another herbal medication after a 15-day washout.

**Setting and participants**

The study was undertaken at Auckland City Hospital. Study subjects were normal healthy volunteers recruited from hospital staff. Specifically excluded were those with a history of cardiovascular disease, bleeding, gastrointestinal ulceration, allergy to aspirin or herbal medications, renal impairment or anaemia. The haematocrit, platelet count, creatinine and von Willebrand factor were measured at baseline. Those with aspirin resistance, defined as a PFA-100 closure time of less than 178 secs, two hours after a dose of aspirin 300 mg, were also excluded. Subjects abstained from medications or herbal supplements that might affect platelet function during the study. The study protocol was approved by the Northern Regional Ethics Committee of New Zealand; all participants gave written, informed consent.

**Randomisation and interventions**

Twelve healthy subjects were assigned to the following herbal medicinal products or to placebo, in random order: garlic (Kordels odourless garlic, Pantech Industrial Complex, Singapore) 10 g, one twice daily; ginger (Lifestream Bioactive ginger, Lifestream International Ltd, Auckland, New Zealand) 800 mg, two twice daily; ginkgo (Kordels ginkgo) 2500 mg, one twice daily; turmeric (Nature’s Way turmeric, Nature’s Way Products, Inc, Springville, Utah, USA) 450 mg, two twice daily; ginseng (Nutralife Korean Ginseng, Nutralife Health & Fitness Ltd, Auckland, New Zealand) 2500 mg, one twice daily. The herbal medication or placebo capsules were
repackaged in identical envelopes before being given to each participant in a double blind manner. The capsules used were not identical to placebo. Randomisation of treatment order was by computer-generated pseudo-random number.

**Outcomes**

The primary study outcomes were:

1. The change in PFA-100 closure time on day five, 12 hours post-dose, comparing each herbal medication with baseline, and
2. The PFA-100 closure time on day six, 22 hours following aspirin, comparing each herbal medication with placebo.

**Statistical methods**

Nonparametric method (descriptive statistics of median and range) was used to describe the PFA-100 closure time. The Wilcoxon rank-sum test was used to assess if there was a statistically significant difference in PFA-100 closure time after five days of herbal medication with baseline, and the PFA-100 closure time on day six, 22 hours following aspirin, comparing each herbal medication with placebo (unadjusted and adjusted by baseline). Analysis was performed by using SAS statistical package, version 9.1.3 (SAS Institute, Cary, NC). Differences were considered statistically significant at $P <0.05$.

**Results**

The PFA closure times before and after each of the herbal medications are depicted in Table 5. None of the herbal medications had an antiplatelet effect detectable by this methodology 12 hours post-dose. There was no evidence that any of the herbal medications reduced the antiplatelet effect of aspirin (Figure 5).
### Table 5. Platelet Function Analyzer 100 closure time (seconds; median & inter-quartiles)

<table>
<thead>
<tr>
<th>Time</th>
<th>Placebo</th>
<th>Garlic</th>
<th>Ginger</th>
<th>Gingko</th>
<th>Tumeric</th>
<th>Ginseng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>115 (103, 128)</td>
<td>133 (97,159)</td>
<td>113 (101, 189)</td>
<td>117 (110, 142)</td>
<td>121 (92, 133)</td>
<td>145 (108, 181)</td>
</tr>
<tr>
<td>After 4 days of herb</td>
<td>114 (103, 130)</td>
<td>127 (90, 145)</td>
<td>127 (134, 143)</td>
<td>119 (105, 133)</td>
<td>106 (92, 127)</td>
<td>121 (109, 139)</td>
</tr>
<tr>
<td>22 hours after herb + Aspirin</td>
<td>300 (166, 300)</td>
<td>300 (231, 300)</td>
<td>300 (300, 300)</td>
<td>300 (165, 300)</td>
<td>300 (130, 300)</td>
<td>300 (188, 300)</td>
</tr>
<tr>
<td>After 4 days of herb vs Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>difference</td>
<td>-7 (-24, 51)</td>
<td>-4 (-23, 3)</td>
<td>20 (-18, 31)</td>
<td>-6.5 (-36, 11)</td>
<td>-4 (-21, 21)</td>
<td>-21 (-63, 10)</td>
</tr>
<tr>
<td>p-value*</td>
<td>0.8105</td>
<td>0.4453</td>
<td>0.4258</td>
<td>0.3145</td>
<td>0.8311</td>
<td>0.1699</td>
</tr>
<tr>
<td>22 hours after herb + Aspirin (Herb vs placebo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>difference adjusted by baseline</td>
<td>-</td>
<td>-11 (-70, 25)</td>
<td>-11 (-111, 146)</td>
<td>4 (-76, 25)</td>
<td>2 (-115, 77)</td>
<td>-37 (-152, 23)</td>
</tr>
<tr>
<td>p-value*</td>
<td>-</td>
<td>0.6221</td>
<td>0.7646</td>
<td>0.7461</td>
<td>0.8501</td>
<td>0.2402</td>
</tr>
<tr>
<td>difference not adjusted by baseline</td>
<td>-</td>
<td>0 (0, 33)</td>
<td>0 (0, 134)</td>
<td>0 (0, 0)</td>
<td>0 (-142, 66)</td>
<td>0 (-52, 31)</td>
</tr>
<tr>
<td>p-value*</td>
<td></td>
<td>1.0000</td>
<td>0.1563</td>
<td>0.6250</td>
<td>0.5469</td>
<td>0.8438</td>
</tr>
</tbody>
</table>

* Signed Rank
Figure 5. Platelet Function Analysis (PFA-100) for each group following herb, herb then aspirin and aspirin alone

Note that some data points are missing due to technical errors with the platelet analyser.
Discussion

None of the five herbal medicinal products evaluated had an effect on ex vivo platelet function, as assessed by the high shear stress PFA-100 point-of-care analyser. The herbal medications evaluated were chosen from those available as most likely to have a clinically-relevant antiplatelet effect. A strength of this study is that the herbal supplements were compared in a standardised manner.

In addition, none impaired the antiplatelet effect of aspirin. An interaction between herbal medication and aspirin might occur in the absence of a detectable antiplatelet effect with the herbal medication itself. Some non-steroidal anti-inflammatory drugs, which reversibly bind to COX-1 and have only a transient antiplatelet effect, are very effective at blocking the irreversible acetylation of platelet COX-1 by aspirin. This interaction has been demonstrated ex vivo in Chapter Two with ibuprofen, indomethacin, tiaprofenic acid and naproxen, using the PFA-100 analyser. Multiple case control series and analyses from randomised trials indicate that this drug interaction is clinically important. (68, 70, 141-144)

Although antiplatelet effects are claimed with a number of herbal medicinal products, many are in vitro studies, and the results are often conflicting. An overview of trials evaluating the effects of garlic on cardiovascular risk factors concluded that garlic significantly reduced platelet aggregation. (145) Sodium 2-propenyl thiosulphate from garlic has been shown to inhibit COX activity(146) and raw garlic appeared a more potent inhibitor of COX-1 than cooked garlic. (147) Some constituents of ginger, especially [8]-Paradol, appear to be potent inhibitors of COX-1,(148) and high dosages of ginger given to rats significantly reduced thromboxane B2 levels. (149) While one study of Danish women taking five grams of raw ginger daily for a week reported reduced thromboxane B2 levels,(150) another study found no effect of either raw or cooked ginger root on thromboxane B2 production in healthy volunteers. (151) The extract EGB from Ginkgo biloba 761 was found to inhibit arachidonic acid-mediated platelet aggregation and thromboxane B2 production in healthy volunteers(152) and in patients with diabetes. (153) However other studies, undertaken in healthy volunteers, found no effect of EGB 761 on platelet function or other coagulation parameters. (154, 155) Curcumin, a major component of the Indian food spice turmeric, reduces thromboxane B2 production in washed platelets,(156) and platelet aggregation induced by both platelet-activating factor and arachidonic acid is inhibited. (157) Platelet-derived growth factor-stimulated vascular smooth
muscle cell proliferation following carotid injury in rats is also reduced by curcumin. (158) Ginseng has been reported to have antiplatelet effects, apparently derived from panaxynol, leading to reduced thromboxane B2 formation. (159)

**Limitations**

A limitation of this study was its small sample size. Although the PFA-100 replicates the high shear substrate found in arterial thrombus formation, any ex vivo platelet function test only partly reflects the complexity of in vivo platelet-thrombus formation. Laboratory light transmittance aggregometry remains the accepted “gold standard”, and might have better assessed platelet function than the point-of-care method employed here. However, aspirin “resistance” assessed with the device is associated with adverse clinical outcomes, and the device was able to detect interactions between NSAIDs and aspirin in chapter two. Platelet function was assessed 12 hours post-dose, at the end of a twice daily dosage interval, as a clinically-useful antiplatelet effect needs to be sustained. However, short-acting platelet inhibition, as found with ibuprofen (Chapter Two), would be missed. Study subjects were assessed on the fifth day of herbal medication treatment; it is possible that longer treatment might have been necessary.

**Conclusion**

On the one hand this study indicates that the herbal medications evaluated are unlikely to have a clinically-useful antiplatelet effect. On the other hand, they can be taken in combination with aspirin, without an attenuation of the important antiplatelet effect of aspirin. The next Chapter evaluates genetic associations with the antiplatelet effect of aspirin.
Chapter 4. Genetic polymorphisms and variability in the antiplatelet effect of aspirin

Chapters Two and Three discussed important interactions between aspirin and commonly used anti-inflammatory and herbal medication. Chapter One presented the case for the presence of non-response to aspirin in the general population. Genetic variation in a population is understood to influence the response to medication and may also affect aspirin response. This Chapter explores the potential link between polymorphisms encoding common platelet enzymes, receptor genes and platelet function in patients taking aspirin.

Introduction

A number of studies have shown that various polymorphisms are associated with aspirin resistance (Table 6).\(^{(42, 71, 72, 160, 161)}\) The most promising are a haplotype within the cyclo-oxygenase I enzyme,\(^{(71)}\) and polymorphisms of the GP IIIa gene and the ADP receptor P2Y1.\(^{(72)}\) As with most genetic studies to date, these results need validating in other cohorts.\(^{(73)}\) This project used an observational study design to assess the prevalence of these genetic polymorphisms in a population undergoing coronary angiography, and to determine whether they influenced the response to aspirin as measured by the VerifyNow point-of-care platelet function analyser.
Table 6. Genetic variants reported to influence the response to aspirin

<table>
<thead>
<tr>
<th>Reference</th>
<th>No.</th>
<th>ASA dose</th>
<th>Gene</th>
<th>Genotype</th>
<th>Platelet Functional test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonzalez-Conejero(42)</td>
<td>22</td>
<td>100mg</td>
<td>COX 1</td>
<td>C50T</td>
<td>Urinary 11-dehydro TxB2</td>
<td>Higher 11-dehydro TxB2 levels before and after ASA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>COX 2</td>
<td>T765C</td>
<td></td>
<td>Lower 11-dehydro TxB2 levels after ASA</td>
</tr>
<tr>
<td>Lepantalo(160)</td>
<td>101</td>
<td>Mean 100mg</td>
<td>COX 1</td>
<td>A842G</td>
<td>Platelet aggregometry</td>
<td>842G carriers more likely to be non-responders</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GPVI</td>
<td>C13254T</td>
<td>PFA-100</td>
<td>C13254T carriers more likely to be non-responders(160)</td>
</tr>
<tr>
<td>Maree(71)</td>
<td>144</td>
<td>75-300mg</td>
<td>COX 1</td>
<td>A842G &amp; C50T</td>
<td>Platelet aggregation</td>
<td>Less responsive to ASA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haplotype</td>
<td>C50C</td>
<td>and serum TxB2</td>
<td>Increased responsive to aspirin</td>
</tr>
<tr>
<td>Jefferson(72)</td>
<td>469</td>
<td>&gt;81mg</td>
<td>P2Y1</td>
<td>-893T</td>
<td>Platelet aggregometry</td>
<td>OR 2.72 (CI 1.12-6.57) of ASA non-response in heterozygotes</td>
</tr>
<tr>
<td>Fujiwara(161)</td>
<td>110</td>
<td>81mg</td>
<td>TxA2R</td>
<td>C924T</td>
<td>Platelet aggregation</td>
<td>Aspirin less effective in homzygotes of C924T &amp; T1018C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GPIbα</td>
<td>T1018C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Methods

Subjects

115 subjects were enrolled into the study. The study protocol was approved by the Northern Regional Ethics Committee of New Zealand. All patients undergoing coronary angiography who were on aspirin were potentially eligible. Exclusion criteria were: a bleeding or platelet disorder, renal failure (creatinine clearance eGFR <30mls/min), anaemia (Hb <115mg/dL), thrombocytopenia (<150 x10⁹/L) and patient treatment with anticoagulants, glycoprotein IIbIIIa inhibitors, non-steroidal anti-inflammatory drugs or herbal supplements that may have interfered with platelet function testing. Subjects were interviewed about their adherence to aspirin therapy for the preceding week. All patients were on both aspirin and clopidogrel.

Platelet function testing

Whole blood was sampled between one and at least eight hours from the last aspirin dose. At angiography arterial blood was sampled through the 6F femoral sheath and transferred immediately to a 3.2% citrate 2ml vacutainer tube (Greiner Vacuette®, Kremsmuenster, Austria), using a 20G needle and syringe. The blood was left at ambient temperature for 10 minutes prior to testing. Platelet function was measured using a point-of-care device, the VerifyNow® rapid platelet function analyser (RPFA) and its corresponding aspirin cartridge (Accumetrics Ltd, San Diego, CA, U.S.A). This device uses fibrinogen coated microbeads and an agonist of arachidonic acid to activate platelets, with light transmittance through whole blood to measure platelet agglutination. The output of the device is the aspirin response unit (ARU); ≥550 denotes aspirin non-response.(38)

Pharmacogenetic analysis

DNA was extracted from whole blood using the QIAamp® DNA Blood Mini Kit (QIAGEN N.V., Netherlands) and stored at -20 degrees C. DNA yield and quality was assessed using the Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A). Genotyping for the Carboxylesterase intronic SNP (IVS10-88) and SNPs involving cyclo-oxygenase I and II genes (rs3842787, rs10306114, and rs20417), glycoprotein VI and Ibα genes (rs1613662 and rs6065), purinergic P2Y1 gene (rs1065776) and thromboxane A2 receptor gene (rs4523) were performed using iPlex® assays on the Sequenom® Autoflex Mass spectrometer and the Samsung 24 pin nanodispensor (Johns Hopkins Court, San Diego, CA, U.S.A).
Genotyping was performed at the Australian Genome Research Facility (University of Queensland, St Lucia QLD 4072, Australia). The call rate for the SNPs was 96.4 percent. The SNPs tested are outlined in Table 7 with the corresponding primer sequences used in the iPLEX assay. The prevalence of these SNPs and functional status, in previously described populations, are outlined in Table 8.

Table 7. Reference sequence accession numbers and Sequenom® primer Sequences

<table>
<thead>
<tr>
<th>Reference</th>
<th>Accession</th>
<th>Sequenom Primer sequences</th>
<th>Extension Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonzalez-Conejero(42)</td>
<td>rs3842787</td>
<td>1°ACGTTGGATGTCTGCAGGGAGTCCTGCTGCT 2°ACGTTGGATGCTGGGTCGCGAGCAGGA</td>
<td>CGCGAGCAGGACGGG GAGCGCC</td>
</tr>
<tr>
<td></td>
<td>rs20417</td>
<td>1°ACGTTGGATGAGGACCAAGTATTATGAGGAG 2°ACGTTGGATGTGTTCTCCGACCTCACCC</td>
<td>TTTTTCTTTGGAAGAG AGG</td>
</tr>
<tr>
<td>Lepantalo(160)</td>
<td>rs10306114</td>
<td>1°ACGTTGGATGCTTCCGATAACTGAGCACCT 2°ACGTTGGATGATTCAATGAGGGAATGCAC</td>
<td>AATCTCCTGTGCTGAGT G</td>
</tr>
<tr>
<td></td>
<td>rs1613662</td>
<td>reverse strand 1°ACGTTGGATGATACTGCCTGCCAGCAATG 2°ACGTTGGATGCCAGGGCAGAAG</td>
<td>CAACAGAACCACCTTC C</td>
</tr>
<tr>
<td></td>
<td>rs1065776</td>
<td>1°ACGTTGGATGAGTAGACCGAGGTGCTGTGGC 2°ACGTTGGATGCTGTTCCTCCAGAGGGGACGAA</td>
<td>ACCCAGACGAACCCACGAG</td>
</tr>
<tr>
<td>Jefferson(72)</td>
<td>rs4523</td>
<td>1°ACGTTGGATGAGTGAGGCGAGGTGGCTGTGCCAGAGG 2°ACGTTGGATGCTGGGAACGAGATCCTGGAC</td>
<td>CCTGAGGCCCTGGGTA</td>
</tr>
<tr>
<td></td>
<td>rs6065</td>
<td>1°ACGTTGGATGAGCTGAGCGCTTCCAGGACCTT 2°ACGTTGGATGGCTCTACCCAGAAGG</td>
<td>CCCTGCCCCCAGGGCT CCTGA</td>
</tr>
</tbody>
</table>
Table 8. Allele frequency of genotypes influencing aspirin response

<table>
<thead>
<tr>
<th>Reference</th>
<th>Gene</th>
<th>SNP</th>
<th>dbSNP accession No.</th>
<th>Genotype</th>
<th>Reported frequency for hetero &amp; homozygotes (CEU HapMap)</th>
<th>This study (n=112)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gonzalez-Conejero(42)</td>
<td>COX 1**</td>
<td>C50T</td>
<td>rs3842787</td>
<td>CT and TT alleles</td>
<td>13%</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>COX 2</td>
<td>T-765C</td>
<td>rs20417</td>
<td>GC and CC alleles</td>
<td>31%</td>
<td>25%</td>
</tr>
<tr>
<td>Lepantalo (160)</td>
<td>COX 1**</td>
<td>A-842G</td>
<td>rs10306114</td>
<td>AG and GG alleles</td>
<td>(19%)*</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>GPIV</td>
<td>C13254T</td>
<td>reverse strand</td>
<td>CT and TT alleles</td>
<td>27%</td>
<td>32%</td>
</tr>
<tr>
<td>Maree(71)</td>
<td>COX 1</td>
<td>A-842G</td>
<td>rs10306114</td>
<td>GG and GC alleles</td>
<td>(19%)*</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>Haplotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jefferson(72)</td>
<td>P2Y1</td>
<td>C893T</td>
<td>rs1065776</td>
<td>TT and CT alleles</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>Fujiwara(161)</td>
<td>TxA2R</td>
<td>T924C</td>
<td>rs4523</td>
<td>TT allele</td>
<td>14%</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>GPIbα</td>
<td>C1018T</td>
<td>rs6065</td>
<td>CC allele</td>
<td>84%</td>
<td>84%</td>
</tr>
</tbody>
</table>

*Data not available on HapMap. Taken from referenced manuscript. **In complete linkage disequilibrium.

**Statistics**

This study was considered a pilot analysis. As no previous genetic study has included the VerifyNow RPFA there was no data to base a power calculation.
Patients were divided into tertiles by RPFA aspirin response, and into responder or non-responder groups. The prevalence of alleles in the upper and lower tertiles and responder groups was compared using the Chi squared test. The effect of each allele was also compared on the average ARU using the student t test. Results are expressed as mean +/- standard deviation, and a p value <0.05 was considered statistically significant. MedCalc version 7.3.0.1 was used for the data analysis.

**Results**

The subjects were predominantly New Zealand European (n=105); the remainder were Maori or Polynesian (n=6), Fijian Indian (n=2), Middle Eastern (n=2) and Southeast Asian (n=1). Patient characteristics are outlined in Table 9. Aspirin non-responders were younger than responders (p=0.02). Most patients (89 of 115, 77 percent) had stable coronary disease; the remainder were two or more days post-myocardial infarction.

The platelet response in the population followed a normal distribution. The platelet response was not influenced by the clinical status of the subjects; stable patients (n=89) had an ARU of 488+/-53, and those with a recent MI (26) had an ARU of 467+/-73 (p=0.11). Aspirin response was not influenced by patient characteristics including clinical presentation, diabetes, smoking status, peripheral vascular disease or body mass index. Most (n=92, 80 percent) of the patients were on 100mg enteric coated aspirin; the remainder were taking 75mg (n=2, 2 percent), 150mg (n=17, 15 percent) or 300mg (n=4, 3 percent). Aspirin response fitted a normal distribution with a mean aspirin response unit (ARU) of 483+/-58. 12 subjects (10 percent) were aspirin non-responders defined by the VerifyNow RPFA (ARU ≥550). One of these subjects claimed to be noncompliant with his medication after further questioning.
Table 9. Baseline characteristics of patients in aspirin pharmacogenetics study

<table>
<thead>
<tr>
<th></th>
<th>Responders (n=103)</th>
<th>Non-responders (n=12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M)</td>
<td>78 (76%)</td>
<td>11 (92%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Age avg(sd)</td>
<td>66 (11)</td>
<td>58 (11)</td>
<td>0.02</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>11 (11%)</td>
<td>2 (17%)</td>
<td>0.9</td>
</tr>
<tr>
<td>DM</td>
<td>19 (18%)</td>
<td>1 (8%)</td>
<td>0.6</td>
</tr>
<tr>
<td>HTN</td>
<td>58 (56%)</td>
<td>6 (50%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Smoker</td>
<td>12 (12%)</td>
<td>3 (25%)</td>
<td>0.4</td>
</tr>
<tr>
<td>Recent MI</td>
<td>23 (22%)</td>
<td>3 (25%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Prior PCI</td>
<td>18 (17%)</td>
<td>5 (42%)</td>
<td>0.09</td>
</tr>
<tr>
<td>PVD</td>
<td>19 (18%)</td>
<td>2 (17%)</td>
<td>0.8</td>
</tr>
<tr>
<td>FHx CAD</td>
<td>38 (37%)</td>
<td>5 (42%)</td>
<td>0.99</td>
</tr>
<tr>
<td>BMI avg(sd)</td>
<td>30 (18)</td>
<td>29 (4%)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

P values calculated using Chi square testing.

One homozygote group, for the thromboxaneA2 receptor C924T (SNP rs4523), was present in 23 (20 percent) and was in Hardy-Weinberg equilibrium. The aspirin response with the rs4523 TT genotype was 486+/−60, compared with 484+/−58 in the remainder (p=0.9). The phenotype/genotype relationship was tested in a number of ways: 1) By comparing the influence of the reported genotypes on the average aspirin response (Table 10), 2) By comparing the frequency of genotypes in the upper and lower tertile of stratified aspirin response (Table 11), 3) By comparing the frequency of genotypes in aspirin responders versus non-responders (Table 12). There was no significant effect of any of the gene variants tested on aspirin response. The gene variants were also assessed additively, by allocating each a score of +1 or 0 for their presence or absence, and plotting the ARU against the number of variants in each individual. There was no relationship between the number of variants and the ARU.
Table 10. Influence of genotype on aspirin response

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Average ARU value (sd)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aspirin non-responder genotype</td>
<td>Aspirin responsive genotype</td>
</tr>
<tr>
<td>COX 1</td>
<td>C50T</td>
<td>502 (61)</td>
<td>482 (58)</td>
</tr>
<tr>
<td></td>
<td>CT and TT alleles (n=17)</td>
<td>CC allele (n=95)</td>
<td></td>
</tr>
<tr>
<td>COX 2</td>
<td>T-765C</td>
<td>472 (47)</td>
<td>489 (62)</td>
</tr>
<tr>
<td></td>
<td>GC and CC alleles (n=27)</td>
<td>GG allele (n=83)</td>
<td></td>
</tr>
<tr>
<td>GPVI</td>
<td>C13254T</td>
<td>475 (56)</td>
<td>489 (59)</td>
</tr>
<tr>
<td></td>
<td>CT and TT alleles (n=36)</td>
<td>CC allele (n=76)</td>
<td></td>
</tr>
<tr>
<td>P2Y1</td>
<td>C893T</td>
<td>463 (47)</td>
<td>486 (59)</td>
</tr>
<tr>
<td></td>
<td>TT and CT alleles (n=6)</td>
<td>CC allele (n=106)</td>
<td></td>
</tr>
<tr>
<td>TxA2R</td>
<td>T924C</td>
<td>486 (60)</td>
<td>484 (58)</td>
</tr>
<tr>
<td></td>
<td>TT allele (n=23)</td>
<td>CT and CC allele (n=89)</td>
<td></td>
</tr>
<tr>
<td>GPIbα</td>
<td>C1018T</td>
<td>476 (59)</td>
<td>485 (58)</td>
</tr>
<tr>
<td></td>
<td>CC allele (n=17)</td>
<td>CT and TT allele (n=94)</td>
<td></td>
</tr>
</tbody>
</table>

P values calculated using Chi square test.
**Table 11. Genotype prevalence in lowest and highest tertile of aspirin response**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Alleles</th>
<th>Lowest tertile ARU (n= 38)</th>
<th>Highest tertile ARU (n=38)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX 1</td>
<td>C50T</td>
<td>CT and TT alleles</td>
<td>3 (8%)</td>
<td>8 (21%)</td>
<td>95% CI = -3 to 29, P = 0.2</td>
</tr>
<tr>
<td>COX 2</td>
<td>T-765C</td>
<td>GC and CC alleles</td>
<td>35 (92)</td>
<td>37 (97%)</td>
<td>95% CI = -5 to 15, P = 0.7</td>
</tr>
<tr>
<td>GPVI</td>
<td>C13254T</td>
<td>CT and TT alleles</td>
<td>14 (37)</td>
<td>12 (32%)</td>
<td>95% CI = -16 to 26, P = 0.8</td>
</tr>
<tr>
<td>P2Y1</td>
<td>C893T</td>
<td>TT and CT alleles</td>
<td>3 (8)</td>
<td>0 (0%)</td>
<td>95% CI = -1 to 17, P = 0.2</td>
</tr>
<tr>
<td>TxA2R</td>
<td>T924C</td>
<td>TT allele</td>
<td>8 (21)</td>
<td>9 (24%)</td>
<td>95% CI = -16 to 22, P = 1</td>
</tr>
<tr>
<td>GPIbα</td>
<td>C1018T</td>
<td>CC allele</td>
<td>28 (74)</td>
<td>32 (84%)</td>
<td>95% CI = -8 to 28, P = 0.4</td>
</tr>
</tbody>
</table>

*P value calculated using Chi square test.*

**Table 12. Genotype prevalence in groups defined by aspirin response**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Alleles</th>
<th>Aspirin responders (n= 100)</th>
<th>Aspirin non-responders (n=12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX 1</td>
<td>C50T</td>
<td>CT and TT alleles</td>
<td>14 (14%)</td>
<td>3 (25%)</td>
<td>95% CI = -14 to 36, P = 0.6</td>
</tr>
<tr>
<td>COX 2</td>
<td>T-765C</td>
<td>GC and CC alleles</td>
<td>27 (27)</td>
<td>1 (8%)</td>
<td>95% CI = 1 to 37, P = 0.3</td>
</tr>
<tr>
<td>GPVI</td>
<td>C13254T</td>
<td>CT and TT alleles</td>
<td>34 (34)</td>
<td>2 (17%)</td>
<td>95% CI = -6 to 40, P = 0.4</td>
</tr>
<tr>
<td>P2Y1</td>
<td>C893T</td>
<td>TT and CT alleles</td>
<td>6 (6)</td>
<td>0 (0%)</td>
<td>95% CI = 1 to 11, P = 0.9</td>
</tr>
<tr>
<td>TxA2R</td>
<td>T924C</td>
<td>TT allele</td>
<td>20 (20)</td>
<td>3 (25%)</td>
<td>95% CI = -21 to 31, P = 1</td>
</tr>
<tr>
<td>GPIbα</td>
<td>C1018T</td>
<td>CC allele</td>
<td>84 (84)</td>
<td>10 (83%)</td>
<td>95% CI = -21 to 23, P = 0.7</td>
</tr>
</tbody>
</table>

*Aspirin non-responders defined as ARU≥550. P value calculated using Chi square test.*
**Discussion**

This study evaluated the effect of genotypes reported to influence aspirin response on the antiplatelet effect of aspirin measured by a point-of-care analyser. None of the genetic variants had an appreciable influence on aspirin response by this method of assessment.

The finding of a normal distribution of antiplatelet effect suggests multiple factors influence the response to aspirin. In contrast an earlier study using the VerifyNow device suggested that the response to aspirin was bimodal, with a discreet subpopulation showing "resistance".(38) Whilst one or more specific gene variants might explain a bimodal distribution of drug response, this has not been validated, and any genetic contribution is likely to be interactive and additive.

The COX-1 enzyme is highly conserved as it is a constitutive essential enzyme.(163) Therefore its gene is not particularly polymorphic and it is unlikely that the response to aspirin is determined by variation in its code. This hypothesis is consistent with the findings from a recent study by Faraday et al, which investigated the heritability of platelet responsiveness to aspirin in 1,880 individuals with premature coronary disease.(164) The response to aspirin, measured by direct COX-1 methods, was not particularly heritable, whereas indirect methods measuring aspirin response were strongly heritable, accounting for 27-77 percent of the variance seen.(164) In comparison, this was significantly higher than the contribution of cardiac risk factors, such as age and sex which only contributed 7 percent and 11 percent to the variance.

The response to aspirin is rapid and relatively uniform when measured by biochemical means, such as serum thromboxane B2.(48, 56) The perceived variability in response to aspirin measured by platelet function testing may therefore reflect platelet aggregation stimulated by alternative pathways independent of COX-1.(40) Therefore assessment of a hereditary influence on platelet response to antiplatelet drugs might be better assessed by platelet function testing, rather than biochemical measures such as thromboxane B2.

Noncompliance to aspirin is a significant reason for aspirin non-response and probably underappreciated in most studies where effective measures of noncompliance (such as pill counts) have not been made. This study was dependent
on clinical history and a formal measure of compliance was not made. However it was discovered that one individual with aspirin non-response, as defined by the platelet function analyser, was noncompliant after further questioning.

**Limitations**

One of the limitations of this study was that the VerifyNow point-of-care device was used to assess platelet function, rather than the gold standard of light transmittance aggregometry (LTA). The VerifyNow instrument predominantly measures the COX-1 pathway of aspirin response and the genetic contribution to this pathway is believed to be limited. Genetic variants in the indirect COX-1 pathway were assessed in this study with an attempt to relate these to VerifyNow platelet function results. The VerifyNow RPFA correlates well with LTA(38) and whole blood platelet aggregation methods have been shown to display a significant degree of heritability(164) leading to the belief that the instrument is not entirely COX-1 specific. The study population size and heterogeneity limits the power to detect more subtle or uncommon effects of genetic variants. Baseline measurements were not performed to compare pre/post drug response as all subjects were already on aspirin.

**Conclusion**

In conclusion, this study failed to show a significant influence of genetic variants, involving a number of components of the platelet cascade, on the response to aspirin. Genetic studies that involve testing multiple genotypes are at considerable risk of false discovery rates; initial results need validating in separate cohorts.(165) Whole genome-wide approaches, when applied to a restricted phenotype such as a drug response, are likely to reveal other possible genetic variants influencing aspirin response. These will need to be assessed individually and in combination with those variants already recognised. Such studies would ideally evaluate clinical events in addition to platelet function analysis.
Chapter 5. The antiplatelet effect of higher loading and maintenance dose regimens of clopidogrel: the Plavix Response in Coronary Intervention (PRINC) trial

Chapter Four explored the genetic influences on platelet response to aspirin. In this current Chapter the antiplatelet drug, clopidogrel, was assessed. This Chapter forms Part I of a two part study. Part II is discussed in Chapter Six. A randomised clinical trial was performed to evaluate the response to standard and higher doses, whilst at the same time looking for associations with genes related to response. Clopidogrel is a prodrug, and requires hepatic biotransformation to have an effect. Since hepatic biotransformation is under the control of polymorphic cytochrome P450 enzymes the drug is an attractive target for pharmacogenetic studies.

Introduction

A loading dose of 600mg clopidogrel, compared with lower dosages, given immediately prior to percutaneous coronary intervention (PCI) achieves greater platelet inhibition and reduces periprocedural myonecrosis.(100, 166) Three studies have examined whether a 900mg loading dose might be even more effective. Two of these studies showed no further platelet inhibition with 900mg compared with 600mg (65, 167), while in the third there was a non-significant trend to greater platelet inhibition only evident six hours from dosing.(168) Clopidogrel drug levels measured in the ISAR-CHOICE study suggested that the ceiling effect with clopidogrel 600mg might be due to saturable intestinal absorption of the drug.(65) Split-dose loading with clopidogrel may overcome this limitation and achieve greater platelet inhibition.(65, 167)

P-glycoprotein is a drug efflux pump mechanism in the gut which may reduce the intestinal absorption of clopidogrel.(82) Verapamil, a vasodilator drug frequently used in the cardiac catheterisation laboratory, inhibits p-glycoprotein in the short-term but upregulates it in the long term. Verapamil also inhibits CYP3A4, potentially interfering with clopidogrel pharmacokinetics.(169)

This study was a randomised, placebo-controlled trial to compare the effect of a clopidogrel 1,200mg split-loading dose with a standard 600mg loading dose, and to
assess a possible interaction between verapamil and clopidogrel. A higher maintenance dose regimen of clopidogrel was also evaluated.

**Methods**

**Study population**

The study protocol was approved by the Northern Regional Ethics Committee of New Zealand and registered with the Australian New Zealand Clinical Trials Registry. 60 consecutive patients undergoing elective percutaneous coronary intervention, who were taking aspirin but not clopidogrel, were enrolled to the study. Exclusion criteria were: a bleeding or inherited platelet disorder, gastrointestinal bleeding or gastric ulcer/duodenal ulcer/gastritis within the last six months, sensitivity/allergy to aspirin/clopidogrel/verapamil, renal failure (creatinine clearance eGFR <30mls/min), anaemia (hemoglobin <115g/L), thrombocytopenia (platelet count < 150x10^9/L), use of a glycoprotein IIbIIIa inhibitor or calcium channel blocker and medications inhibiting CYP3A4. Patients on warfarin were eligible if the INR was less than 1.5 at study entry and warfarin could be withheld for the seven day study duration.

**Study design**

The study design had two phases: a 2x2 factorial, randomised, placebo-controlled, double-blind study over the first 24 hours, followed by a one week randomised, placebo-controlled, double-blind study. Randomisation was by computerised pseudo-random number. Clopidogrel was repackaged into gelatine capsules packed with lactose powder to match the placebo capsules.

Patients were first randomised in a 2x2 manner to receive either 5mg intra-arterial verapamil or placebo at baseline, and either placebo or 600mg clopidogrel, two hours from the standard loading dose of clopidogrel given at baseline (Figure 6). Verapamil 5mg approximates the dose used in clinical practice. All received 600mg clopidogrel at the start of the PCI procedure, 10 minutes after administration of verapamil or placebo. Starting the next day, all patients were separately randomised to receive clopidogrel 75mg or 150mg once daily for one week, followed by 75mg once daily thereafter. Adherence to the treatment regimen was assessed by phone interview and pill count.
**Blood sampling**

Arterial blood was sampled through a 6F femoral sheath and transferred immediately to 3.2% citrate 2ml vacutainer tubes (Greiner Vacuette®, Greiner, Kremsmuenster, Austria), using a 20G needle and syringe. After sheath removal blood was drawn by venepuncture directly into vacutainer tubes. The collection tubes were inverted four times to mix the anticoagulant and left for 10 minutes at ambient temperature (24°C) before testing. Platelet function was tested at baseline, two, four and seven hours from the first clopidogrel loading dose, and at seven days.

**Figure 6. Study protocol for the PRINC trial**

**Platelet function analysis**

Platelet function was measured using the VerifyNow point-of-care rapid platelet function analyser (RPFA) and its P2Y12 cartridge (Accumetrics Ltd, San Diego, CA, U.S.A). This device uses fibrinogen coated microbeads, an agonist of adenosine diphosphate (20mM ADP), and light transmittance through whole blood, to measure platelet agglutination. The P2Y12 cartridge result correlates favourably with light transmittance aggregometry,(35) and has reportedly increased sensitivity to the
P2Y12 receptor due to the addition of prostaglandin E2 (22nM) to the reaction chamber. Platelet inhibition is reported as the percentage change in the platelet response unit (PRU) from a baseline BASE unit, derived from a second channel run in parallel with the ADP channel using the agonist iso-TRAP (Thrombin Receptor Activating Peptide).

Antiplatelet drug “resistance” may be assessed as residual post-treatment activity in the target pathway of an antiplatelet agent. Non-response to clopidogrel was defined as less than 10 percent maximal inhibition at seven hours.

**Endpoints**

The study primary endpoints were RPFA values at two, four, and seven hours for verapamil, four and seven hours for clopidogrel loading and seven days for the clopidogrel maintenance dose. The seven hour value was considered the peak loading dose effect.

Secondary endpoints were plasma troponin T (Roche troponin T assay, reference value <0.01 μg/L) and creatine kinase at seven hours, and safety outcomes including death, myocardial infarction, bleeding events and adverse drug reactions. Significant bleeding was defined as any intracranial bleeding, haemoglobin decrease of greater than 5g/dL, bleeding requiring transfusion, femoral haematoma greater than 10cm diameter, or femoral pseudoaneurysm.

**Statistical analysis**

With the 2x2 design, it was estimated that 120 patients (30 in each group) would approximately provide 80 percent power to detect one standardised difference between groups in the percentage platelet inhibition, at a significance level of 0.05. An interim analysis was pre-specified once 60 patients were enrolled. As verapamil had no effect, the verapamil/placebo results were combined, giving 60 subjects to compare the 600mg and 1200mg dosages over the first 24 hours, and the 75mg and 150mg dosages at one week.

Chi square likelihood ratio tests were used to compare categorical outcomes between the different intervention groups; Fisher exact tests were used where more than 25 percent of the expected counts in a table were less than five. The Mann Whitney U test was applied to compare continuous measures between groups.
Analysis of covariance was used to adjust for baseline PRU and age when comparing percentage platelet inhibition in the 600mg and 1200mg clopidogrel groups, and to adjust for baseline PRU, age and diabetes in the 75mg and 150mg clopidogrel groups. Equal variances tests were applied. The effect of the loading dose on platelet inhibition at one week was non-significant and the results at one week, across loading doses, were pooled into just 75mg and 150mg groups. A kinetic model on log scale of hours was used to describe the growth rate of percentage platelet inhibition over time. The model was validated by undertaking linear regression on each patient’s percentage platelet inhibition versus the natural log of hours after medication. Coefficient of determinants R² were summarised and compared across two other two kinetic models (Ln(%inhibition)~Ln(hours) and %inhibition~ exp(hours)). Two sided tests were used in all analysis and a p value < 0.05 was considered statistically significant. The software used for the analysis was SAS institute (version 9.1) and R (version 2.1.1).

Results

Patient characteristics

Patients were well matched between treatment groups with similar baseline demographics, pre-existing illnesses, smoking rates and current medications (Table 13). The average age was greater in the verapamil group and there were more with diabetes in the clopidogrel 150mg group. Baseline PRU and percentage platelet inhibition did not differ significantly at baseline or two hours after all patients received the first clopidogrel 600mg dose. The treatment group numbers differed as a block randomisation schedule was not used and enrolment was stopped at 60 participants.
<table>
<thead>
<tr>
<th>Demographics</th>
<th>n=60</th>
<th>clopidogrel 600g and placebo n=23</th>
<th>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age mean(std)</td>
<td>68(10)</td>
<td>64(10)</td>
<td>70(10)</td>
<td>0.06</td>
</tr>
<tr>
<td>Male</td>
<td>50(83%)</td>
<td>19(83%)</td>
<td>31(84%)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Caucasian</td>
<td>57(95%)</td>
<td>22(96%)</td>
<td>35(95%)</td>
<td>0.3</td>
</tr>
<tr>
<td>BMI mean(std)</td>
<td>29(4)</td>
<td>28(4)</td>
<td>29(5)</td>
<td>0.4</td>
</tr>
<tr>
<td>current smoker</td>
<td>6(10%)</td>
<td>3(13%)</td>
<td>3(8%)</td>
<td>0.7</td>
</tr>
<tr>
<td>baseline PRU</td>
<td>346.2(58)</td>
<td>335(45)</td>
<td>353(64)</td>
<td>0.1</td>
</tr>
<tr>
<td>Demographics</td>
<td>n=60</td>
<td>clopidogrel 600g and placebo n=23</td>
<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
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<tr>
<td>Demographics</td>
<td>n=60</td>
<td>clopidogrel 600g and placebo n=23</td>
<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
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<tr>
<td>Demographics</td>
<td>n=60</td>
<td>clopidogrel 600g and placebo n=23</td>
<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
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<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
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<tr>
<td>Demographics</td>
<td>n=60</td>
<td>clopidogrel 600g and placebo n=23</td>
<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
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<tr>
<td>Demographics</td>
<td>n=60</td>
<td>clopidogrel 600g and placebo n=23</td>
<td>Clopidogrel 600g and clopidogrel 600g at two hours after PCI n=37</td>
<td>P value</td>
</tr>
</tbody>
</table>

**Clinical Characteristics**

| ASA | 59(98%) | 23(100%) | 36(97%) | >0.9 |
| Statin Y | 56(95%) | 21(95%) | 35(95%) | >0.9 |
| Beta blocker | 47(80%) | 16(73%) | 31(84%) | 0.3 |
| ACE | 33(56%) | 13(59%) | 20(54%) | 0.7 |
| DM | 11(18%) | 4(17%) | 7(19%) | >0.9 |
| HTN | 34(57%) | 15(65%) | 19(51%) | 0.3 |
| CHF | 2(3%) | 1(4%) | 1(3%) | >0.9 |
| Prior CABG | 6(10%) | 1(4%) | 5(14%) | 0.4 |
| Recent NSTEMI | 4(7%) | 2(9%) | 2(5%) | 0.6 |
| Recent STEMI | 3(5%) | 2(9%) | 1(3%) | 0.6 |
| Prior PCI | 12(20%) | 5(22%) | 7(19%) | >0.9 |
| PVD | 13(22%) | 5(22%) | 8(22%) | >0.9 |
| FHx CAD | 24(40%) | 12(52%) | 12(32%) | 0.1 |
| Ejection fraction mean (Std) | 68(16) | 63(24) | 71(11) | 0.3 |
| Multiple stents | 9(15%) | 2(9%) | 7(19%) | 0.5 |
| DES | 21(35%) | 7(30%) | 14(38%) | 0.6 |
All participants completed the treatment allocated in the first stage of the protocol. Three outlier RPFA values were observed at baseline, but were included in the analysis. These indicated either sampling error, or inadvertent administration of clopidogrel, prior to PCI. Three outlier values in the post-loading phase, thought related to overfilled citrate tubes, were also included. 15 subjects (12 in the 150mg group and three in the 75mg group) did not have the one week follow-up RPFA because they lived more than two hours from the tertiary PCI centre.

**Primary outcomes**

Platelet inhibition at two hours did not differ in the group receiving verapamil compared with placebo (20.9+/−23.1% versus 17.0+/−17.5%, p= 0.4). There was also no difference seen at four or seven hours (Table 14).

Platelet inhibition at four hours differed significantly between the 600mg loading dose and the 1,200mg split loading dose (23.7+/−21.6% versus 42+/−26.6%, P=0.03). This difference was sustained at seven hours (28.7+/−21.9% versus 48.9+/−30.3%, P=0.03) (Figure 7) but not at seven days (35.7+/−27.3% versus 44.5+/−25.8%, P=0.3). Analysis of the four and seven hour results using ANCOVA, to adjust for baseline differences in age, baseline PRU and any interaction between drugs and dose, showed that the treatment group differences remained significantly different (p=0.01) (Table 14).

The rate of change over time in platelet inhibition in the 1,200mg split dose group was significantly greater than in the 600mg group. Linear percentage inhibition change by log(hours) was 43 percent versus 14 percent, respectively (P=0.0001) (Table 15).
Table 14. Percentage platelet inhibition [mean (STD)] in patients receiving clopidogrel and placebo, compared with those receiving clopidogrel and verapamil

<table>
<thead>
<tr>
<th></th>
<th>placebo</th>
<th>verapamil</th>
<th>unadjusted p value*</th>
<th>adjusted p value**</th>
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<tr>
<td>Baseline</td>
<td>7.4(15.0)</td>
<td>2.2(3.9)</td>
<td>0.3</td>
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</tr>
<tr>
<td>2 hours</td>
<td>17.0(17.5)</td>
<td>20.9(23.1)</td>
<td>0.6</td>
<td>0.2</td>
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<tr>
<td>4 hours</td>
<td>32.9(27.1)</td>
<td>37.6(25.5)</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>7 hours</td>
<td>40.4(30.5)</td>
<td>42.1(27.8)</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>7 days</td>
<td>40.8(23.7)</td>
<td>41.1(28.6)</td>
<td>0.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*Mann Whitney U test

**Analysis of covariance, adjusted by baseline platelet response unit (PRU) and age

Table 15. Percentage platelet inhibition growth rate in patients receiving clopidogrel 600mg at baseline, compared with clopidogrel 600mg at baseline and a second dose of clopidogrel 600mg at two hours

<table>
<thead>
<tr>
<th></th>
<th>Clopidogrel 600mg + placebo</th>
<th>Clopidogrel 600mg + 600mg 2 hours after PCI</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 2 hours</td>
<td>16(0,30)</td>
<td>13(3,39)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>At 4 hours</td>
<td>14(2,24)</td>
<td>43(29,62)</td>
<td>0.0001</td>
</tr>
<tr>
<td>At 7 hours</td>
<td>15(3,20)</td>
<td>24(12,37)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

*Mann Whitney U test

The data presented are Median (IQR). The unit is % change / Ln (hour)
Figure 7. Temporal dose response showing mean platelet inhibition in patients receiving 600mg and 1200mg clopidogrel loading doses

The points represent the mean platelet inhibition and the bars represent the 95% confidence intervals. The unadjusted p value is from Mann whitney U test. This result was adjusted by baseline platelet response unit (PRU) and age level using analysis of covariance.

At one week a significant difference in platelet inhibition was seen between the group receiving 75mg of clopidogrel, compared with 150mg once daily (28.8+/-19.4% versus 49.8+/-27.7%, P = 0.01) (Figure 8). 150mg once daily was required to maintain a steady state after a 1200mg loading dose and conversely 75mg once daily was required to maintain steady state after 600mg (Figure 9).
Figure 8. Mean platelet inhibition in patients receiving 75mg and 150mg once daily clopidogrel at baseline and after one week

- △ Clopidogrel 75mg/day
- ○ Clopidogrel 150mg/day

Unadjusted p value is from Mann whitney U test. This result was adjusted by baseline PFA level, age and diabetes using analysis of covariance.
Numbers in each group: 600mg+75mg n=10, 1200mg+75mg n=12, 600mg+150mg n=13, 1200mg+150mg n=25.

Secondary outcomes

Troponin T levels at seven hours were significantly higher in patients considered non-responders to clopidogrel, compared with those who were responsive (median 0.05 [quartiles 0.01, 0.29] versus median 0.01 [quartiles 0.01, 0.03], p=0.05). However, there was no significant difference in seven hour troponin T between the 1200mg and 600mg loading dose groups (median 0.04 [inter quartiles 0.01,0.23] versus median 0.01 [inter quartiles 0.01,0.03],p=0.5).
In the 1200mg group there were three gastrointestinal adverse reactions; one patient vomited, one experienced indigestion, and another developed diarrhoea the next day. This was thought due to the lactose vehicle in the gelatine capsules.

There were two significant bleeding events, both femoral hematomas at the groin puncture site and both in study subjects in their 80s. Neither required blood transfusion. One subject was in the 1200mg dose and the other in the 600mg dose group; P2Y12 platelet inhibition at the time of bleeding was 28 percent and 25 percent, respectively. There were no spontaneous bleeding episodes. Adherence to treatment at one week was 89 percent.

**Discussion**

A clopidogrel 1200mg loading dose achieves more rapid and complete platelet inhibition, if administered as two 600mg doses given two hours apart, than 600mg. Greater platelet inhibition was evident within two hours of the second 600mg dose. This finding is consistent with the recent PREPAIR study which assessed the effect of reloading with a further 600mg clopidogrel 18 to 24 hours after an initial loading dose.(171) VASP guided iterative reloading with clopidogrel doses up to 1.8 grams have also shown to increase platelet inhibition in selected resistant patients.(172) Until now the ceiling loading dose of clopidogrel appeared to be 600mg. Staggered dosing may more effective by overcoming the saturable nature of intestinal absorption of the parent drug.(65) Another possible explanation is that circulating platelets have longer exposure to the primary metabolite of clopidogrel. This primary metabolite has a short half life, and is created by the hepatic biotransformation of the parent drug by cytochrome P450 3A4, 1A2, 2B6, 2C19 and 2C9.(60, 61, 173)

Although a larger trial is needed to confirm the clinical efficacy and safety of this regimen, other platelet inhibition and periprocedural myonecrosis serves as a surrogate endpoint.(100) In Clopidogrel for the Reduction of Events During Observation (CREDO), an early and sustained antiplatelet effect, achieved by dosing more than six hours before PCI, was necessary to reduce the combined risk of death, MI, or urgent target vessel revascularization.(174) Intracoronary Stenting and Antithrombotic Regimen: Rapid Early Action for Coronary Treatment (ISAR-REACT 2) demonstrated that clopidogrel 600mg given two hours or more pre-intervention was insufficient to achieve optimal platelet inhibition in high-risk, troponin T positive,
acute coronary syndrome patients. In that study, adding abciximab reduced adverse events.(175) It is possible that a clopidogrel regimen with a higher loading and maintenance dose might have achieved a similar outcome.

Optimal loading dose regimens of clopidogrel are important because pre-treatment is not always logistically possible or clinically desirable. Clopidogrel loading prior to angiography in patients who subsequently need early coronary bypass graft surgery is a problem, as it is associated with a 50 percent increase in major peri-operative bleeding.(7)

Another major finding is that, in an unselected population, a maintenance clopidogrel dose of 150mg daily achieves greater chronic platelet inhibition than the standard regimen of 75mg daily. This is consistent with a study by Kastrati et al showing that reloading with 600mg clopidogrel, at the time of coronary intervention daily achieves greater platelet inhibition in patients on clopidogrel 75mg daily.(176) The Optimising antiplatelet therapy in diabetes mellitus (OPTIMUS) study also showed greater platelet inhibition with 150mg than 75mg clopidogrel daily. That study selectively enrolled patients with diabetes who had demonstrated a suboptimal response to clopidogrel 75mg daily, as assessed by light transmittance aggregometry.(177) It is unclear whether this increased antiplatelet effect will translate into a reduction in adverse clinical events, without an undue increase in bleeding. The CURRENT/OASIS-7 study has shown efficacy in increasing the maintenance dose of clopidogrel for the first week after PCI but the risk:benefit ratio is patient dependent and may be borderline.(178)

Drug-drug interactions are a common cause of hospital morbidity. Atorvastatin and erythromycin interact with clopidogrel by affecting hepatic biotransformation. Although the ex vivo antiplatelet effect of clopidogrel is reduced, non-randomised clinical studies to date have not demonstrated worse clinical outcomes.(64) Verapamil has mixed and unexpected effects on the intestinal p-glycoprotein efflux pump. Short term dosing inhibits p-glycoprotein activity, potentially enhancing clopidogrel absorption, whereas longer term dosing upregulates p-glycoprotein and may have the opposite effect. Verapamil also has an inhibitory effect on cytochrome P450 3A4 metabolism, which could reduce the biotransformation of clopidogrel. A single dose of verapamil did not impair the antiplatelet effect of clopidogrel.
The extent of platelet inhibition at seven hours with the split 1200mg loading dose was similar to that achieved with 150mg clopidogrel daily for one week, supporting the idea to use such a regimen in future clinical trials. Although this study lacked sufficient power to determine whether the 1200mg loading dose reduced post-PCI troponin T elevation, it did confirm previous reports that those who were unresponsive to clopidogrel were significantly more likely to have periprocedural myonecrosis.

The TRITON-TIMI 38 trial reported that prasugrel given as a 60mg loading dose followed by 10mg/day reduced post-PCI ischemic events compared with clopidogrel 300mg loading dose and 75mg/day, although bleeding was increased with prasugrel.(179) More recently PRINCIPLE-TIMI 44 found that the same prasugrel regimen produced greater platelet inhibition than clopidogrel 600mg loading followed by 150mg/day.(180) How prasugrel would compare with a clopidogrel 1200mg loading regimen remains to be seen. Even with a 1200mg loading dose the range of platelet response appears wider with clopidogrel than prasugrel. Thirteen percent of patients in the present study had less than 10 percent inhibition at seven hours; these patients may particularly benefit from prasugrel.

**Limitations**

The clopidogrel loading dose of 1,200mg and maintenance dose of 150mg daily appeared to be safe and well tolerated. The small study population precludes an accurate safety assessment. Platelet function was only assessed with the point-of-care platelet function analyser. The VerifyNow device lacks dynamic range compared with light transmittance aggregometry and loses sensitivity below inhibition levels of 20 percent.(181) However, results from the VerifyNow device correlate well with those of light transmittance aggregometry (35, 181) through most of the platelet reactivity range. The results also correlate well with specific markers of P2Y12 platelet activity such as vasodilator-stimulated phosphorylation (VASP).(46) Pharmacokinetic measurements would have enhanced the study. However the active metabolite of clopidogrel is difficult to measure, requiring a derivatising agent to protect the thiol ester.(182) The numbers in the treatment groups in this study were uneven because block randomisation was not used and the study was stopped after the planned interim analysis.
Conclusion

This Chapter demonstrated that it is possible to improve the response to clopidogrel by administering higher doses of the drug. The adverse consequences of increasing the dose could not be assessed due to the small sample size. Ideally increasing the dose would be performed only in patients who were non-responders. In the next Chapter, using the same patients in this study, various methods are applied in an attempt to identify non-responders to clopidogrel.
Chapter 6. The pharmacogenetics and pharmacodynamics of clopidogrel response: an analysis from the PRINC (Plavix Response in Coronary Intervention) trial

This chapter is part II of the same study described in Chapter Five. The aim in this Chapter is to investigate the methods of identifying non-responders to clopidogrel. Due to the time dependency and acuity of acute coronary syndromes it would be ideal for non-responders to be identified early, possibly even prior to treatment. Pharmacogenetics is a method that could stratify patients very early in a treatment course. Due to the novel design of the study in Chapters Five and Six the effect of genetics on both loading and maintenance doses could be observed. In addition as two dosing strategies were used some inferences can be made about alternative treatment approaches for genetic non-responders. The results of this study were considered novel at the time of its completion and led to the filing of a patent.

Introduction

Clopidogrel, an adenosine diphosphate receptor (P2Y12) blocker, is clinically used to prevent thrombotic events.\(^{(24)}\) The response to clopidogrel shows a wide interindividual variation\(^{(46)}\), however, 5-30 percent of patients do not respond to clopidogrel therapy.\(^{(2, 47)}\) Clopidogrel is a pro-drug that requires conversion to its active thiol derivative and is catalysed predominantly by cytochrome P450 (CYP) 3A4 and 3A5 with contributions from 2C19, 2C9, and 1A2 enzymes.\(^{(57-59)}\) Several studies have evaluated the impact of polymorphisms in genes encoding enzymes involved in clopidogrel metabolism (CYP2C19, CYP2C9, CYP3A4 and CYP1A2), as well as polymorphisms of P-glycoprotein transporter protein coding gene (ABCB1), on clopidogrel response (Table 16).

The activity of CYP3A4, measured by radiolabelled erythromycin breath testing, correlates with the response to clopidogrel.\(^{(183)}\) CYP3A4 expression may be variable due in part to polymorphisms in the gene coding this enzyme including \textit{CYP3A4*1B}, \textit{CYP3A4*3} and \textit{CYP3A4*4}.\(^{(184-186)}\) These variants are uncommon in Caucasians \(^{(187, 188)}\) with only one CYP3A4 polymorphism (IVS10+12G>A) to date reported to be associated with a reduced response to clopidogrel.\(^{(77, 189)}\) Expression of the CYP3A5 enzyme occurs only in subjects carrying at least one
CYP3A5*1 allele, which is present in six percent of Caucasians. (190) Subjects carrying the CYP3A5*1 allele have been reported to have greater platelet inhibition and fewer atherothrombotic events after percutaneous coronary intervention (PCI) than those who do not have this allele. (59)

A number of well-described polymorphisms exist within the CYP2C19 and CYP2C9 genes. The CYP2C19*2, *3 and *4 alleles display a loss of function of this enzyme, while CYP2C19*17 is associated with ultrarapid enzyme activity. (191) CYP2C19*2 carriers reportedly have a reduced antiplatelet response to a 300mg loading dose (58) and to a 75mg daily maintenance dose of clopidogrel. (78, 189) Similarly, CYP2C9*2 and CYP2C9*3 code for enzymes with decreased activity compared with CYP2C9*1*1, and the CYP2C9*2 allele is said to be associated with a reduced response to a clopidogrel 300mg loading dose. (58)

The Intracoronary Stenting and Antithrombotic Regimen: Choose between three High Oral Doses for Immediate Clopidogrel Effect ISAR-CHOICE study supports the idea that the apparent ceiling antiplatelet effect with clopidogrel 600mg could be due to saturable intestinal absorption of the drug, (65) mediated by the p-glycoprotein efflux pump. A polymorphism of the ABCB1 gene (C3435T) influences plasma levels of clopidogrel and its active metabolite; patients with 3435T/T genotype having lower Cmax and AUC values for both clopidogrel and its active metabolite compared to 3435C/T and 3435C/C carriers following 300 or 600mg single loading doses. (82) However, the polymorphism has not been related to the antiplatelet activity of clopidogrel.
<table>
<thead>
<tr>
<th>Allele</th>
<th>SNP</th>
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<th>Functional Effect</th>
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<td>1236C&gt;T</td>
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<tr>
<td>CYP2C9*1</td>
<td>-</td>
<td>-</td>
<td>Normal enzyme function</td>
<td>Brandt et al.(58)</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*2</td>
<td>3608C&gt;T</td>
<td>rs1799853 Reduced enzyme function</td>
<td>negative</td>
<td>Brandt et al.(58)</td>
<td></td>
</tr>
<tr>
<td>CYP2C9*3</td>
<td>42614A&gt;C</td>
<td>rs1057910 Reduced enzyme function</td>
<td>negative</td>
<td>Brandt et al.(58)</td>
<td></td>
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<tr>
<td>P2Y12 H2</td>
<td>i-T744C</td>
<td>rs2046934 Increased platelet response to ADP</td>
<td>none</td>
<td>Von Beckerath et al.(65)</td>
<td></td>
</tr>
<tr>
<td>CES2</td>
<td>g.275A&gt;G</td>
<td>rs1464602 Reduced midazolam clearance</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Intronic SNP (IVS10-88)</td>
<td>rs3893757</td>
<td>Reduced mRNA gene expression</td>
<td>-</td>
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<td></td>
</tr>
</tbody>
</table>

ABCB1 = P-glycoprotein gene, CYP = Cytochrome P450, CES = Carboxylesterase.
Dash denotes information not available. *1 normal variants are denoted by the absence of variant SNPs.

A haplotype of the P2Y12 gene, denoted H2, consists of five SNPs (i-C139T, i-T744C, i-ins801A, C34T and G52T).(76) As the SNPs occur in complete linkage disequilibrium they can be tagged by the intronic SNP (i-T744C).(193) Haplotype H2 is associated with increased platelet responsiveness to ADP but does not appear to influence the response to clopidogrel.(76, 194) Plasma carboxylesterases break down the active metabolite of clopidogrel.(52) Although the carboxylesterase gene
(CES) is well conserved a polymorphism has been described that decreases mRNA transcription. (195)

This study evaluated the impact of polymorphisms in a number of genes to determine whether they influence response to clopidogrel therapy as part of a randomised, placebo-controlled trial comparing the antiplatelet effect of a split-dose 1200mg clopidogrel loading dose with a standard 600mg loading dose and comparing a 150mg daily with a 75mg daily maintenance dose, in patients undergoing PCI.

**Methods**

The full details of the study protocol, baseline characteristics of the patients and additional pharmacotherapies are described in the previous chapter.

**Genotyping**

DNA was extracted from whole blood using the QIAamp® DNA Blood Mini Kit (QIAGEN N.V., Netherlands) and stored at -20 ºC. Genes of interest were identified using a candidate gene approach. The CYP3A4*1B(188), CYP3A4*3(187), CYP3A4*4(184), and CYP2C19*17(191) polymorphisms were identified by PCR-RFLP methods previously described, while the CYP3A5*3, CYP3A5*2, CYP3A5*6, CYP2C19*2, CYP2C19*3, CYP2C19*4; CYP2C9*2, CYP2C9*3 and ABCB1 1236C>T, 3435C>T, 2677G>A/T polymorphisms were investigated by real-time PCR using TaqMan kits (Applied Biosystems, Foster City, CA, U.S.A.) and ABI PRISM 7000 Sequence Detection System. Genotyping for the P2Y12 H2 haplotype and carboxylesterase intronic SNP (IVS10-88) were performed using iPLEX® assays on the Autoflex Mass spectrometer (Sequenom®, San Diego, CA, U.S.A). Genotyping using the Sequenom® Mass spectrometer was performed at the Australian Genome Research Facility (University of Queensland, St Lucia QLD, Australia).

**Statistical Analysis**

The sample size of 60 was determined by the number needed to detect differences between the treatment regimens evaluated in the Chapter Five. Genotyping was undertaken as a secondary analysis. A receiver operator characteristic was used to assess the predictive value of percentage platelet inhibition at two hours, compared with seven hours. Kruskal Wallis test was used to compare three or more genotype groups and Mann Whitney test was used to compare two genotype groups for platelet inhibition as a percentage of the baseline platelet activity using Graphpad.
Prism 4 (GraphPad Software, Inc. San Diego, California USA). ANCOVA was used to assess the influence of genotyping for CYP2C19 and CYP2C9 polymorphisms and clopidogrel dose on the variance in maximal platelet inhibition using SAS institute (version 9.1) and R (version 2.1.1) software. Statistical adjustment for multiple hypothesis testing was not used.

**Results**

The difference in platelet inhibition, between the treatment groups, is reported in the previous chapter.

**Prediction of response with phenotyping**

A receiver operator characteristic analysis found that lack of platelet inhibition at two hours predicted clopidogrel resistance at seven hours (p=0.02). There were fewer non-responders in the 1,200mg group than the 600mg group; two of 37 in the high dose group (five percent), compared with six of 26 in the low dose group (26 percent), had seven hour platelet inhibition less than 10 percent. The AUC for these ROC curves was 0.90. Platelet inhibition of less than two percent at two hours was the best predictor of non-response (inhibition less than 10 percent) in all patients, regardless of dose, at seven hours (sensitivity 100%, and specificity 88%) (Figure 10).
Platelet inhibition of less than two percent at two hours predicted non-response at seven hours (inhibition less than 10 percent), with a sensitivity of 100 percent, and specificity of 88 percent.

**Prediction of response with genotyping**

The frequency of the tested genotypes in our study population and previously reported frequencies in Caucasians are outlined in Table 17.

**CYP3A4/5 polymorphisms**

None of the patients carried the CYP3A4*1B, *3, *4 or CYP3A5*2 and *6 variant alleles, while three patients had the CYP3A5*1*1 genotype and three patients had the CYP3A5*1*3 genotype. However, no significant difference was observed in platelet inhibition in association with CYP3A5 genotype (data not shown).
Table 17. Frequency of detectable alleles in our study population and those reported in Hapmap database(162)

<table>
<thead>
<tr>
<th>Allele</th>
<th>SNP</th>
<th>dbSNP Reference Sequence No.</th>
<th>SNP frequency Cohort versus Hapmap (CEU)</th>
<th>Cohort* homozygotes</th>
<th>Hapmap homozygotes</th>
<th>Cohort* heterozygotes</th>
<th>Hapmap heterozygotes</th>
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<tr>
<td>ABCB1*2</td>
<td>1236C&gt;T</td>
<td>rs1128503</td>
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<td></td>
<td>3435C&gt;T</td>
<td>rs1045642</td>
<td>0.22</td>
<td>0.24</td>
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<td></td>
<td>2677G&gt;T/A</td>
<td>rs2032582</td>
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<td>0.11</td>
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<tr>
<td>CYP3A5*1</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>CYP3A5*3</td>
<td>6986A&gt;G</td>
<td>-</td>
<td>0.92</td>
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<tr>
<td>CYP2C19*1</td>
<td>80161A&gt;G</td>
<td>rs3758581</td>
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<td>-</td>
<td>0.48</td>
<td>-</td>
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</tr>
<tr>
<td>CYP2C19*2</td>
<td>19154G&gt;A</td>
<td>rs4244285</td>
<td>0</td>
<td>0.05</td>
<td>0.27</td>
<td>0.2</td>
<td></td>
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<tr>
<td>CYP2C19*4</td>
<td>1A&gt;G</td>
<td>rs28399504</td>
<td>0</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
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</tr>
<tr>
<td>CYP2C19*17</td>
<td>-806C&gt;T</td>
<td>rs12248560</td>
<td>0.07</td>
<td>0.02</td>
<td>0.27</td>
<td>0.4</td>
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<tr>
<td>CYP2C9*1</td>
<td>1188T&gt;C</td>
<td>-</td>
<td>0.65</td>
<td>-</td>
<td>0.33</td>
<td>-</td>
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<tr>
<td>CYP2C9*2</td>
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<td>rs1799853</td>
<td>0.02</td>
<td>0</td>
<td>0.25</td>
<td>0.21</td>
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<tr>
<td>CYP2C9*3</td>
<td>42614A&gt;C</td>
<td>rs1057910</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>P2Y12 H2</td>
<td>i-T744C</td>
<td>rs2046934</td>
<td>0.03</td>
<td>0.03</td>
<td>0.25</td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

*Note that 3 patients were not Caucasian in this cohort

CYP2C19 and CYP2C9 polymorphisms

None of the patients carried the CYP2C19*3 allele. The frequency of the CYP2C19*17 allele was 20 percent in this study population, which is similar to that reported in Swedish and Ethiopian subjects (18 percent) and higher than in a Chinese population (four percent).(191) CYP2C19*1*1 carriers had greater platelet inhibition two hours after a 600mg dose (median, range; 23%, 0-66%), compared with CYP2C19*2 or *4 carriers (10%, 0-56%, p=0.029) and CYP2C19*17 carriers (9%, 0-98%, p=0.026) (Figure 11).
CYP2C19*1*1 carriers had similar platelet inhibition after a 1200 and 600mg loading dose at four hours (43%, 13-97% and 35%, 0-65%, p=0.3) (Figure 12a) and seven hours (63%, 15-98% and 29%, 0-75%, p=0.05) (Figure 12b), and after a 150 and 75mg daily maintenance dose (46%, 18-97% and 32%, 24-64%, p=0.2) (Figure 12c).

In contrast, CYP2C19*2 or *4 carriers had greater platelet inhibition with the higher 1200mg loading dose compared with 600mg at four hours (37%, 8-87% and 14%, 0-22%, p=0.002) (Figure 13a), and tended to show a similar trend at seven hours (42%, 7-94% and 22%, 0-51%, p=0.09) (Figure 13b). Similarly, platelet inhibition was significantly greater with the 150mg than the 75 mg daily maintenance dose regimen (51%, 15-86% and 14%, 0-67%, p=0.042) (Figure 13c).
Figure 12. Platelet inhibition (percent of baseline) at a) four hours, b) seven hours and c) seven days after clopidogrel loading in CYP2C19*1*1 carriers.
Figure 13. Platelet inhibition (percent of baseline) at a) four hours, b) seven hours and c) seven days after clopidogrel loading in CYP2C19*2 or *4 carriers.

a)

b)

c)
CYP2C9 genotype did not influence the response significantly, except CYP2C9*1*3 carriers having reduced platelet inhibition compared to CYP2C9*1*1 carriers at seven hours with 600 mg clopidogrel dose (9%, 8-11% and 31%, 0-96%, respectively, p=0.045).

Individuals were aggregated based upon their CYP2C19 and CYP2C9 genotype status as either predictive of response (CYP2C19*1*1 or CYP2C9*1*1 or *2 carriers) or predictive of poor-response (CYP2C19*2 or *4 or *17 or CYP2C9*3), as has been described previously.(58) Despite the different dosing regimens of clopidogrel, platelet inhibition at seven hours was significantly influenced by having a genotype predictive of poor-response (p=0.04, Mann Whitney). Two individuals who were compound heterozygotes for both CYP2C19 *2 and CYP2C9*3 genotypes had platelet inhibition of 8 percent and 11 percent at seven hours, respectively.

ANCOVA was used to compare the influence of genetics which expressed as number of variants with that of clopidogrel dose on platelet inhibition at seven hours. The similar F values of 8.3 for clopidogrel dose (p=0.006) and 5.9 for genetics (p=0.02) suggesting that the clopidogrel dose had a greater influence on variance of maximal platelet inhibition than genetics.

**ABCB1 polymorphisms**

No significant influence of ABCB1 polymorphisms were observed in any of the time intervals in our study.

**P2Y12 H2 Haplotype**

Patients homozygous for the P2Y12 H2 haplotype denoted by the insertion variation i-T744C were uncommon; only two subjects were CC homozygotes. Although neither had a high baseline PRU value, platelet inhibition two hours after 600mg clopidogrel was less than two percent in both individuals.

**CES**

The carboxylesterase intronic SNP (IVS10-88) was not present in this cohort.
**Discussion**

Patients carrying the CYP2C19*2, CYP2C19*4 and CYP2C19*17 alleles have a decreased antiplatelet response to clopidogrel compared to CYP2C19*1*1 carriers. Patients carrying any of these alleles displayed both a reduced early response to clopidogrel loading and a reduced sustained response after one week of maintenance therapy. CYP2C19 appears to be important in the first step of biotransformation of the parent drug into the active metabolite, and a loss of function in this enzyme system leads to a reduction in the plasma levels of the active metabolite.(58)

The CYP3A4 variant alleles are rare in the Caucasian population and due to lack of patients carrying the variants analysed in our study the impact of CYP3A4 polymorphisms on clopidogrel response could not be observed. Moreover, in the 6 patients who carried CYP3A5*1 allele no significant impact of CYP3A5 expression was observed on clopidogrel response in this study.

The finding of reduced platelet inhibition in those with the CYP2C19*17 allele is unexpected given that *17 codes for an enzyme with higher activity than *1.(191) Our finding was contrary to an expected increased response to clopidogrel, however one homozygote in the study did demonstrate a hyper-response. It is possible that the interacting confounding factors and the low prevalence of *17 homozygotes reduced the ability to perceive a difference in response. The CYP2C19*17 variant is less well studied however a number of clinical studies have now demonstrated either improved efficacy of standard dose clopidogrel in *17 patients(196, 197) with ACS or increased bleeding particularly in *17/*17 homozygotes.(198, 199)

At the time this was the first study to evaluate the impact of CYP2C19*4 and *17 on clopidogrel response. The relationship between the CYP2C19*4 and *17 variants is complex as both can occur on the same chromosome in a cis form. As the *4 variant encodes a partial loss of function, the presence of this alongside the *17 ultrametaboliser variant on the same chromosome may partially negate the effect of *17.(200) CYP2C19*2 and *17 may also occur in a cis form, however as the *2 variant codes for a null allele *17 is unable to upregulate *2 on the same chromosome.(201)
Several studies have shown that the CYP2C19*2 allele is associated with a reduced antiplatelet response to clopidogrel therapy. (58, 78, 189) Hulot et al. (78) found that heterozygote CYP2C19*2 subjects had no significant change in platelet function after seven days of clopidogrel 75mg daily, as assessed by both 10μmol/L ADP light transmittance aggregometry and vasodilator stimulated phosphorylation (VASP). In another normal subject cohort platelet inhibition after clopidogrel 75mg daily for one week, measured by 20μmol/L ADP aggregometry, was significantly less in the subjects carrying the CYP2C19*2 allele. (189) Brandt et al. (58) found that the CYP2C9*2 and *3 alleles, in addition to the CYP2C19*2 allele, influence the plasma levels of clopidogrel active metabolite as well as the response. These variants are not in linkage disequilibrium and, when considered together, reportedly accounted for two thirds of poor-responders. Subsequent to the publishing of this study several clinical outcome studies have reported an increase in cardiovascular events in patients carrying the CYP2C19*2 and *3 variants. (79-81)

Of note the third generation thienopyridine, prasugrel, is not as dependent on either the CYP2C19 or CYP2C9 enzymes for metabolic activation. (58) Pharmacogenomics has been proposed as a method to individualise the use of this novel agent. (202)

Patients given a split 1200mg clopidogrel loading dose and a 150mg daily maintenance dose had greater platelet inhibition than 600mg and 75mg, respectively. Interestingly, the higher clopidogrel loading and maintenance dose regimens achieved greater platelet inhibition in CYP2C19*2 or *4 carriers compared to lower doses, while this dose-dependency was not observed in CYP2C19*1*1 carriers. In other words, those with the genotype predictive of poor-response may specifically benefit from higher-dose clopidogrel. However, the numbers in this study were small and this finding needed to be validated. The aim of Chapter Seven is to validate this finding in a dose escalation study.

The CYP2C19*2 allele is present in 12.7 percent of Caucasians, 18.2 percent of African Americans and 28.9 percent of East Asians. (203) The higher frequency of the CYP2C19*2 allele in Chinese suggests that there may be inter-ethnic differences in clopidogrel response. While a ethnicity-based treatment approach, such as targeting the antihypertensive drug Bidil to African Americans, has yet to be widely accepted (204), potential differences between ethnic groups in drug response may need to be considered when evaluating trial data.
Drugs that are competitively metabolised or inhibit the activity of CYP2C19 also have the potential to reduce the activity of clopidogrel. This drug interaction has been shown with omeprazole (189, 205, 206) but this has not translated into outcome studies.(207) Other drugs which may theoretically interact with clopidogrel via CYP2C19 include phenytoin and fluoxetine. The significance of clopidogrel drug interactions remains controversial.

**Limitations**

The major limitation of this study was the population size, which particularly impacts assessment of the influence of the less common genotypes. Since this study was an observational study confounding factors such as interacting medication may have contributed to the findings. As there were only two subjects with compound heterozygosity for CYP2C19 and CYP2C9 and a poor response to clopidogrel, there is a suggestion that a combination of genotypes may be more predictive of clopidogrel response than one genotype. However this remains a hypothesis with initial supportive data. A single study has suggested a gene-gene interaction between the P2Y12 haplotype and the CYP2C19*2 variant.(208) While a poor response to clopidogrel has been associated with an increase in peri-procedural myonecrosis during PCI,(100) it remains to be shown whether these genotypes will predict those at increased risk for vascular atherothrombotic complications whilst on clopidogrel, and whether some of those complications will be prevented by an individualised, higher-dose treatment regimen, targeting carriers of CYP2C19*2 and *4 genotypes.

The finding of reduced platelet inhibition in some patients with the CYP2C19*17 polymorphism was unexpected given that the polymorphism codes for an ultrametaboliser variant. One homozygote however had an exaggerated response to clopidogrel. The small sample size and confounding factors, such as drug interactions, may have accounted for the unexpected finding of reduced response in many of the CYP2C19*17 carriers.

Larger clinical trials will be needed to confirm the merits of a genotype-focused dosing approach over individualised treatment based upon either laboratory or point-of-care platelet function analysis.
Conclusion
In summary, this study provides evidence for the impact of CYP2C19 genotype on the platelet response to clopidogrel, and shows that higher-dose clopidogrel regimens can increase the degree of platelet inhibition in patients with genotypes predictive of poor-response. Personalised therapy targeting patients who carry the CYP2C19 variants predictive of poor response may help to improve clinical outcomes and the cost-benefit ratio of treatment.
Chapter 7. Pharmacogenetic testing for clopidogrel using the rapid INFINITI analyser: A dose escalation study

In Chapter Six it was shown that the response to clopidogrel is influenced by genetic polymorphisms in the cytochrome P450 CYP2C19 gene. Carriers of the CYP2C19*2 and *4 polymorphisms have a reduced response to clopidogrel. CYP2C19*17 carriers may have an improved response to clopidogrel or increased risk of bleeding due to hyperresponse. The results in Chapter Six suggested that increasing the dose of clopidogrel might overcome genetic non-response to the drug. In this chapter a dose escalation study was performed, to validate this finding. Genotyping in this study was performed with a rapid genetic analyser which may have clinical utility, since it is capable of returning a result in eight to 12 hours. Rapid turnaround in results is a critical component to the clinical application of clopidogrel pharmacogenetics.

Introduction

As mentioned in previous chapters clopidogrel shows wide population variability,(46) and up to five to 30 percent of patients may not respond to the drug.(183, 209) Clopidogrel is a pro-drug that requires conversion to its active thiol derivative and this is catalysed by the cytochrome P450 enzyme system. As shown in Chapter Six whilst (CYP) 3A4, 3A5, 2C19, 2C9, and 1A2 may be relevant in this conversion,(62, 77, 210) only loss of function polymorphisms within the 2C19 gene have been associated with a reduced clinical response to clopidogrel.(211-214)

Recent attention has been drawn to the clinical importance of the CYP2C19*2 genetic variant.(212) Most studies investigating this have shown a consistent increase in the risk of stent thrombosis in genetic non-responders taking clopidogrel.(212-215) In the TRITON TIMI-28 study (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction) there was a higher incidence of the composite of ischaemic events and cardiovascular death in those carrying the CYP2C19*2 allele who received clopidogrel. In contrast CYP2C19*2 allele carriers receiving prasugrel did not face the same risk of ischaemic events or death and interestingly did not display a trade-off with increased bleeding on prasugrel.(216) Alternatively loss of function allele carriers may respond to higher doses of clopidogrel.(129, 217)
Individualising treatment, using genotyping, may allow cost effective targeting of treatment or introduction of new therapies. This might improve outcomes without increasing adverse events. This study assessed genotyping in a cohort in which a clopidogrel dose was escalated over a week. A genotyping platform capable of rapid results turnaround was used.

**Methods**

**Study population**

The study protocol was approved by the Northern Regional Ethics Committee of New Zealand and registered with the Australian New Zealand Clinical Trials Registry. Patients were individuals who had undergone elective percutaneous coronary intervention greater than two weeks before enrolment. Exclusion criteria were: myocardial infarction requiring PCI <2 weeks before testing, a bleeding or inherited platelet disorder, gastrointestinal bleeding or gastric ulcer/duodenal ulcer/gastritis within the last six months, sensitivity/allergy to aspirin/clopidogrel, renal failure (creatinine clearance eGFR <30mls/min), anaemia (haemoglobin <115g/L), thrombocytopenia (platelet count < 150x10⁹/L).

**Study design**

Open label dose escalation study with molecular randomisation.

**Hypothesis**

The primary hypothesis was that an increased clopidogrel dose would increase platelet response in *CYP2C19*<sup>*2*</sup> allele carriers. The secondary hypothesis was that carriers of the *CYP2C19*<sup>*2*</sup> allele would have a greater change in platelet inhibition, with an increased dose of clopidogrel over one week, than wildtype individuals. A post-hoc analysis included the addition of *CYP2C9*<sup>*3*</sup> allele carriers to *CYP2C19*<sup>*2*</sup> carriers, to assess the change in response of a higher dose compared to wildtype. An interaction with proton pump inhibitors was also assessed.

**Study protocol**

Forty patients already taking 75mg daily of clopidogrel had a baseline platelet function test performed, followed by a dose increase to 150mg daily for one week.
Blood Sampling

Venous blood was sampled using a vacutainer technique and collected into 2ml 3.2% citrate tubes (Greiner Vacuette®, Greiner, Kremsmuenster, Austria), using a 20G needle and syringe. The collection tubes were inverted four times to mix the anticoagulant and left for 10 minutes at ambient temperature (24°C) before testing. Platelet function was tested at baseline and at seven days. Whole blood for DNA extraction was taken in EDTA (ethylenediaminetetraacetic acid) tubes.

Platelet function analysis

Platelet function was measured using the VerifyNow point-of-care rapid platelet function analyser (RPFA) and its P2Y12 cartridge (Accumetrics Ltd, San Diego, CA, U.S.A). This device uses fibrinogen coated microbeads, an agonist of adenosine diphosphate (20mM ADP), and light transmittance through whole blood, to measure platelet agglutination. The P2Y12 cartridge result correlates favourably with light transmittance aggregometry,(35) with reported increased sensitivity to the P2Y12 receptor due to the addition of prostaglandin E2 (22nM) to the reaction chamber.(46) Platelet inhibition is reported as the percentage change in the platelet response unit (PRU) from a baseline BASE unit, derived from a second channel run in parallel with the ADP channel using the agonist iso-TRAP (Thrombin Receptor Activating Peptide). A lower PRU indicates greater platelet inhibition but this is not controlled for the effect of thrombin.

Genotyping

Genomic DNA was extracted from whole blood using a MiniAmp extraction kit (Qiagen, Netherlands). Multiplex amplification of each sample was performed in an individual well of a 24 well plate using an Eppendorf™ Mastercycler™. Template and Platinum Taq Polymerase™ (Life Technologies) were added to an analyte-specific amplification mix (AutoGenomics, Inc.). After amplification, the plate was placed in the INFINITI® analyser where detection primer extension occurred, followed by hybridisation of detection primers to individual oligonucleotides arrayed on the BioFilmChip™. After hybridisation, the Biofilmchips were washed and scanned in the INFINITI optics module.

*6 and *1 polymorphisms respectively. These tests were performed after the second platelet function test, and the results were retrospectively assessed. CYP2C19*2 and CYP2C9*3 but excluding CYP2C19*17 carriers were classified as poor metabolisers (PMs). Combined carriers of CYP2C19*17 and *2 and wildtype/wildtype were considered intermediate metabolisers (IMs). CYP219*17 carriers without CYP2C19*2 or CYP2C9*3 alleles were classified as ultrametabolisers (UMs).

**Statistics**

The numbers of patients required to reach statistical power were calculated using figures for platelet inhibition effected by higher doses of clopidogrel.(217) It was estimated that a study of 40 patients would provide 90 percent power to detect the influence of genotypes on platelet function at a significance level of 0.05. The primary output of interest from the VerifyNow instrument was platelet inhibition but where a result using this variable was not statistically significant the platelet response unit has been reported. The Students t-test was applied where the data was parametric is reported in mean values +/- standard deviation when paired and mean and 95% confidence interval (CI) when unpaired. The Wilcoxon rank sum test was used for the nonparametric platelet percentage inhibition data and reported as median and range. The software used for analysis was Medcalc version 7.3.0.1 (Mariakerke, Belgium).

**Results**

40 patients were enrolled. One patient did not complete the study protocol due to non-attendance at one week. There were no bleeding episodes. One DNA sample was insufficient for analysis. Baseline Characteristics of the patients are outlined in Table 18. The allelic frequencies of the polymorphisms are outlined in Table 19. A number of individuals were compound heterozygotes, but they were insufficient in number to evaluate gene-gene interactions. Platelet inhibition at baseline for each allelic group is shown in Figure 14.

Platelet inhibition on average increased over one week, mean +8.6 +/-13.5% (P = 0.0003) (Figure 15). Carriers of the CYP2C19*2 allele had significantly reduced platelet inhibition at baseline (median 18%, range 0-72%) compared to wildtype (wt/wt) (59%, 11-95%, P = 0.01) and at one week (p=0.03).

A paired comparison of baseline results with results at seven days showed that platelet inhibition could be increased in CYP2C19*2 allele carriers with an increased
dose of clopidogrel. A mean increase in platelet inhibition of (mean +9+/−11%, P = 0.03) and reduction in platelet reactivity (mean -26+/−38 PRU, P = 0.04) was seen in CYP2C19*2 carriers with a higher dose (Figure 16).

Table 18. Baseline characteristics for patients in dose escalation trial

<table>
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<th>n=40</th>
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<td>Caucasian†</td>
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<td>Age (years)</td>
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</tr>
<tr>
<td>No. given 600mg loading dose</td>
<td>39 (98%)</td>
</tr>
<tr>
<td><strong>Past Medical History</strong></td>
<td></td>
</tr>
<tr>
<td>Type II Diabetes</td>
<td>8 (20%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>20 (50%)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>17 (43%)</td>
</tr>
<tr>
<td>Congestive Heart Failure</td>
<td>2 (5%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>4 (10%)</td>
</tr>
<tr>
<td>Coronary Bypass Surgery</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>Percutaneous Coronary Intervention</td>
<td>35 (88%)</td>
</tr>
<tr>
<td>Family History of Coronary Artery Disease</td>
<td>7 (18%)</td>
</tr>
<tr>
<td><strong>Interacting Medication</strong></td>
<td></td>
</tr>
<tr>
<td>Omeprazole</td>
<td>13 (33%)</td>
</tr>
<tr>
<td>Other CYP3A4, 2C19 drug</td>
<td>0</td>
</tr>
</tbody>
</table>

Values given as number and percentage or mean and standard deviation. † Other ethnicity included Maori (n=2), Fijian Indian (n=2), Chinese (n=1).
Table 19. Allelic frequencies of detected variants in dose escalation trial

<table>
<thead>
<tr>
<th>Gene</th>
<th>Star Nomenclature</th>
<th>dbSNP Reference Number</th>
<th>No. of alleles in cohort</th>
<th>Allelic Frequency (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19</td>
<td>*2</td>
<td>rs4244285</td>
<td>13</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>*3</td>
<td>rs1057910</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>*17</td>
<td>rs12248560</td>
<td>18</td>
<td>46%</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>*2</td>
<td>rs1799853</td>
<td>6</td>
<td>15%</td>
</tr>
<tr>
<td></td>
<td>*3</td>
<td>rs1057910</td>
<td>8</td>
<td>21%</td>
</tr>
</tbody>
</table>

Figure 14. Baseline platelet inhibition in dose escalation trial

Platelet function demonstrated for each genotype group.
Figure 15. Platelet function at baseline (75mg) and seven days (150mg)

Platelet function at baseline in patients on 75mg once daily clopidogrel and after one week of 150mg daily. Variable responses but overall a mean increase in platelet inhibition is demonstrated.

Figure 16. Platelet function in CYP2C19*2 carriers at baseline and seven days

Platelet function at baseline in CYP2C19*2 carriers and after one week of 150mg daily clopidogrel. An improvement in platelet inhibition is seen in most patients.

Together CYP2C19*2 and CYP2C9*3 loss of function carriers had a greater change in platelet inhibition with 150mg daily than wt/wt (Δmean -10% 95% CI -20 to -0.1, P = 0.05) (Figure 17).
Figure 17. Change in percentage platelet inhibition in CYP2C19*2 carriers

*Box plots represent median, range and 25th and 75th quartile. See text for definition of PM, IM status.*

Omeprazole (n=12) or lansoprazole (n=1) were being taken by n=13 (33 percent) of individuals. Patients on these proton pump inhibitors, which are metabolized by CYP2C19, had lower percentage platelet inhibition (median +14%, average rank 16 to 22, P=0.18) and a higher platelet reactivity (mean -51 PRU, 95% CI -7 to 110, P = 0.08) suggesting a drug-drug interaction, leading to a reduced response to clopidogrel. After one week of therapy those taking these PPIs still had lower platelet inhibition than those not on PPIs (mean -10 percent inhibition, 95% CI -8 to 27, P = 0.3). However compared to baseline those on a PPI had a significant increase in platelet inhibition after one week of a higher dose (mean +9 +/-10 %, P = 0.007), showing that the influence of the PPI could be overcome. Those on PPIs had a trend towards a greater change in platelet inhibition than those not on PPIs (Δmedian 15 percent inhibition, 95% CI -3 to 16 versus Δmedian seven percent inhibition, 95% CI -2 to 13; P=0.3) (Figure 18). Only one CYP2C19*2 carrier was on omeprazole.
Discussion

This study addressed the question of whether increasing a dose of clopidogrel from 75mg once daily to 150mg once daily for one week increases clopidogrel's antiplatelet response in genetic non-responders. The population in this study can be considered in a stable phase of coronary disease, given they were tested, on average, 9 weeks from PCI. The results show that platelet inhibition can be increased in individuals carrying the loss-of-function CYP2C19*2 allele by giving higher doses of clopidogrel. This supports the study in Chapter Six.(217) However despite a dose of 150mg once daily there is still a strong influence of this variant on platelet function and the small improvement in platelet inhibition may not translate into clinical significance. There was also a trend to a reduced antiplatelet response to clopidogrel in those taking omeprazole, a drug metabolized also by the CYP2C19 enzyme. This drug-drug interaction may also be overcome by increasing the dose of clopidogrel.

Several studies have linked an increased risk for stent thrombosis and major adverse cardiac events to carriers of the CYP2C19*2 polymorphism.(211-215) One conflicting result however has been demonstrated in the FAST-MI study, which showed an increased event rate in *2 homozygotes but a reduced event rate in heterozygotes, compared to wildtype homozygotes.(214) The reason for this paradoxical finding is not clear.
With the availability of the third generation thienopyridine prasugrel the potential for targeting treatment appears appealing, however increasing the dose of clopidogrel in some individuals may be sufficient to make them responders. A study looking at just phenotypic non-responders, using vasodilator-stimulated phosphoprotein (VASP) showed an increase in suppression of platelet function with an increased clopidogrel dose, independent of the CYP2C19*2 allele. Another study, using doses up to 2.4 grams, in a widely spaced iterative loading protocol has shown the existence of a core group of phenotypic non-responders. Whether this profound non-response is genetically determined is unknown. The Clopidogrel and Response Variability Investigation (CLOVIS) study has shown loading with clopidogrel 900mg is sufficient to overcome non-response in *2 heterozygotes but not homozygotes. In addition a maintenance dose of clopidogrel 225mg provides equivalent platelet inhibition in *2 heterozygote compared to clopidogrel 75mg in wildtype patients.

This study has shown that a practical method of clopidogrel loading, using 1,200mg split over two hours, results in a mean increase of +20 percent platelet inhibition (VerifyNow, Accumetrics, San Diego), seven hours after loading, compared to 600mg (p = 0.03).

The functional relevance of rarer CYP2C19 variants needs further investigation. The CYP2C19*3 allele, coding for a truncated enzyme protein, is particularly prevalent in some populations, such as East Asians and those of East Asian descent such as Pacific Peoples and Maori. This variant however has not been detected in many of the studies performed to date. Together the *3 and *2 variant account for 99 percent of all poor metabolisers in East Asians and both would need to be considered if clinical genotyping for clopidogrel was being considered in Asian countries. This study has shown that the rare loss of function CYP2C19*4 allele has an effect on the platelet response to clopidogrel. This variant is more common in those of Ashkenazi descent and ignoring this variant in a genotyping panel can lead to misclassification of phenotypic non-responders. Figure 14 shows that genotyping alone will misclassify some individuals with the *2 allele, who have satisfactory platelet inhibition and vice versa. The biotransformation of clopidogrel requires a two-step CYP dependent process. Gene-gene interactions require further study as a combination of SNPs may allow better risk stratification for cardiovascular events and further enhance treatment decisions. Pharmacogenetics is unlikely to encapsulate the whole picture and response
The prediction is likely to be enhanced by the incorporation of other phenotype measures (Figure 19).(215, 226) This might include metabolomics or other biomarkers.

**Figure 19. Clopidogrel response incorporating static and variable factors**

![Diagram of Clopidogrel response incorporating static and variable factors]

The importance of the CYP2C9*3 allele (2C9 being related to 2C19) with respect to the clopidogrel biotransformation, platelet response and clinical outcomes is uncertain. Pharmacokinetic/pharmacodynamic data suggest that it is an important variant(173) however a larger pharmacokinetic study with clinical outcome data has shown no effect.(212) This may be due to sequence homology or 2C9 primer issues leading to genotyping errors.(227)

The interaction between proton pump inhibitors and clopidogrel has received recent attention.(206) Both omeprazole(206, 228-230) and lansoprazole(225) have been shown to reduce the responsiveness to clopidogrel, whereas pantoprazole(228, 230, 231) and esomeprazole(228, 231) (the S-enantiomer of omeprazole) have not. In this study the patients taking omeprazole or lansoprazole had a trend towards a lower
than average platelet response suggesting a drug-drug interaction. In this study a higher dose of clopidogrel was able to partly overcome the clopidogrel-PPI interaction.

Rapid genotyping will be necessary for pharmacogenomics to be useful in the clinical setting. The INFINITI analyser provides a rapid turnaround time in eight hours. Other technologies offer faster genotyping, but some are not capable of multiplexing beyond a certain number of SNPs.(232) Other examples of rapid genotyping include High Resolution Melt (HRM) curve analysis, used in warfarin pharmacogenetic studies(233) and direct nucleic acid detection using gold nanoparticles. Direct detection with nanoparticles has the advantage of a PCR-free environment, two hour result turnaround and may only require a single drop of blood to perform an analysis.(234)

On the flip side of non-response to clopidogrel is the potential for an excessive antiplatelet response and bleeding. Dual antiplatelet therapy is associated with a reduction in cardiovascular event rates but also a rise in bleeding events compared to aspirin alone.(235) One of the limiting factors to the acceptance of prasugrel has been the concern regarding bleeding events. Patient characteristics such as being ≥75 years of age, weight less than 60kg or presence of previous ischaemic stroke are risk factors identified with bleeding events on prasugrel.(179) Treating diabetics, patients undergoing stenting, and patients undergoing primary percutaneous coronary intervention with prasugrel, instead of clopidogrel, may be cost beneficial.(216, 236, 237) In Chapter Fourteen a pharmacogenomic algorithm incorporating some of this information is proposed.

**Limitations**

There are a number of limitations of this study, mainly related to lack of statistical power. Genotyping was also performed retrospectively. Despite showing the ability to enhance platelet response in CYP2C19*2 allele carriers with higher doses of clopidogrel this may not be clinically significant. There was no measure of treatment adherence in this study. Tachyphylaxis may have influenced the variability in baseline response.
Conclusion

In conclusion this study has shown that higher doses of clopidogrel, for some individuals, may improve the antiplatelet response. Further study is required to assess whether there is a therapeutic window for thienopyridines, balancing effectiveness and bleeding. Targeting treatment based on non-response using either phenotyping, genotyping or both may be more cost effective than a "one-size-fits all" strategy. Integrating important clinical, genetic and phenotypic data is complex and decision support software may be required to optimize prescribing. This is discussed in more detail in Chapter Fourteen. (222) Prospective studies, applying pharmacogenetics before treatment is given, are required to prove the cost-effectiveness and clinical usefulness of this approach.
Chapter 8. Addressing ethnic disparities in New Zealand with Personalised Medicine

In Chapter Seven a study was performed to demonstrate the effect of increasing the dose of clopidogrel in patients carrying non-responder gene variants. This Chapter reviews the field of pharmacogenetics and the population significance of clopidogrel pharmacogenetics. Pharmacogenomics arguably may allow cost-effective rationing of costly drug treatments and may reduce ethnic disparities (Appendix A).

Pharmacogenetics has long held the promise of individualising pharmacological therapy using genetic biomarkers. Within the last few years pharmacogenetic tests predicting adverse reactions to the antiepileptic drug carbamazepine and HIV medication abacavir have entered routine clinical practice. With ever growing healthcare costs, modest incremental benefits from new pharmaceuticals and the world-wide economic downturn, a crisis is being realised. There has never been a greater need to ration treatment to those who have the most to gain from it.

Cardiovascular medicine is well positioned to benefit from rapid advances in the field of pharmacogenetics. Potential genomic biomarkers for clinically used drugs include a genetic test for warfarin to predict the treatment maintenance dose, for simvastatin to predict the likelihood of myopathy/myositis, for bucindolol to predict potential efficacy in heart failure and for clopidogrel to predict increased recurrent thrombotic events, including stent thrombosis.

Elucidation of the genetic markers predicting response to clopidogrel, the second most-prescribed drug in the world, is of particular importance as a reduced antiplatelet effect with clopidogrel is associated with adverse clinical outcomes including cardiovascular death, myocardial infarction, stroke and additional healthcare costs. Clopidogrel is attractive for pharmacogenetic study as it is a pro-drug that requires conversion to an active derivative, catalyzed by cytochrome P450 (CYPs). The functional polymorphisms within the CYP genes have been relatively well characterised, with those of interest including 3A4 and 3A5, 2C19, 2C9, and 1A2 enzymes. A number of studies have shown that the loss of function allele CYP2C19*2 is associated with adverse vascular outcomes in those taking clopidogrel. While other rarer variants such as the CYP2C19*3 and *4 alleles are
also associated with reduced function of the enzyme, the CYP2C19*17 variant is associated with ultrarapid enzyme activity and may increase the response to clopidogrel. (191) In contrast the third generation thienopyridine prasugrel is not as dependent on the CYP2C19 and CYP2C9 enzymes for biotransformation into its active metabolite. (212)

Genotypes that code for a phenotypic poor response to clopidogrel are more frequently found in some ethnic groups than others. The CYP2C19*2 loss of function variant occurs in 13 percent of Caucasians, 18 percent of African Americans and 29 percent of East Asians. It also occurs in higher frequency in Maori (24 percent) than NZ Europeans (15 percent). (220) CYP2C19*3 is four to five times more frequent in Pacific Peoples and Maori (1.8 percent) than Europeans (0.4 percent). (220, 221) This variant codes for a truncated protein and, together with the *2 allele, accounts for 99 percent of poor metabolisers in related Asian populations. (223)

These ethnic disparities have two potential important clinical consequences. Firstly, these differences should be considered when interpreting trial data. For example, the largest trial evaluating clopidogrel and its effect on mortality was undertaken in 46,000 Chinese patients presenting with ST elevation myocardial infarction (COMMIT-CCS trial). (245) While the response to clopidogrel found in this study might reasonably be extrapolated to a Maori and Pacific Island population, the magnitude of benefit observed may have been greater in other ethnic groups with a lower prevalence of CYP2C19*2.

Taking this hypothesis one step further, it is possible that therapy guided by genomics may help reduce the disparity in treatment outcome in populations such as Maori where cardiovascular disease is highly prevalent and clinical outcomes on treatment are poor.

Pre-determining poor-responders to clopidogrel may aid in optimising antiplatelet therapy in these patients by either giving a higher dose of clopidogrel or using alternative therapy such as prasugrel or ticagrelor. Whilst prospective clinical trials are necessary to assess this theoretically-attractive approach, pharmacogenetic data from TRITON (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction), and PLATO, gave some insights. Patients with the reduced-response allele CYP2C19*2 on clopidogrel treatment had a higher incidence of vascular events, stent thrombosis
and death, whereas those with the same variant on prasugrel had no increased events and, interestingly, no increase in bleeding.(121, 216)

Although this individualised genetic approach to therapeutics may improve the patient’s response to treatment, it does not address the lifestyle changes that need to be implemented to prevent disease, issues such as reduced access to healthcare resources, and socio-economic, educational or cultural influences on treatment choices. Further understanding of the molecular basis of disease may well bring us effective tailored preventative therapies targeted at currently unmodifiable risk factors. We can only hope that these are affordable to the healthcare system, and accessible to disadvantaged ethnic groups.

Genotyping prior to drug administration may be of particular importance for drugs like clopidogrel, which is often started in the acute setting with the need for a rapid and effective antiplatelet effect. Genotyping may also help predict the clinical importance of drug interactions, such as with omeprazole(246, 247) and other CYP2C19 inhibitors. Although phenotyping, using platelet function testing, provides a more integrated assessment of drug response,(248) testing can only be performed after a drug is administered. In addition genomics can provide information on many more dimensions of health than just the response to one drug. There is some evidence that combining genotyping and phenotyping may be more effective in predicting clinical outcomes than either alone.(249)

The pharmacogenetic tests discussed above are now officially endorsed by the US Food and Drug Administration, through the policy direction of the Critical Path Initiative.(250) The aim of the Critical Path Initiative is to reduce the expanding cost of pharmaceutical products. Recent analysis has shown that at a simple three SNP test for warfarin is not cost effective under current average test prices.(251) However the costs of genotyping are reducing exponentially and the era of the $1,000 genome is not far away.

A therapeutic wall appears to have been hit for population-based medicine, with many mainstays of preventative treatment under question due to adverse reactions. With the rapid reduction in diagnostic testing using a companion diagnostic to guide therapy appears appealing and could be cost-effective. However this will only be achieved if the incremental cost of the diagnostic test can be recouped (Figure 20).(252)
With appropriate safeguards in place, a once in a lifetime genetic test should soon be part of every patient’s medical record. Busy physicians will need to integrate this companion diagnostic information into their day-to-day clinical decision-making, when they use the information from clinical trials, patient co-morbidities and potential drug interactions to apply evidence-based practice in the individual patient.

**Figure 20. Microeconomics of Personalised Medicine**

a) Displays current expenditure on a pharmaceutical agent, with substantial portion of spending wasted on treating non-responders. b) Future expenditure based on personalised approach where the therapeutic diagnostic (‘theranostic’) constitutes a fraction of total expenditure. The objective of the targeted approach is to maximise the benefit of next generation pharmaceuticals and minimise potential harm. A cost effectiveness analysis is required prior to adoption of the new model, taking into account savings from prevented events. (252) (adapted from Personalised Medicine: The Emerging Pharmacogenomics Revolution. A 2005 monograph by Price Waterhouse Coopers)
**Chapter 9. Molecular archiving in the era of genomic medicine**

Chapter Eight summarised the findings from previous work and placed its relevance into the field of pharmacogenomics. Adequate collection and storage of DNA and biological material carries great significance. This Chapter discusses the storage of biological material for research and clinical purposes. Archiving of samples for molecular analysis was a crucial part of each of the previous research projects, and also for those projects in subsequent Chapters.

**Introduction**

We now live in the Post-Human Genome Project era. A wealth of information has been delivered into the hands of clinicians and human health scientists. The limiting step for the translation of this data into clinical practice is the linking of disease phenotype with genotype and understanding the complex interactions between the genome and the environment. One of the main obstacles to establishing this link in complex diseases is the difficulty with managing the vast amount of data coming from clinical studies. Whole genome association studies and gene expression analysis, using high density microarrays, deliver hundreds of thousands of data points on each individual patient. This “curse of dimensionality” is being overcome with the establishment of bioinformatics as a new discipline in statistics.

What is thus far becoming clear is that large datasets derived from small numbers of patients do not produce the results required to reach statistical significance. The rate of false discoveries is high. Large collaborative biobanks have been established to overcome this problem. The deCODE and Utah population database are two early examples of biobanks that have delivered a wealth of information over several years.(253) The establishment of the United Kingdom biobank, funded by the Wellcome trust, will dwarf both of these by enrolling 500,000 individuals into a database that hopes to address complex diseases like cancer, heart diseases, diabetes, arthritis, forms of dementia and more. (254)

Smaller biobanks are however still important for the study of rare orphan diseases, such as long QT syndrome.
What follows here is an outline of the issues related to collection and storage of biological samples for future study.

**Biological samples**

The field of Molecular Medicine is rapidly evolving and it would be naïve to specify now the most important biological tissue to preserve. Therefore it makes sense to preserve as much as is practically possible, considering the increased cost, issues of physical space, and ongoing maintenance that such a process involves.

Samples may come from a variety of sources with issues arising from the storage of particular tissue and in preparation for a specific method of analysis. It is possible to collect complete nucleic acid samples, of both DNA and RNA in one collection (Table 20). A method to collect DNA, RNA proteins and metabolites in a single blood tube does not exist as yet, however dried blood spot testing comes close to achieving this goal.

**Table 20. Tissue sample types; collection and biobanking**

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal tissue</td>
<td>• Can be obtained remotely by surface post without the need for specialised collection&lt;br&gt;• Non-invasive</td>
<td>• Ethical considerations of informed consent cannot be addressed easily without face to face contact&lt;br&gt;• Mail-back response may be poor&lt;br&gt;• Subjects may not provide sufficient tissue&lt;br&gt;• DNA yields may be low if specialised collection swabs are not supplied&lt;br&gt;• Tissue may be subjected to unfavourable environments enroute to</td>
</tr>
<tr>
<td></td>
<td><strong>Whole blood</strong></td>
<td><strong>Blood spot</strong></td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>• Provides high yield, high quality DNA &amp; RNA</td>
<td>• Provides moderately high yield, high quality nucleic acids, including DNA and RNA.</td>
</tr>
<tr>
<td></td>
<td>• Yields can be enriched by obtaining buffy coat</td>
<td>• If collected on prepared collection medium (i.e.</td>
</tr>
<tr>
<td></td>
<td>• mtDNA retrievable</td>
<td>• Does not allow for paired sampling of other biological tissue e.g. plasma</td>
</tr>
<tr>
<td></td>
<td>• Plasma can be sampled simultaneously to pair phenotypic data with genomic data</td>
<td>• RNA may not be stable at room temperature for</td>
</tr>
<tr>
<td></td>
<td>• Differential methods can be applied to isolate blood components (e.g.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lymphocytes using a Ficoll® centrifuge gradient medium, or platelets using</td>
<td></td>
</tr>
<tr>
<td></td>
<td>standard centrifugation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Peripheral leucocytes may be used as surrogate for disease tissue in living</td>
<td></td>
</tr>
<tr>
<td></td>
<td>subjects e.g. gene expression profiling in inflammatory conditions</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Organ tissue | Whatman® FTA elute paper) it is highly resistant to endogenous enzymes and other environmental stressors  
  • Can be stored in solid state at room temperature for many years (13 years according to Whatman®) under appropriate conditions. Chapter Ten demonstrates DNA recovery out to nearly three times that length) | more than two weeks (which exceeds whole blood)  
  • Relatively rapid subsequent extraction at a core lab may be necessary to retrieve RNA  
  • Sample is exposed and prone to contamination |
|---|---|---|
| Organ tissue | • May be source tissue for disease  
  • High yield of DNA & RNA  
  • May be suitable for proteomic analysis (requires protease inhibitors)  
  • mtDNA retrievable  
  • Laser capture microdissection allows the collection of highly focused RNA sampling | May be difficult or risky to obtain e.g. endomyocardial biopsy  
  • Nucleic acids can be obtained retrospectively from formalin and fixed specimens. This mostly involves DNA but increasingly RNA is being recognised as retrievable e.g. late viral PCR assays  
  • May only be obtainable post-mortem e.g. brain biopsy |
| Plasma | • May contain platelets and therefore mtDNA and mtRNA retrievable  
  • When collected onto | Limited nuclear DNA & RNA content |
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Characteristics</th>
<th>Analysis Capabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whatman Protein 903 card</strong></td>
<td>Suitable for proteomic analysis</td>
<td>Metabolomic data may be obtainable</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td>Non-invasive</td>
<td>DNA &amp; RNA detectable in urine however cannot be relied upon for sourcing genomic DNA</td>
</tr>
<tr>
<td></td>
<td>May be useful as diagnostic test or marker of disease in context of renal diseases</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metabolomic data may be obtainable</td>
<td></td>
</tr>
<tr>
<td><strong>Faeces</strong></td>
<td>Non-invasive</td>
<td>DNA &amp; RNA detectable in faeces however cannot be relied upon for sourcing genomic DNA</td>
</tr>
<tr>
<td></td>
<td>May be useful as diagnostic test or marker of disease in context of intestinal diseases</td>
<td></td>
</tr>
<tr>
<td><strong>Hair and skin</strong></td>
<td>Can be retrieved post-mortem</td>
<td>Mostly used by forensic teams investigating criminal cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Often requires low-copy PCR to achieve satisfactory nucleic acid quantities for analysis</td>
</tr>
<tr>
<td><strong>Saliva</strong></td>
<td>Can be collected remotely</td>
<td>Limited capability for testing proteins and metabolites due to stability issues</td>
</tr>
<tr>
<td></td>
<td>Commercially available collection systems exist</td>
<td>Highly dynamic medium</td>
</tr>
<tr>
<td></td>
<td>High yield DNA available, suitable for genechip analysis</td>
<td></td>
</tr>
<tr>
<td><strong>Breath</strong></td>
<td>Noninvasive</td>
<td>Difficult to store, though</td>
</tr>
<tr>
<td>Can be performed remotely</td>
<td>can be collected in milar balloons</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td>Contains numerous small molecules</td>
<td>Contains dead space air if collected without fractionation</td>
<td></td>
</tr>
</tbody>
</table>

*Endorsed by UK Biobank (254)*

If collected appropriately, e.g. using a prepared medium (such as Whatman® FTA elute paper) blood spots have the ideal profile for collection. Extensive experience has been obtained using Guthrie cards in the postnatal testing of infants for monogenic diseases and diseases of inborn errors of metabolism.

### Stabilisation

A number of methods can be used to stabilise samples prior to nucleic acid extraction. Collection of samples usually occurs at ambient temperatures but for some types of analysis rapid chilling or stabilisation is required e.g. plasma for proteomics. Samples should not be exposed to direct sunlight. Handling should be minimised to prevent contamination. Stabilisation media exist for immediate preservation of nucleic acids, such as Whatman® FTA elute paper. This paper is embedded with proprietary factors that lyse cells to release nucleic acids, prevent bacterial growth and trap inhibiting substances, such as haemoglobin, in a matrix.(255) This medium also allows one step extraction, using a punch card system, and elutes into DNAse/RNase free water. The presence of the inhibitors in this process means that the extracted nucleic acids are suitable for many downstream applications that require high fidelity samples.(256) The added advantage of this system is that other biological tissues can be stored on the same medium e.g. buffy coat, buccal swab or homogenised organ tissue. The storage and retrieval of DNA from standard Whatman® paper is discussed in Chapter Ten.

An alternative to this method is plasma and buffy coat extraction from whole blood. Ideal sampling of whole blood should be performed using a CPDA (yellow top) vacutube which optimises DNA and RNA retrieval.(257) Tissue and blood can be
preserved in RNAlater™ which recently has not only been shown to preserve RNA, but also yields DNA when an extraction process is applied.\(^{(258, 259)}\)

Samples specifically taken for gene expression can be stabilised in RNAlater™ which has proven to be an extremely effective method of halting transcription and stabilising RNA for prolonged periods, even at room temperature.\(^{(258)}\) An alternative to this approach, in circumstances where immediate processing and extraction are not available, is to use a prepared blood collection tube such as the Qiagen PAXGene™ system,\(^{(260)}\) Applied Biosystem’s Tempus™ tubes\(^{(261)}\) or Ambion’s Ribopure™.\(^{(262)}\) Using this collection tube cellular transcription can be halted until a formal extraction process can be performed some hours later.

**Protein Preservation**

Since proteins can be highly labile, the quality of sample collection can have a drastic impact on downstream protein identification. Samples collected in BD P100™ (Becton, Dickinson and Company, New Jersey, USA) tubes preserve the proteome accurately. These tubes separate red blood cells immediately from plasma and protease inhibitors capture the plasma proteome, before enzymatic degradation can occur.\(^{(263)}\) This has the advantage of preserving a physiological state at a certain point in time; however it may not reflect the type of samples that are collected clinically. BD P100™ tubes are still a research tool and are expensive. The advantage of short-term storage at ambient, or minus 20 degrees centigrade, with BD P100 tubes make these ideal for field studies but paired sample collection with EDTA (ethylene diaminetetraacetic acid) tubes may be more appropriate, to compare collection methods and gain clinical reproducibility (In Chapter Eleven clinical samples for proteomics were collected in EDTA tubes).\(^{(264)}\) The degradation of proteins in EDTA tubes results in a peptidome (endogenous peptides) which may have clinical value in itself.\(^{(265)}\)

Other experimental conditions, occurring in the collection and processing phase, can affect the outcome of a proteomic study. For some proteins the method of sampling, ambient conditions and time to analysis will have an effect on results, for example TNF\(\alpha\) and IL-2 are particularly labile at usual ambient temperatures.\(^{(266)}\)

Sample handling after collection, prior to mass spectral analysis, can also alter eventual results. For instance depletion of proteins will eliminate a set of proteins in
which valuable data may be derived. Structural alteration to common, high abundance proteins such as albumin (modified by ischaemia), may be an important finding in certain disease states.(267) Depletion alters the physiological proteome in such a way that these types of changes may be over-looked. These issues are discussed further in Chapter Eleven.

Proteomics has been used in the evaluation of cardiac ischemia in animals(268), and humans,(269) and in models of preconditioning.(270) It has also been used in the evaluation of drug response around the time of coronary angioplasty.(271) Proteomes from body fluids, other than plasma, have been investigated e.g. urine(272) and saliva.(273) However there are particular issues with each body fluid, particularly the salivary proteome, which is extremely dynamic.(274)

**Metabolite Preservation**

Metabolomics has been an attractive alternative to proteomics due to the limited number of possible metabolites and the belief that metabolites reflect the final composition of all metabolic pathways. Of note however is the impact external influences, such as gut flora and diet, can have on metabolome variation.(275, 276) Proteomic and metabolomic approaches can be amalgamated to provide a complete gestalt view of human physiology. Integration of this data is described in Chapter Eleven.

As with proteomics, metabolites are influenced by cellular activity, therefore quenching needs to occur quickly to preserve a metabolic state at a particular time-point and to minimise the effects of ex vivo blood collection.(277) There is no commercially available blood tube for the collection of metabolites as there is with the P100 proteomics tube. Metabolites have been stored and extracted from dried blood spots effectively for years in screening programs for inborn errors of metabolism. This method is also used by the pharmaceutical industry in pharmacometabolomics studies.(278)

**DNA Extraction**

Traditional methods of DNA extraction use hazardous chemicals and solvents such as chloroform and phenol. Using these substances in a laboratory requires specific safety measures and handling protocols. A fume hood is necessary when handling these compounds and this may increase the cost for any low budget operation.(279)
Other methods of extraction include salting out methods, and matrix elution. (280) Newer technologies such as magnetic bead extraction systems avoid any toxic chemicals and are used in robotic high throughput extraction processes without multiple pipetting steps. Robotic systems provide consistent extractions which have benefits for massively paralleled downstream applications. (281) Though initially expensive they become cost effective in a high throughput environment.

Extraction from solid tissue poses some added challenges. Steps to prepare the sample are required which include homogenisation and cellular lysis. (282) Added steps increase the chances of disruption of RNA, particularly by shearing forces from the homogeniser. Increased chances of contamination also become an issue.

**Storage**

Adequate storage must be secure, effective and robust over time to respect the donors who have supplied samples and the efforts invested by researchers.

**Archiving**

Any archival system must include a systematic storage and retrieval system. Common sense dictates that this requires a marker system with identifiers for each sample e.g. barcoding or numbering. If samples are to be stored at low temperature, possibly with other solvents, then the identifying markers must be resistant to smudging and dissolution etc. The registration of each sample entering and exiting the system must be centrally stored, preferentially on a computer based system that can be backed up, if necessary. The physical location of each sample should be registered to allow the rapid location of specimens.

The archival system should de-identify samples to respect the privacy of donors and allow blinding of researchers to analysis results. For the same reasons the database including clinical data should be kept separately with a method to link clinical information to tissue samples that cannot be easily accessed. A separate electronic key, held by a curator of the database, would be ideal to prevent tampering.

Software driving these systems should be encrypted or password protected to further ensure privacy and confidentiality to donors.
**Room temperature storage**

Storage at room temperature is the ideal method for nucleic acid storage but may not be suitable for other biological tissues. Low temperature storage carries a number of disadvantages, with the possibility of freezer failure being the most critical. Costs of running, maintenance and the physical space required for freezers limits their capabilities. Because of these concerns there is a move towards room temperature storage which overcomes a lot of these problems.

**Security**

Archived samples must be protected physically from accidental or intentional damage. Therefore basic protection against fire, water and theft must be considered. Minimising access to only a few individuals reduces the chances of unexpected events. Replicates or split samples stored at a separate location also reduce the chances of loss of samples.

**Ownership/Curatorship**

In the lifetime of a project researchers may enter and leave. Ownership of samples ultimately should always remain with the donor; however the ownership of a collection is a complicated issue that needs to be predefined before banking begins. An independent curator, who controls access, may be necessary to manage the biobank. An independent bioethics committee ideally should also oversee the biobank and regulate access.

**Intellectual property**

With any research there is the potential for new discoveries that could lead to novel diagnostics or therapeutics. For the further development after discovery it may be necessary for researchers to capture the intellectual property with patents. This then allows for capital to be raised for further development. Many biobanks set up for community and nonprofit purposes have processes developed for intellectual property. The P3G consortium is an example of a group who help with the development of such processes. Some biobanks set a fee for industry or researchers to access samples with the knowledge that information discovered could lead to novel products.
Ethics/Consenting/Privacy

As with the collection of any data or biological samples the rights, autonomy, cultural and gender specific issues for donors must be respected. The deCODE database of the Icelandic population is a case example of where this has come into dispute. This database was originally established as a publicly owned database but after many samples had been collected it transformed into a privately owned entity. The company now collaborates with a number of pharmaceutical and diagnostics companies and is developing lead compounds and diagnostic tests for commercial use. The population of Iceland has retained some intellectual property of any discoveries made as drugs forthcoming from deCODE’s research must be offered to the Icelandic people free of charge. These issues are raised in Chapters Twelve, Thirteen and Fifteen.
Chapter 10. Posthumous diagnosis of long QT syndrome from the neonatal screening card

In this Chapter a clinical application of genomic medicine is explored. The channelopathies are a group of inherited cardiac arrhythmias caused by mutations in ion channel genes. This Chapter describes a case series of patients with long QT syndrome, where a posthumous genetic diagnosis was made from dried blood spots on Whatman® paper. The collection and use of these dried blood spots has relevance to the issues discussed in the previous Chapter. Since 1969 all newborns in New Zealand have undergone screening for inborn errors of metabolism, through collection of blood onto Whatman® paper. This screening process uses metabolomics, which is discussed in Chapter Eleven. The applied use of both genomics and metabolomics in testing neonatal cards is of particular interest as these broad methods span both hereditary and environmental factors. Once biomarker profiles for preclinical disease are identified these methods could be applied on a population-wide basis for screening programs in the future. Some of the issues surrounding the retrospective use of these neonatal screening cards are discussed in this Chapter as well as in Chapter Twelve.

Introduction

When a young person dies a natural sudden and unexpected death and the standard autopsy is uninformative, the grieving family is universally desperate for an explanation. A molecular autopsy can deliver a diagnosis of cardiac channelopathies such as long QT syndrome (LQTS), Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia in 40 percent of cases. Such diagnoses enable effective screening and management of the deceased's family, with the aim of preventing other sudden deaths in the same family.

Obtaining DNA posthumously is problematic if appropriate samples are not collected at the time of the postmortem. Stored formalin-fixed, paraffin-embedded tissue blocks may not yield DNA of sufficient quality for testing as DNA can be degraded by the process and will also undergo degradation over time. In some instances, tissue blocks are not archived adequately for retrieval. Pathologists and coroners may also be under pressure to avoid unnecessary tissue storage after public concern...
over long-term organ or tissue retention. New best-practice recommendations for the investigation of young natural sudden death were recently developed by a clinical and scientific collaboration of Australia and New Zealand known as TRAGADY (the Trans-Tasman Response Against Sudden Death in the Young). These stipulate that tissue or blood suitable for DNA extraction should be stored. However, not all practitioners adhere to these guidelines, and many families still seek a diagnosis for deaths occurring many years ago. Neonatal screening (Guthrie) cards provide a potential source of DNA in countries where the cards are stored long term and are available for retrieval.

**Figure 21. A neonatal screening (Guthrie) card**

*Four drops of blood are taken from all newborn infants onto the newborn screening (Guthrie) card in the first week of life (permission to use image obtained from the National Testing Centre of New Zealand)*

In New Zealand, blood spots on Guthrie cards have been collected from newborns since 1969. Standardized cards of untreated Whatman no. 903 paper are used to collect four heel-prick blood spots (Figure 21). These are used to screen for diseases of inborn errors of metabolism by the National Testing Centre. The cards are then stored indefinitely at ambient temperature and are available on request to the individual to whom the sample belongs or to other individuals who have the authority to access according to a strict protocol (such as the coroner or next of kin). They may also be requested for the purposes of research after approval by the program advisory committee and an Ethics Committee. Genetic testing of blood spots from Guthrie cards occurring years or decades after collection has not been well-described. However, the practice of DNA storage using
blood spots on Whatman no. 903 paper is well established. This paper is used by existing commercial biobanking products, although the paper is altered and contains preservative agents for nucleic acid storage.(290)

With the increased awareness of cardiac channelopathies as a cause of young sudden death, and in particular LQTS, families and their clinicians may seek a molecular genetic diagnosis months or years after the death has occurred.

Since most pathologists have not, until recently, stored blood or tissue suitable for DNA extraction, it would be valuable to know whether adequate DNA for testing could be obtained from Guthrie cards, which may be decades old. This Chapter describes the investigation of a series of young sudden unexplained deaths up to 13 years after the death occurred, where no tissue suitable for DNA extraction had been retained at autopsy. An attempt to obtain DNA from Guthrie cards in these patients and analyse the LQTS-associated genes was made.

**Methods**

This audit describes 21 cases where the neonatal screening card was accessed for DNA extraction to find the cause of a young sudden and hitherto unexplained death. These families were being investigated by the New Zealand Cardiac Inherited Diseases Group (CIDG), who were approached by the family, by a family doctor, or through Coronial Services, between two and 13 years after the death. CIDG has a national coordinator who keeps a record of all investigated cases. Families and individuals in whom an inherited heart disease is identified are registered (with their informed consent) with the CIDG national registry, which has ethical approval from the Multi-Regional Ethics Committee. This registry, in collaboration with the clinical genetic services, also facilitates family screening. Postmortem investigations had been negative in each case, and no tissue suitable for DNA extraction had been retained. Family cardiological investigations had either failed to make a diagnosis of inherited heart disease or had revealed some suspicion of LQTS.

In 19 cases, LQTS could not be proven unequivocally by electrocardiogram (ECG) in a first-degree relative, so a genetic screen of the long QT genes was performed on the Guthrie card of the deceased. In two additional cases, LQTS was identified in other family members, and a genetic diagnosis had been made in a relative. In these, only the familial mutation was analysed in DNA isolated from the card of the sudden death victim.
Residual blood from newborn screening testing, stored by the New Zealand newborn metabolic screening program, was released for DNA retrieval at the request of an appropriate relative of the deceased. Informed consent from the next of kin was obtained to test for LQTS. The tests were performed either at the University of Auckland or in a clinical laboratory (LabPlus) at Auckland City Hospital.

The Guthrie cards in this series were stored in ambient conditions in Auckland, New Zealand, with annual temperature variations from 7°C to 32°C (45°F to 90°F) and relative humidity 60 to 80 percent.

Four 3-mm hole punches were made in one of the four blood spots on the Guthrie cards. DNA was extracted from the punches using two different methods. The QIAamp DNA mini kit (n = 6; Qiagen Inc., Hilden, Germany) was used at the LabPlus clinical diagnostics laboratory (Auckland City Hospital, New Zealand), and the Masterpure extraction kit (n = 15; Epicentre, Madison, WI) was used at the University of Auckland, both according to the manufacturers' recommendations. Average DNA yield was 50ng/μL in an elution volume of 100 μL and was of sufficient quality for polymerase chain reaction (PCR). (Figure 22)

Most DNA samples were initially analysed using the Transgenomic 2100 WAVE DNA fragment analysis system (Transgenomic, Omaha, NE) and Navigator version 1.5.1 software (2003, Transgenomic, Omaha, NE). Amplicons encompassing the coding exons of the KCNQ1, KCNH2, and SCN5A genes (LQT1, 2 and 3, respectively) that exhibited variant denaturing high-performance liquid chromatography (dHPLC) profiles were purified using the Qiaquick PCR purification kit (Qiagen, Inc.). The purified amplicons were subjected to DNA sequencing using the Big Dye Ready reaction kit. version 3.1 (Applied Biosystems, Foster City, CA), with subsequent electrophoresis in an ABI Prism 3130XL Genetic Analyser. In the case of the six most recent samples, all coding exons of the above genes, together with those of the KCNE1 and KCNE2 genes (LQT5 and 6, respectively), were subjected to direct DNA sequencing only without prescreening using dHPLC. The reporting of variants follows Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen/) and used the following accession numbers: NM_000218.2 (KCNQ1), NM_000238.2 (KCNH2), NM_198056.2 (SCN5A), NM_000219.3 (KCNE1), and NM_172201.1 (KCNE2).
DNA was extracted from a Guthrie card, and amplicons encompassing the coding exons of long QT genes 1, 2, 3, 5, 6, and 7 were PCR amplified. The products were electrophoretically separated in an agarose gel. The DNA ladder is on the right-hand side of the gel.

Any novel unclassified variants that were identified were assessed in 100 control chromosomes using restriction fragment length polymorphism analysis if a suitable restriction enzyme was available. Candidate mutations that failed to generate restriction fragment length polymorphism changes were assessed by dHPLC of controls versus mutation positive profiles to test the frequency of the abnormal profile in the control population.

Testing of Guthrie cards for cascade screening was carried out by direct DNA sequencing of the relevant exon. The SALSA MLPA P114 LQT kit (lot 0805, MRC-Holland, Amsterdam, The Netherlands) was used to identify a large deletion (KCNH2 ex6–14del) in a deceased family member of an affected proband. This methodology has previously been discussed and reported this mutation.(291)

All of the families that were investigated in this study were registered with the consent-based Cardiac Inherited Disease Registry, which has national ethical approval. Approval for the present study was obtained from the Northern Regional Ethics Committee.
Results

Guthrie cards were retrieved up to 13 years after death. Deaths occurred at less than one year in one individual, one to 18 years in 18 cases, and 19–35 years in two further cases. Although a sufficient quantity and quality of DNA was eventually obtained from all of the Guthrie cards, much more time and technical skill was required than was needed for routine screening of DNA samples extracted from whole blood. Standard PCR conditions needed to be amended for several amplicons. PCR cycle number was increased from 30 to 40 cycles in most cases, and several more repeat steps were required throughout all stages of the methodology.

A full diagnostic screen was performed in 19 cases, revealing long QT gene variants in six (32 percent) cases. Cascade testing for familial mutations was performed on two cards with a positive result in both. The results are listed in Table 21, and the clinical histories and details of first-degree relatives are described briefly below.
Table 21. Clinical features and molecular genetic diagnosis made from DNA extracted from neonatal screening (Guthrie) cards of 21 victims of SUDY

A. Full screen

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Gender</th>
<th>Age of Guthrie card, years</th>
<th>Age of Death</th>
<th>Trigger/circumstance before SCD</th>
<th>Gene</th>
<th>Gene variant</th>
<th>Amino acid substitution</th>
<th>LQT type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>15</td>
<td>12 years</td>
<td>Exercise/previous symptoms while swimming</td>
<td>KCNQ1</td>
<td>727C&gt;T&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R243C</td>
<td>LQT1</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>8</td>
<td>2 years</td>
<td>Gastroenteritis</td>
<td>KCNH2</td>
<td>3140G&gt;T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R1047L</td>
<td>LQT2</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>4</td>
<td>21 months</td>
<td>Sleep</td>
<td>KCNH2</td>
<td>243G&gt;C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Q81H</td>
<td>LQT2</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>11</td>
<td>6 years</td>
<td>Emotional stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>10</td>
<td>19 months</td>
<td>Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>20</td>
<td>13 years</td>
<td>Exercise</td>
<td>KCNQ1</td>
<td>1363C&gt;T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H455Y</td>
<td>LQT1</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>23</td>
<td>13 years</td>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>8</td>
<td>2 years</td>
<td>Febrile illness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>11</td>
<td>6 years</td>
<td>Bathing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>22</td>
<td>16 years</td>
<td>Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>14</td>
<td>8 years</td>
<td>Emotional stress with history of epilepsy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>19</td>
<td>4 years</td>
<td>Bathing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>15</td>
<td>12 years</td>
<td>Asthma exacerbation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>7</td>
<td>2 years</td>
<td>Mild febrile illness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>39</td>
<td>34 years</td>
<td>Reading newspaper</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>32</td>
<td>28 years</td>
<td>Startled by alarm clock</td>
<td>KCNH2</td>
<td>1861A&gt;C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S621R</td>
<td>LQT2</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>20</td>
<td>17 years</td>
<td>Exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>4</td>
<td>19 months</td>
<td>Lower respiratory tract infection one week prior</td>
<td>KCNE1</td>
<td>112A&gt;G&lt;sup&gt;c&lt;/sup&gt;</td>
<td>S38G</td>
<td>LQT5</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>3</td>
<td>6 months</td>
<td>Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Cascade test

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Gender</th>
<th>Age of Guthrie card, years</th>
<th>Age of Death</th>
<th>Trigger/circumstance before SCD</th>
<th>Gene</th>
<th>Gene variant</th>
<th>Amino acid substitution</th>
<th>LQT type</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>F</td>
<td>26</td>
<td>13 years</td>
<td>In bathroom</td>
<td>KCNH2</td>
<td>exon 6-14del&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td>LQT2</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>9</td>
<td>8 months</td>
<td>Sleep</td>
<td>KCNQ1</td>
<td>436G&gt;A&lt;sup&gt;c&lt;/sup&gt;</td>
<td>E146K</td>
<td>LQT1</td>
</tr>
</tbody>
</table>

Note: Panel A lists those undergoing a diagnostic screen of the long QT (LQT) genes. Panel B lists those in whom a genetic diagnosis of LQTS was made in another family member and mutation analysis was carried out on the deceased's Guthrie card. F: female; M: male; SCD: sudden cardiac death

<sup>a</sup>Variant previously described in a patient with LQTS

<sup>b</sup>Variant with putative association based on in vitro testing

<sup>c</sup>Variant with putative association but in vitro testing not available

<sup>d</sup>Novel variant
Cases undergoing a diagnostic screen of the LQT genes

Cases in whom the genetic variants are considered likely to be of aetiological significance in the cause of death

Case 1

This 12-year-old boy was diagnosed with, and treated for, epilepsy during life and had a classical history of arrhythmic syncope and seizures during or after swimming. He died suddenly warming up for a hockey game. Screening of the Guthrie card revealed a missense mutation in the KCNQ1 (LQT1) gene, c.727C>T (p.R243C). (292) He was the index case for this series and has been described elsewhere. (293) The mother was asymptomatic but had a baseline QTc of 450 ms, which failed to shorten with exercise; genetic testing subsequently revealed she was a carrier for the mutation and commenced beta-blocker therapy. An only sibling of the proband had a borderline QTc of 450 ms that shortened appropriately with exercise; genetic testing was negative and reassurance was provided.

Case 3

This 21-month-old female infant died during sleep. A few weeks before death, she had been treated in the children's emergency department and diagnosed with a febrile convulsion. The evening before her death, she had another event during which she had become pale and lifeless and had tonic-clonic movements, possibly associated with a low-grade fever. The parents phoned the hospital and were advised to keep her cool, but when she was checked a few hours later she was deceased in her bed. Full screening of the Guthrie card revealed a novel missense mutation in the KCNH2 (LQT2) gene, c.243G>C (p.Q81H). This mutation occurs in the intracellular amino-terminal domain of the KCNH2 gene-encoded protein that is critical for channel deactivation. Functional studies of other mutations in the amino-terminal region appear to decrease the K+ current during cardiac repolarization, resulting in a prolonged cardiac action potential. (294) The father tested positive as a carrier of the mutation but was asymptomatic and had a QTc of 420 ms. Beta-blockers were not prescribed, but advice was given regarding the avoidance of QT-prolonging medications and of loud noise at night. Cautious cascade screening has been offered to the wider family to evaluate the cosegregation of phenotype and genotype, and in vitro functional testing is planned.
**Case 6**

This 13-year-old boy collapsed suddenly while playing soccer. A premorbid ECG showed a QTc of 600 ms. Suitable DNA was not retained at autopsy, and full screening of the proband's Guthrie card revealed a novel mutation in the KCNQ1 (LQT1) gene, c.1363C>T (p.H455Y), for which in vitro testing has confirmed likely pathogenicity.(295) His 43-year-old asymptomatic mother, with a QTc of 490 ms, is a carrier of the H455Y mutation and is on beta-blockade. A sister who is also an asymptomatic mutation carrier with a QTc of 420 ms also elected to take a beta-blocker.

**Case 16**

This 28-year-old woman died soon after activation of an alarm clock. A missense mutation was identified in the KCNH2 (LQT2) gene, c.1861A>C (p.S621R). This mutation is located in the pore region of the KCNH2 gene-encoded protein and most likely affects ion transfer across the potassium channel and prolongs ventricular depolarization.(296) An ECG ordered some months earlier by the family doctor was reported in the notes as normal but in retrospect showed overt QT prolongation. ECGs performed on the father and sister showed QTcs of 417 and 360 ms, respectively, and they are genotype negative. The daughter (age three) of the proband had a QTc of 500 ms and tested positive for the mutation. She is being treated with a beta-blocker. Several other more distant relatives have been identified as affected gene carriers.

**Cases with rare variants of uncertain significance**

**Case 2**

This girl died at the age of two years. She was observed to have bradycardia in utero and shortly after delivery developed 2:1 AV block, QTc 480 ms, and a pacemaker was implanted. She developed polymorphic ventricular tachycardia and ventricular fibrillation at the time of pacemaker implantation. At 20 months of age, she developed congestive heart failure with a dilated cardiomyopathy. She died suddenly during an episode of gastroenteritis. Full screening of her Guthrie card revealed a missense variant in the KCNH2 (LQT2) gene, c.3140G>T (p.R1047L). This rare variant lies within the domain that is essential for channel physiology, but the variant protein appears electrophysiologically indistinguishable from a wild-type channel.(297) Both parents and a sibling were asymptomatic and had a normal QTc. The family was
provided with reassurance based on the current literature, but will be monitored as the clinical relevance of this variant remains unclear.

**Case 18**

This 19-month-old boy was found face down deceased in his bed. There had been a preceding history of wheezy breathing. An ECG performed on day one after birth showed a QTc of 400 ms. The cause of death could not be determined from the postmortem. DNA samples retrieved from formalin-fixed paraffin-embedded tissue blocks were inadequate for genetic testing owing to the presence of inhibitory factors. Full screening of the DNA from the Guthrie card revealed a missense variant in the KCNE1 (LQT5) gene, c.112A>G (p.S38G). This has been recognised to be a relatively common polymorphism. The parents of the proband had normal QTc intervals on ECG; however, the father had an incomplete right bundle branch block. He proceeded to pharmacological challenge of the cardiac sodium channel with flecainide to investigate for Brugada syndrome, which was negative. This variant was felt unlikely to be of pathological significance, and cascade screening was not offered.

**Cascade screening performed using the Guthrie card DNA**

**Case 20**

This 13-year-old female died suddenly in the bathroom. A 27-year-old female sibling was found to have a history of seizures with exercise and had been treated with sodium valproate, despite a normal electroencephalogram. An ECG showed a QTc of 566 ms, and she was diagnosed with LQTS. Beta-blockers were started, and an implantable cardioverter-defibrillator was implanted. Initial testing of the 27-year-old’s DNA showed no mutation; however, subsequent gene dosage analysis using MLPA (multiplex ligation-dependent probe amplification) detected the presence of a large heterozygous deletion of exons 6–14 in the KCNH2 gene (KCNH2 ex6–14del). This novel deletion was considered highly likely to be implicated with LQTS, but its exact functional relevance has not been established. After this discovery, MLPA performed on the Guthrie card of the deceased 13-year-old girl revealed the same deletion and gave a cause for her sudden death.
Case 21

This eight month-old girl died during sleep, with no preceding illness. Subsequently, a two year-old sibling also died during her sleep. Frozen spleen was preserved from this second autopsy, and DNA was extracted, revealing a missense mutation in the KCNQ1 (LQT1) gene, c.436G>A (p. E146K). This variant has previously been associated with the LQTS and described as a mutation. Interestingly, polyphen analysis of this variant predicts the effect of this mutation to be benign. In vitro testing results are not available. DNA from the Guthrie card of the deceased eight month-old revealed that the same variant was present. Further family phenotype-genotype cosegregation study was inconclusive. Two asymptomatic siblings have ECGs in the borderline range; one is positive, the other negative, and the father is an asymptomatic carrier with a QTc in the upper normal range. Wider family screening is underway observing carefully for phenotype-genotype cosegregation in addition to in vitro testing prioritisation.

Discussion

This Chapter describes a case series of 21 individuals in whom DNA of sufficient quality was obtained from residual material from the newborn screening Guthrie cards to perform molecular diagnostic testing. Full screening of genes linked to LQTS was performed successfully in 19 cases, and cascade screening in two cases. From this analysis, genetic variants were found in eight individuals, six of which indicate that LQTS was the likely cause of death. These results have provided a valuable diagnosis to the families of the sudden death victims, clinicians, forensic pathologists, and coroners. In terms of the latter, the certified cause of death has been altered significantly. For example, two cases were initially described as death due to seizure disorders.

Increasingly, population-based studies are showing that sudden natural death is far more common than previously predicted, with an incidence of more than 20 per million per year in one to 35-year-olds. It is only by accurately assigning cause of death that meaningful data can be obtained to inform health service provision. The molecular autopsy can work alongside family heart screening, which itself can identify cause of death in 40 percent to 53 percent of autopsy-negative sudden death cases.
The oldest card tested in this series was 39 years old. Obtaining adequate quality DNA from Guthrie cards is clearly feasible, and when archived, these cards could potentially act as a DNA “bank” for future testing. As a source of DNA, the newborn screening Guthrie cards are usually superior to paraffin-embedded formalin-fixed tissue blocks.\(^{286, 287}\) The accuracy of the results in the present study is also partly confirmed by finding the same mutations in living relatives. Furthermore, the diagnostic hit rate of 21 percent (four pathogenic LQTS mutations among 19 full screening sudden unexplained deaths in the young [SUDY] cases) is similar to other postmortem case series. Tester and Ackerman\(^{285}\) found 10 LQTS-associated mutations among 49 postmortem negative SUDY victims (20 percent). Tan et al\(^{301}\) and Behr et al\(^{302}\) found that 12 percent and 23 percent had definite LQTS in their series, respectively, by cardiological screening of family members. However, extracting and purifying the DNA in this setting was time-consuming and technically challenging. Dried blood spot storage would not be recommended at postmortem if the primary aim is to store a sample for DNA analysis. In accordance with best-practice autopsy guidelines,\(^{288}\) it is better to extract and store DNA (preferably from spleen or liver) early after the postmortem and to store blood or tissue at minus 80°C or in a medium capable of protecting cellular RNA, such as Ambion RNAlater™ (Applied Biosystems, Victoria, Australia).\(^{288}\)

Furthermore, identification of a definite proband among living family members using clinical tests would allow analysis of DNA from fresh whole blood, and the Guthrie card sample could be tested with much greater ease for the family mutation only, as in the final two cases of the present report. This approach could equally apply to other inherited heart conditions such as catecholaminergic polymorphic ventricular tachycardia, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy, all of which may escape diagnosis by standard postmortem examination.\(^{301, 303}\)

Testing residual material from stored neonatal screening cards for population genetics studies is well described, and the success of these large-scale studies, with standardised methods, suggests that testing archived cards up to 25 years old is possible.\(^{304, 305}\) Storage conditions in these studies are relevant. Amplicon lengths of up to 480 bp have been genotyped in samples stored for 25 years in a moderate climate (Washington State) and up to 1039 bp in tropical climates for 10 years.\(^{306}\) The longest amplicon in the series reported in the present study was 574 bp. Importantly, improvements in technology such as whole genome amplification
may allow testing of Guthrie cards that have previously been deemed inappropriate for the isolation of DNA for subsequent testing.

**Conclusion**

Neonatal blood spot collection and storage is already standard practice in many countries for biochemical screening. The present study shows that these cards provide a backup archival DNA resource for genetic interrogation in deceased individuals for disorders that have clinical implications for living family members. Neonatal cards carry significant value for research purposes. Ethical issues, for this type of research, need to be addressed as informed consent for future research is not obtained at the time these cards are collected. In New Zealand approximately two million neonatal cards are stored for a current population of 4.2 million. Since DNA can be retrieved from even the oldest of these cards this collection is of significant potential value to New Zealanders. Privacy concerns have led to discussions over the destruction of these cards.(307)

Chapters Twelve and Thirteen describe population genomic studies, using DNA material within a research database. The value of stored biological material is significant and future uses cannot always be predicted. New Zealand is a unique environment where potentially large population genomic studies could be performed over a lifetime, with regular assessments of phenotype. Whilst many biomarker and genome wide association studies have focused on established disease, in the future more focus will need to be placed on well populations with subclinical disease. Plotting trajectories for well patients will enable targeting of invasive screening programs. These issues are discussed in more detail in Chapter Twelve.
Chapter 11. An integrated network analysis of metabolomic and proteomic profiling in cardiac ischaemia

Chapter Nine discussed varying methods for biosample collection. Proteomic and metabolomic research is the natural extension to genomics, as these methods assess the expression of the genome, its interaction with other endogenous elements and the environmental. This Chapter describes a project which used metabolomic profiling, and integrated proteomics. Targeted metabolomic profiling is used in population screening for inborn errors of metabolism, discussed in Chapter Ten. It can also be used to demonstrate acute disease processes as in this Chapter. The objective in this Chapter was to identify mechanistic pathways involved with the acute physiological insult of coronary angioplasty. This Chapter also discusses the value of metabolomics and proteomics for biomarker discovery.

Introduction

The heart is highly metabolic and consumes more energy than any other organ.(308) Unsurprisingly, cardiac energetics and metabolism is an area of intense study. Metabolomic profiling is ideally suited for the analysis of cardiac metabolism in healthy and diseased states. Metabolomics is a relatively newer ‘omic technology that takes a high-throughput, global approach and screens for thousands of metabolites using either targeted or untargeted methods.

Cardiac metabolomics is still an emerging field with significant research activity in the examination of metabolic diseases, such as diabetes.(309-311) Early work in the field was hindered by a lack of validation studies,(312, 313) however independent consistencies are now emerging, which can be taken as an encouraging sign. Whilst many metabolomic studies are in the preclinical space, clinical metabolomics is showing promise as a diagnostic and prognostic tool for complex diseases. Due to its agnostic approach, and breadth of observation, its strength is in identifying novel pathways, not previously related to disease.

Cardiac ischaemia is a logical target for applied clinical metabolomics. Work by Lewis et al has demonstrated a small number of metabolites that could potentially be used as a diagnostic tool for myocardial infarction.(268)
This Chapter describes a metabolomic profiling of plasma, taken from the coronary sinus immediately prior to coronary angioplasty and at several subsequent time points following the procedure. The purpose for this was to characterise mechanistic alterations in cardiac metabolism at the time of PCI. Intracoronary metoprolol was administered to a number of patients in addition to the standard metoprolol treatment all patients were given. The purpose of this was to investigate the metabolic effects of metoprolol in a subgroup of patients. The hypothesis of this study was that this method would identify alterations in pathways related to cardiac energetics, apoptosis due to ischaemia, coagulation, inflammation and preconditioning. A targeted approach using GC-MS was undertaken and an attempt to merge proteomic data with metabolomic data in pathway analysis was made.

**Methods**

This study was approved by the Northern Regional Ethics Committee for New Zealand. 33 patients gave informed consent to be enrolled into the trial. Patients with a first non ST elevation myocardial infarction and serum troponin T >0.1mmol/l undergoing coronary angiography between 24 hours and six days following were screened. Eligible patients with a single identifiable culprit lesion either of the left anterior descending (LAD) or dominant right coronary artery (RCA) and who were to undergo adhoc PCI were randomised.

A double blinded 2:1 LAD to RCA randomisation through a pseudorandom number generation program was followed. The RCA venous return drains directly in to the right ventricle and therefore this was considered a control for LAD angioplasty, draining into the coronary sinus. Patients were also randomised 1:1 to receive intracoronary metoprolol at the beginning of the procedure.

Exclusion criteria included: acute myocardial infarction within the preceding 24 hours, haemodynamic instability (including cardiogenic shock, systolic blood pressure <100mmHg, uncontrolled heart failure or significant LV impairment (EF <35 percent)), significant (moderate-severe) valvular disease, renal impairment (creatinine >0.16mmol/l), occluded vessel or extensive angiographic thrombus on diagnostic angiography and contraindication to beta blockade (including: asthma, current use of bronchodilator therapy, 2nd / 3rd degree AV block, known sick sinus syndrome or baseline bradycardia <50bpm).
At the time of the intervention the majority of patients were taking an oral beta-blocker, which was continued during the study. All non-study medications including use of heparins, direct thrombin inhibitors, clopidogrel and glycoprotein IIb/IIIa inhibitors were administered at the discretion of the leading physician.

**Study procedure**

Baseline angiography was performed in the usual manner. A catheter was advanced through the coronary sinus and into the great cardiac vein to ensure selective sampling of LAD territory drainage. Catheter position was confirmed by contrast injection. Baseline blood samples were simultaneously taken from the great cardiac vein and ascending aorta. Following crossing of the culprit coronary lesion with an angioplasty guide wire, intracoronary metoprolol or placebo (randomised 1:1) were administered through the guide catheter (30μg/kg in two 5ml doses, or 10ml 0.9% saline, over two minutes). A post-beta blocker sample was then obtained from the great cardiac vein one to two minutes after study drug administration. Angioplasty was then performed with a mandated initial predilatation of 60 seconds, unless otherwise indicated on clinical grounds. Beginning 10 seconds after the first balloon deflation a further blood sample from the great cardiac vein was taken. Following PCI and at least 20 minutes after the first and five minutes after last balloon inflation, final blood sample from the great cardiac vein (CS), ascending aorta (AO) and femoral vein were drawn.

Blood sampling was done at baseline (pre-PCI) and 18 ± 2 min from the first balloon inflation (post-PCI), using multipurpose catheters placed in the coronary sinus. Blood was collected into 5 ml EDTA (ethylenediaminetetraacetic acid) vacutainer tubes (Becton, Dickinson and Company) and centrifuged at RT at 3310 x g for five mins, within two minutes of collection for proteomics and within 15 minutes for metabolomics. For proteomics one ml of plasma was pipetted into microtubes containing pepstatin A (Sigma Aldrich) and bestatin (Sigma Aldrich) to give a final concentration of 8 μmole/L of pepstatin A and 16 μmole/L of bestatin. All samples were snap frozen in dry ice methanol slurry.
Laboratory Procedures

Metabolomics

Extraction

The plasma samples were aliquoted into an eppendorf tube (250 μL) followed by the addition of 20 μL of internal standard (10 mmol solution of 2,3,3,3-d4 DL-alanine). The samples were then freeze-dried. Metabolites were then extracted according to an optimised protocol developed by the Villas-Bôas laboratory; 500 μL of cold methanol: water (4:1 v/v) at -30 °C was added to the freeze-dried sample. The sample was then mixed vigorously for 30 s using a vortex mixer and then centrifuged for 15 min at 10,000 g. The supernatant was then collected and sample stored at -80 °C. The samples were then freeze-dried after the addition of 3 mL of cold bi-distilled water (4 °C).

Sample derivatisation

The methyl chloroformate (MCF) derivatization was performed according to Villas-Bôas et al. (314) In summary, the dried samples were resuspended in 200 μL of sodium hydroxide solution (1 M) and mixed with 34 μL of pyridine and 167 μL of methanol. 20 μL of MCF was added to the reagent mixture followed by vigorous mixing for 30s using a vortex. Another 20 μL of MCF was added to the reactive mixture followed again by vigorous mixing for another 30 s. To separate the MCF derivatives from the reactive mixture, 400 μL of chloroform was added to the mixture and then mixed vigorously for 10 s followed by the addition of 400 μL of sodium bicarbonate solution (50 mM) and vigorous mixing for an additional 10s. The upper aqueous layer was discarded and the chloroform phase was subjected to GC-MS analysis.

GC-MS analysis

GC-MS analysis was undertaken according to the parameters established by Villas-Bôas & Bruheim. (315) In summary the samples were injected under pulsed splitless mode. The oven temperature was initially held at 45°C for 2 min, the temperature was then raised with a gradient of 9°C/min until 180°C was reached. This temperature was held for 5 min. the temperature was then raised with a gradient
of 40°C/min until a temperature of 220°C was reached and held for 5 min. Then the
temperature was raised with a gradient of 40°C/min until a temperature of 240°C was
reached and held for 11.5 min. Lastly, the temperature was raised with a gradient of
40°C/min until 280°C was reached and this temperature was held for 2 min. The flow
through the column was held constant at 1.0 mL of He/min. The injection volume was
2 μL. The inlet temperature was 290 °C, the interface temperature 250 °C and the
quadrople temperature was 200 °C.

**Metabolite identification**

The approach for metabolite identification was developed by the Villas-Bôas
laboratory[8] and is summarised as follows; metabolites in the samples were
identified using our in-house MCF MS library of derivatised metabolites.(277) The
library contains MS spectra obtained from ultra-pure standards that have been
derivatised, with the mass spectrum saved in the AMDIS 2.65 software
(www.amdis.net). The relative level of the metabolites was based on the base peak
height. Values were normalised by the base peak height of the internal standard (d4-
alanine). A metabolite profile for each sample was thus built up and subjected to
further analyses as described below.

**Proteomics substudy (n=8) sample preparation and analysis**

**Depletion of plasma samples**

To allow for detection of moderately abundant plasma proteins, plasma samples
were depleted of the 12 most abundant proteins using the ProteomeLab IgY-12 High
Capacity SC Spin Column Kit (Beckman Coulter), according to the instructions
provided by the manufacturer. Depleted samples were stored at -80 °C until
analysed.

**Sample preparation and liquid chromatography-tandem mass spectrometry
(LC-MS/MS)**

Depleted samples were prepared for iTRAQ labeling according to our previously
devised procedure (316) with some modification. Defrosted 2 ml samples were
concentrated to 1 ml using a Savant SPD121P SpeedVac Concentrator (Thermo
Savant, Holbrook, NY), supplemented with dithiothreitol (DTT) to a final concentration
of 10 mM and incubated at 57 °C for one hr. Iodoacetamide (IAM) was then added to
20 mM and the samples were incubated for at RT for one hr in the dark before inactivation of IAM by addition of excess DTT. Protein concentrations were determined using the Bradford method (BioRad). Sample volumes corresponding to 100 μg were taken to fresh low protein-binding microcentrifuge tubes (Axygen Inc., CA) and supplemented with 2 μg trypsin. Digestion was performed at 37 °C for 16 hrs. Digested plasma protein from each preparation was then labelled with 4-plex iTRAQ reagent, according to the manufacturer’s description. The labels were rotated between the runs to reduce potential labelling bias or interference.

Prior to LC-MS/MS, four paired samples with different labels (pre-PCI and corresponding post-PCI from two patients) were combined, allowing for analysis of all 16 patient samples over four separate LC-MS/MS runs. The combined pools of iTRAQ-labelled samples were then fractioned by on-line cation exchange using 15 salt steps and the resulting LC effluent was directed into the ion spray source of a QSTAR XL hybrid mass spectrometer (Applied Biosystems, Foster City, CA) set to scan from 300 to 1600 m/z and with the top three most abundant multiply charged peptides selected for MS/MS analysis (80–1600 m/z). Protein Pilot 1.0 software (Applied Biosystems, Foster City, CA) was then used with the “rapid” search effort to search the output data against the human IPI v3.27 database with carboxamidomethyl cysteine as fixed modification and trypsin as the enzyme specificity. The ratio of false-positive identifications was estimated by performing an identical search of the data against the same database with all protein sequences reversed. In cases where the protein names in the database were insufficient (e.g. “unnamed protein”) a BLAST search (using the software available online) based on the amino acid sequence of the identified protein was made. In most cases it found an identical protein with an established name.

Processing of the ProteinPilot output

Changes in relative protein abundance were assessed manually to avoid known software problems. ProteinPilot peptide summaries for all matched peptides were initially saved and used to normalise the total level of iTRAQ labelling for each label within each run, after removal of any spectra where the peptides ended with a C-terminal proline, which is known to interfere with the 116 label (316), all spectra shared between proteins, spectra matched with confidence = 0, spectra matched to proteins with an unused score less than two, and all spectra with insufficient labelling (raw iTRAQ label area sum less than 40 for each two-sample comparison). The
levels of residual depletion targets (expressed as the percentage of the total iTRAQ signal matched to depletion targets), was then assessed as a rough guide for depletion efficiency. For the 16 depleted samples, on average 31.1% ±1.5% (S.E.M.) of the total iTRAQ signal originated from proteins that should theoretically no longer have been present in the samples.

The most completely depleted sample contained 19.3 percent depletion targets, while the least depleted sample contained 39.2 percent. In order to bypass variations introduced by uneven depletion efficiency, all spectra matched to any of the depletion targets were excluded prior to normalization, as were spectra matched to introduced pig trypsin. Given the observed tendency toward more missed cleavages in the post-PCI samples compared to pre-PCI samples, normalization was performed using separate correction factors for spectra matched to correctly cleaved peptides and spectra matched to peptides with missed cleavages. Individual protein ratios (post-PCI versus pre-PCI) within each run were then calculated from log-transformed sums of the corrected raw area values for each protein. Finally, overall relative abundance (post-PCI versus pre-PCI) for each protein was calculated as the average of the previously obtained protein ratios (a maximum of eight ratios for each protein across four runs).

**Statistics and Bioinformatics**

**Proteomic data analysis**

In order to evaluate changes at the individual level, an initial analysis was performed using log ratios of each patient's post-PCI versus pre-PCI values (average for each protein), obtained for all 77 proteins that were found in at least four comparisons, as described above. Significance was determined using a two-tailed Student's t-test assuming unequal variance, p<0.05 was considered significant and p<0.10 was considered trending. Further to this first pass analysis proteins found to change with p<0.10 were subjected to a second test in order to predict the relevance of these findings across patients, i.e. their potential usefulness as biomarkers. For this, protein log ratios were obtained for each post-PCI versus the non-corresponding pre-PCI value analysed within the same LC-MS/MS run. Significance was then determined using an unpaired two-tailed Student’s t-test assuming unequal variance, p<0.05 was considered significant and p<0.10 was considered trending. In addition, due to the
small sample size, the consistency of behaviour in response to treatment was observed, along with the above mentioned statistical significance.

**Metabolomic data analysis**

The metabolomic data was initially transformed using Principal Component Analysis (PCA) before bioinformatic methods were applied. A signal to noise (SNR) method was used to rank data from most important to least important based on SNR.

\[
SNR(X) = \frac{|mean(x_a) - mean(x_b)|}{std(x_a) + std(x_b)}
\]

(Where \(x_a\) are the values of variable \(X\) belonging to class A and \(x_b\) are the variables of belonging to class B)

The data was pre-processed (missing values filled in, normalisation) and then split several times (three to six folds cross validation) into a training part (e.g. 70 percent) and test part (e.g. 30 percent). Features were extracted and ranked from the training part and then these features and the training data were used to build a personalised model (PM) for every sample from the test data. The accuracy of classification was established as average for all folds and a set of ranked features (potential global markers), that are mostly selected during the folds, was obtained. Methods included both inductive and transductive techniques. Six discriminatory techniques were used to separate the data. These techniques can be grouped into globalised, localised and personalised methods. The two globalised approaches used were; Multi-Linear Regression and Support Vector Machines. The two localised techniques used were Evolving Classification Function and Radial Basis Function and the two personalised methods were the Weighted k-Nearest Neighbour (WKNN) and Weighted WKNN (WWKNN) methods.

**Functional analysis**

The proteins and metabolites that featured commonly between the first principal component and the bioinformatic methods, used above, were mapped into the Metacore network database (GeneGo, MI, USA). Genes known to interact with or affected by the key metabolites from comparative toxicogenomics studies were also included in the analysis.
Results

Proteomics

One-hundred-and-fifty-one unique endogenous non-depletion targets were found in the forward searches. Of these, 77 proteins were found in at least four paired comparisons and were analysed for significant changes in abundance in response to treatment. The results of the reversed searches suggested a false discovery rate of 2.5% ± 2.1% above the chosen cut-off (unused score ≥ 2 assigned by ProteinPilot). None of the “reversed matched” proteins were found in more than one reversed search.

The initial analysis looking at changes within patients identified 31 proteins as significantly different between pre- and post-PCI samples (p<0.05), a further 11 were trending towards significance (p<0.10) and 23 of the proteins changed with p<0.10 also exhibited absolute consistency, i.e. all comparisons (maximum eight per proteins) showed changes in the same direction. A second analysis of the 42 proteins with p<0.10 incorporated a certain degree of individual variability by comparing post-PCI samples to pre-PCI samples from a different patient analysed within the same run. Here, 27 proteins were identified as significantly different (p<0.05) and a further four were trending towards significance (p<0.10) (Table 22). This suggests that seven of the proteins were highly variable at baseline. Of the 31 proteins with p<0.10 in the second analysis, 9 were found to be consistently decreased and five consistently increased in response to PCI.

Combining the information from the two analyses displayed in Table 23 highlighted 13 proteins exhibiting absolute consistency and which changed with p<0.10 in both analyses. Of these, four decreased by PCI had calculated ratios (post-PCI Vs pre-PCI, paired and crossed) consistently lower than <0.6, and a further three proteins were increased with all ratios > 1.4.

Prior to normalization, the iTRAQ label associated with peptides with missed tryptic cleavages sites was substantially higher in the post-PCI samples compared to the corresponding pre-PCI samples (133% ± 10%, p=0.01). This could be related to the 1.7-fold higher levels of the endogenous trypsin inhibitor bikunin alpha-1-microglobulin/bikunin in the post-PCI samples (p<0.05 in the paired comparison, n=8).
Western blotting

Obtaining reliable antibodies for most of the proteins of interest was difficult and therefore restricted confirmation of the proteomic results by western blotting to ceruloplasmin (CP). The behavior of CP levels in non-depleted plasma was largely consistent with the proteomic findings in that seven of the eight patients showed a marked decrease in CP levels post-PCI (Fig 23A). The inconsistent sample (patient six, post-PCI) was almost three standard deviations removed from the rest of the post-PCI samples (anti-CP staining intensity normalised to protein loading) and showed additional unusual features in immunoreactive bands and was therefore excluded from statistical analysis. Densitometry scans of western blotting results and total protein per lane, as assessed by Ponceau S staining, showed that the average paired CP immunoreactivity ratio (post Vs pre-PCI) normalized to protein loading was 0.58 ± 0.08, p<0.01 using a unpaired student’s t test (Fig 23B, compare 0.35 from global proteomics).
Figure 23. Western blot for ceruloplasmin in total plasma of all eight patients (abbreviated 1-8). Panel A demonstrates reduced intensity of the immunoreactive band with the correct molecular mass (arrow) after PCI for all patients except 6. Note also the otherwise unusual pattern for this patient (no immunoreactive band at >62 kDa and an additional high molecular weight (>188 kDa) not found in any other samples). B) Densitometry and normalisation to total protein per lane, as described in the Expanded Methods section, showed significantly lower levels of immunoreactivity post-PCI compared to pre-PCI. ** indicates p<0.01 (two-tailed paired t test).
Table 22. Proteins changed with p<0.10 when statistical analysis was performed on ‘paired’ ratios (post-PCI values versus pre-PCI value from the same patient) or ‘crossed’ ratios (post-PCI from one patient versus pre-PCI value from the other patient analyzed within the same LC-MS/MS run). In bold are proteins which exhibited absolute consistency, i.e. all internal ratios either < or > 0 in both comparisons. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>IPI Accession</th>
<th>No of comparisons</th>
<th>Relative abundance (pre-PCI: post-PCI)</th>
<th>Confidence Interval</th>
<th>t test (p)</th>
<th>Consistency</th>
<th>Relative abundance (pre-PCI: post-PCI)</th>
<th>Confidence Interval</th>
<th>t test (p)</th>
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<td><strong>Carrier proteins</strong></td>
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<td>0.50</td>
<td>0.39 - 0.65</td>
<td>*</td>
<td>6↓/2↑</td>
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<td>0.41</td>
<td>0.32 - 0.53</td>
<td>*</td>
<td>4↓</td>
<td>0.41</td>
<td>0.21 - 0.78</td>
<td>&gt;0.10</td>
<td>3↓/1↑</td>
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<td>0.20 - 0.35</td>
<td>**</td>
<td>5↓</td>
<td>0.25</td>
<td>0.20 - 0.32</td>
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<td>0.35 - 0.55</td>
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<td>1.56</td>
<td>1.26 - 1.92</td>
<td>&lt;0.10</td>
<td>2↓/6↑</td>
<td>1.49</td>
<td>0.79 - 2.82</td>
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<td>0.29 - 0.47</td>
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<td>6↓</td>
<td>0.37</td>
<td>0.27 - 0.50</td>
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<td>0.24</td>
<td>0.18 - 0.32</td>
<td>**</td>
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<td>0.24</td>
<td>0.15 - 0.38</td>
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<td>No of comparisons</td>
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<td>Confidence interval</td>
<td>t test (p)</td>
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<td>Confidence interval</td>
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<td>0.06 – 0.11</td>
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<td>0.29 – 0.52</td>
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<td>0.07 – 0.25</td>
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**Growth factors**

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**Miscellaneous**

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| N-Acetyl-    | IPI00394992   | 8                | 1.55 | 1.24 | 1.94 | <0.10 | 1↓/7↑ | 1.55 | 1.33 | 1.80 | * | 2↓/6↑ |
| muramoyl-L-alanine amidase |
Table 23. Min and max ratios (paired and crossed comparisons) for the 13 proteins exhibiting absolute consistency and changed with p<0.10 in both analyses, as displayed in Table 22. In bold are four decreased proteins where the highest individual post-PCI Vs pre-PCI ratio (paired or crossed) was below 0.6, and three increased proteins where the lowest post-PCI Vs pre-PCI ratio (paired or crossed) was at least 1.40.

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<td></td>
<td></td>
<td>0.19</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Alpha-2-antiplasmin</td>
<td>IPI00029863.4</td>
<td>0.24</td>
<td>Min</td>
<td>0.07</td>
<td>Max</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.92</td>
<td></td>
<td>0.24</td>
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<tr>
<td>Kallistatin</td>
<td>IPI00328609.3</td>
<td>0.17</td>
<td>Min</td>
<td>0.09</td>
<td>Max</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>0.47</td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Heparin cofactor 2</td>
<td>IPI00292950.4</td>
<td>0.12</td>
<td>Min</td>
<td>0.07</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td>Isoform HMW of Kininogen-1</td>
<td>IPI00032328.2</td>
<td>0.06</td>
<td>Min</td>
<td>0.03</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.13</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Apolipoprotein(a)</td>
<td>IPI00739995.2</td>
<td>3.70</td>
<td>Min</td>
<td>2.19</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.52</td>
<td></td>
<td>3.70</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>IPI00022229.1</td>
<td>0.42</td>
<td>Min</td>
<td>0.22</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.75</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Alpha-1B-glycoprotein precursor</td>
<td>IPI00745089.2</td>
<td>2.14</td>
<td>Min</td>
<td>1.22</td>
<td>Max</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.20</td>
<td></td>
<td>2.14</td>
</tr>
</tbody>
</table>
Metabolomics

The CS dataset has a total of 63 samples which contain 32 patient samples taken before coronary angioplasty and 31 patient samples taken approximately twenty minutes after. The CS dataset had a total of 38 attributes. 42 percent of the variance in the results obtained was accounted for by 31 metabolites identified by PCA. 21 of these metabolites were in the first PCA (Table 24, Figures 24 and 25) and were cross-referenced with the metabolites identified by the highest signal to noise ratio (top five noted in Table 25) in the coronary sinus plasma dataset. 12 common metabolites were identified through this method (Table 26). These metabolites were used for the functional analysis.
Table 24. Metabolites identified in first Principal Component (PCA1)

<table>
<thead>
<tr>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminobutyric acid</td>
</tr>
<tr>
<td>Methyl-2-oxopentanoic acid</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Hydroxybutyric acid</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Creatinine</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Oleic acid</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Pyroglutamic acid</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Myristic acid</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Lactic acid</td>
</tr>
</tbody>
</table>

Table 25. Top five features from coronary sinus dataset, ranked by signal to noise ratio (SNR)

<table>
<thead>
<tr>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>*Myristic acid</td>
</tr>
<tr>
<td>3-Methyl-2-oxopentanoic acid</td>
</tr>
<tr>
<td>Caprylate</td>
</tr>
<tr>
<td>Caprinate</td>
</tr>
</tbody>
</table>

*SNR on AO dataset (Myristic acid is the first feature)

Table 26. Common 12 features seen between PCA1 and SNR ranking of coronary sinus plasma

<table>
<thead>
<tr>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminobutyric acid</td>
</tr>
<tr>
<td>Methyl-2-oxopentanoic acid</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Hydroxybutyric acid</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Oleic acid</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Myristic acid</td>
</tr>
</tbody>
</table>
Figure 24. Principal components analysis score plot for coronary sinus samples at baseline and post procedure

The PCA analysis indicates that 41 percent of the variance in these data can be explained by two PCs, and that there is some grouping that can be seen for the samples based on the treatment time.

Figure 25. 3D score plot for coronary sinus samples at baseline and post procedure

In the scores plot of the first three PCs, describing 48 percent of the variance in the data, the separation of samples into groups can be seen more clearly.
**Potential biomarker analysis**

**Proteomics**

14 proteins were selected from the list of candidate proteins identified by proteomic analysis, as described earlier (Table 27). Proteins were selected heuristically as representative of the different pathways identified e.g. inflammation, clotting etc and were considered of interest for future biomarker study.

**Table 27. Significant proteins identified, based on fold-change increase with PCI**

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERPINA7 Thyroxine-binding globulin</td>
<td>IPI00292946</td>
</tr>
<tr>
<td>CP Ceruloplasmin</td>
<td>IPI00017601</td>
</tr>
<tr>
<td>GPX3 Glutathione peroxidase 3</td>
<td>IPI00026199</td>
</tr>
<tr>
<td>SOD3 Extracellular superoxide dismutase [Cu-Zn]</td>
<td>IPI00027827</td>
</tr>
<tr>
<td>B2MG BETA-2-MICROGLOBULIN</td>
<td>IPI00004656</td>
</tr>
<tr>
<td>CFB Isoform 1 of Complement factor B (Fragment)</td>
<td>IPI00019591</td>
</tr>
<tr>
<td>ANT3 ANTITHROMBIN III VARIANT</td>
<td>IPI00032179</td>
</tr>
<tr>
<td>AMBP AMBP protein</td>
<td>IPI00022426</td>
</tr>
<tr>
<td>LPA Apolipoprotein</td>
<td>IPI00029168</td>
</tr>
<tr>
<td>ECM1 Extracellular matrix protein 1</td>
<td>IPI00003351</td>
</tr>
<tr>
<td>IGF2 Isoform 1 of Insulin-like growth factor II</td>
<td>IPI00001611</td>
</tr>
<tr>
<td>C1RL Complement C1r-like protein</td>
<td>IPI00009793</td>
</tr>
<tr>
<td>APOH Beta-2-glycoprotein 1</td>
<td>IPI00298828</td>
</tr>
<tr>
<td>A1BG Alpha-1B-glycoprotein</td>
<td>IPI00022895</td>
</tr>
</tbody>
</table>
**Metabolomics**

Discriminatory analysis using bioinformatics was applied to systemic blood taken from the aorta. Three methods were used, including wKNN classification model, a SVM classification method and an evolving spiking neural network classification model (eSNN). After 1,000 iterations of the algorithm a discriminatory accuracy of 81 percent was made. 11 features were identified using these models, five were common with the metabolites identified by PCA1 and SNR from the coronary sinus dataset (Table 28).

**Table 28. Potential biomarkers: common five metabolites identified by bioinformatic analysis of aortic blood and cross-referenced with coronary sinus data**

<table>
<thead>
<tr>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-2-oxopentanoic acid</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
<tr>
<td>Oleic acid</td>
</tr>
<tr>
<td>*Myristic acid</td>
</tr>
</tbody>
</table>
**Functional network analysis**

The overall findings from the proteomic analysis were consistent with activation of both the classical and alternative complement pathways (Figure 26). Looking at the acute-phase proteins as a whole, several positive acute-phase reactants were found at lower levels (alpha-1-antichymotrypsin, prothrombin, plasminogen and ceruloplasmin). There was however little consistency in the response since retinol-binding protein, a negative acute-phase protein, was also found at lower levels post-PCI. Post-PCI there were lower levels of several pro-coagulation proteins and higher levels of antithrombin III and beta-2-glycoprotein 1 (Figure 26). Decreased fibrinolysis is further suggested by 3.7-fold increased levels of apolipoprotein(a). Several proteins involved in lipid transport and metabolism were also found at lower levels post-PCI. In combination, these findings may prove important for understanding the systemic response post-PCI.

Cross-referencing proteins, metabolites and putative genes identified a number of pathways of interest (Figure 27, 28 and Legend). These included; steroid, lipid, cholesterol and sterol metabolic processes as well as vLDL particle assembly. General biomarkers fell into the categories of glucose metabolism disorders, hyperinsulinism, nutritional and metabolic disorders. The top five diseases associated with the key metabolites and interacting genes included; myocardial ischaemia, glucose metabolism, metabolic disorders, vascular diseases and eye disease. Statistically significant pathways and process networks showed glutathione metabolism, and EDG3 and EDG1 signalling pathways to be involved as well as networks related to hypoxia and oxidative stress, signal transduction ESR2, inflammation and complement system, immune response – phagocytosis, cell adhesion (platelet-endothelium-leukocyte interactions), translational selenium pathway, cell cycle G1-5 growth factor regulation, cell adhesion glycoconjugates, signal transduction WNT signalling and cytoskeleton regulation and rearrangement. The pharmacometabolomic effects of metoprolol were examined. Statistically significant differences were demonstrated between patients receiving either metoprolol or placebo. Quantitatively lysine, oleic acid, dimethyltetradecanoate and stearic acid appeared to be lower in patients receiving IC beta blocker. Metoprolol had the highest impact on dimethyltetradecanoate, the methyl ester of myristic acid. The key metabolites identified were linked in the network analysis to the action of metoprolol (Figures 27, 28).
Figure 26. Functional context of some of the proteins found in human plasma. A shows the two main complement pathways of the innate immune response. Both pathways ultimately lead to assembly of the membrane attack complex, which results in lysis of invading pathogens or, as shown here, autoimmune lysis of non-invasive cells. B illustrates the main events of the clotting cascade, which cumulates with the formation of cross-linked fibrin fibrils at the site of injury. Also shown are some key enzymes governing fibrinolysis. HCF2, Heparin cofactor 2; LPA, (apo)lipoprotein A. Colors represent change in response to PCI (paired comparisons): red, increased (p<0.05); pale red, increased (p<0.10); blue, decreased (p<0.05); light blue, decreased (p<0.10); white, p>0.10; grey, detected in less than 4 comparisons.
Figure 27. Processes identified relating to administration of metoprolol
**Discussion**

In this exploratory experiment a number of metabolites were identified, that had reasonable discriminatory value in distinguishing samples taken before and after
induced cardiac ischaemia. Although these metabolites were not organ specific only a small number of them were required to provide discriminatory confidence of 81 percent. This finding was strengthened by the fact that the same metabolites were seen not only in blood exiting the heart in the coronary sinus but was also seen in systemic blood in the aorta. This finding therefore shows a general consistency in not only patient response but also analytical methodology (from sample collection, extraction through to detection and quantitation). Furthermore the metabolites identified, when merged with proteomic data demonstrated pathways and disease mechanisms consistent with the perturbation being examined, cardiac ischaemia.

The overall findings are consistent with activation of both the classical and alternative complement pathways in that early factors were increased while inhibitory factors as well as several later complement components were decreased, possibly due to their assembly into the membrane attack complex (Fig 27A). Looking at the acute-phase proteins as a whole, several positive acute-phase reactants were found at lower levels (alpha-1-antichymotrypsin, prothrombin, plasminogen and ceruloplasmin). There was however little consistency in the response since retinol-binding protein, a negative acute-phase protein, was also found at lower levels post-PCI. Capacity for blood clotting was uniformly lower post-PCI, as shown by lower levels of several pro-coagulation proteins and higher levels of antithrombin III and beta-2-glycoprotein 1 (Fig 27B). This is very likely to be an effect of heparin treatment and not caused by ischemia per se. Decreased fibrinolysis is further suggested by 3.7-fold increased levels of apolipoprotein(a). Several proteins involved in lipid transport and metabolism were also found at lower levels post-PCI. In combination, these findings may prove important for understanding the systemic response post-PCI.

The second analysis, where post-PCI samples were compared to pre-PCI samples from a different patient in order to introduce individual variability, was largely consistent with the initial analysis, although eleven of the proteins which had been found to change (p<0.10) in the paired comparison here showed p>0.10. Amongst these were five proteins related to immunity, suggesting particularly variable baseline levels of proteins in these pathways.

A core set of 14 proteins may be of interest for future investigation using a larger test group (Table 27). In particular, the seven proteins highlighted in bold in Table 22 displayed favourable features suggesting they might be useful as biomarkers. However, the levels of at least five of these proteins are key factors modulating blood
clotting and were probably altered in response to heparin treatment rather than ischaemia. Specifically, heparin appears to block the binding of alpha-1-antichymotrypsin (318) (ACT, down to 8 percent of pre-PCI levels, p<0.0001, n=8) to certain proteases, and changes in ACT levels may thus be linked to heparin administration rather than PCI-induced ischemia. Thyroxin levels are clearly affected by heparin (319), (320) and so the lower levels of thyroxin-binding globulin (26 percent of pre-PCI, p<0.01, n=5) may also be induced by heparin treatment. Similarly, heparin cofactor 2 (12 percent of pre-PCI, p<0.01, n=4), HMW kininogen (6 percent of pre-PCI, p<0.01, n=4) and apolipoprotein(a) (370 percent of pre-PCI, p<0.01, n=4) are also clearly linked to anticoagulation and inhibition of fibrinolysis (321), (322). Elevated apolipoprotein(a) has however also been found to correlate with propensity for heart disease (323), (322) and may thus be increased independently of heparin treatment.

Unrelated to blood clotting and heparin, increased levels of the complement C1r-like protein (247 percent of pre-PCI levels, p<0.10, n=4) suggests either increased activation of the classical complement pathway (324) or increased cleavage of prohaptoglobin (325) following increased plasma haptoglobin levels associated with the inflammatory response.

The multifunctional CD44 antigen (510 percent of pre-PCI samples, p<0.05, n=4) is a hyaluronan receptor primarily involved in cell-cell and cell-matrix interactions. It is noteworthy that both apolipoprotein(a) and CD44 appear to bind to the C-terminal heparin-binding domain of fibronectin (322), (326). It is thus plausible that part of the reason for their observed higher plasma levels post-PCI was due to dislodgment of these proteins from fibronectin in response to heparin treatment. CD44 antigen is however also known to be up-regulated in response to ischaemic injury of the kidneys as well as myocardial infarct also without heparin administration and may mediate infarct healing through regulation of inflammatory and fibrotic response (327), (328, 329). mRNA levels for CD44 antigen were also found to increase in infarcted mouse myocardium, suggesting increased production rather than redistribution of the protein, with peak levels six hrs after reperfusion and higher mRNA levels sustained also after 72 hrs (329). This is the first observation of increased CD44 protein levels in plasma in response to myocardial ischemia.

The metabolites identified are best visualised through the use of a network analysis. In a sense this visualization of network events can be considered a form of functional...
holography, demonstrating whole systems effects from measurement of only one part. (330) From a mechanistic perspective the most interesting metabolite identified was myristic acid which featured strongly in the coronary sinus and aortic dataset during the signal to noise ranking, PCA and SVM processing. Myristic acid has previously been shown to act as a protective agent against reperfusion injury in vitro. (331) This may be as a result of preconditioning. Interestingly preconditioning can be induced by a small molecule, and can be transferred between animals and species, using dialysate from treated animals. (332-334)

The pharmacometabolomics of metoprolol were examined in this study. Statistically significant changes were seen in response to administration of the drug. Metoprolol is a carnitoyl palmitoyltransferase I inhibitor. Carnitine palmitoyltransferase I is an essential enzyme involved in the beta-oxidation of long chain fatty acids. Inhibition by metoprolol shifts metabolism towards increased utilisation of glucose for energy and reduced beta-oxidation of fatty acids. (335) This results in a reduction in oxygen requirements of the heart and protection from ischaemia. In this study a reduction in ischaemia related metabolites was expected in the group administered metoprolol.

**Limitations**

Although plasma proteomics is a powerful technique there are still many unresolved issues to be addressed. As already noted, depletion of high abundance proteins is necessary to enable detection of lower-abundance proteins. High-quality species-specific depletion columns are now commercially available which efficiently remove most of the high abundance proteins. In order to avoid skewing of the quantification by residual depletion targets, all spectra matched to depletion targets, prior to normalization of the dataset, were excluded.

This experiment was performed on a small sample size (n=33) with the potential for variability due to uncontrollable factors. The inability to perform multiple parallel MS experiments on the same samples limits the reproducibility of results. Patients were recruited in a clinical setting however the angioplasty procedure was performed in a consistent protocolised fashion. An additional limitation was the fact that the effects of pharmacological agents (heparin and glyceryl trinitrate) and contrast administered in conjunction with PCI cannot be easily distinguished from that of ischaemia induced by balloon inflation during PCI. For this reason, the current investigation must be viewed as investigating the overall effects of PCI, rather than the effects of ischaemia induced in conjunction with PCI. Although the sample size was small the strength of
this study is that each of the 33 patients acted as their own controls. A control group of patients receiving heparin and undergoing angiography without PCI would have been ideal. A recent study by Brunner et al has demonstrated that heparin has an effect on non-esterified fatty acids which needs to be taken in context with the findings of this study.(336)

The metabolites identified demonstrated pathways of interest for the time-point at which they were collected however as biomarkers a time course study would need to be performed to confirm the sustained presence of the biomarkers so confidence could be drawn to their use within a window following a symptom.

The limitations of using metabolomics in clinical practice are numerous and worth noting as there are still many technical aspects to the technology that need addressing. Firstly although metabolites are relatively stable their production and degradation is not and highly dependent on enzymatic activity. It is therefore essential that samples collected for metabolomics are processed and quenched quickly.(277) There is as yet no standard for this method for human plasma. There are a number of analytical methods available which involve either destructive e.g. MS or non-destructive NMR methods. They each have their own advantages and disadvantages and provide differing coverage to the metabolome. Metabolites themselves are highly dependent on the individual state of an organism and diet, concomitant drugs and gut flora are also known to alter the metabolome.(337)

**Conclusion**

Metabolomic profiling has the potential to revolutionise laboratory diagnostics, however there will need to be significant validation of methodology and biomarker profiles before this technique enters the hospital laboratory. Metabolite analysis has some advantages over proteomics in that the number of metabolites in the metabolome is smaller and the metabolites themselves are not prone to denaturing. Quantitatively metabolites are known to change by orders of magnitude greater than protein or gene expression and this makes them attractive as biomarker candidates. Individual metabolites themselves however are rarely, if ever, organ specific and it is likely that panels of metabolites identifying response patterns will need to be created as the future “biomarkers” for disease states.
This mechanistic study has demonstrated a method for integrating metabolomic and proteomic profiling of plasma to demonstrate networks and pathways associated with disease. To identify novel biomarker profiles of disease a larger population would need to be tested. An emerging literature is suggesting that this is a viable method for both the diagnosis of cardiac ischaemia (338, 339) and in reclassification of cardiovascular risk in patients with stable coronary disease (340). Ironically the metabolite profiles associated with cardiovascular risk in stable disease include acylcarnitines which are also measured in metabolite profiling for inborn errors of metabolism, discussed in Chapter 10. Since high throughput newborn screening has been present in clinical laboratories for over a decade it may be possible for the same methods to crossover into adult screening for chronic disease. Efficient screening and health monitoring will be realised through the linking of molecular data with electronic medical records. This is discussed in the next Chapter.
Chapter 12. A longitudinal study of a 9p21.3 SNP using a national electronic healthcare database

Chapter Ten discussed the value of having banked biological material for retrospective diagnosis of disease. This Chapter presents a proof of principle study demonstrating the ability to link genomic data to a national electronic healthcare database. This study demonstrates the significant potential in performing longitudinal population studies in New Zealand. This should place New Zealand in a good position to maximise on the benefits of personalised medicine in the future.

Introduction

The aim of personalised medicine is to make the application of medicine more predictive, preventive, personalised and participatory.(341) The field encompasses a wide range of sciences but genomics and pharmacogenomics have received the greatest attention. Genome-wide association studies (GWAS) have attempted to identify the common genetic variants which underlie complex diseases and demonstrated a number of tag SNPs of interest. These SNPs may have a number of clinical uses including prognostic biomarkers, treatment decision markers and may aid in the individualised assessment of disease risk. Although the predictive ability of these genetic variants is low, GWAS studies have provided novel insight into biology. Deep re-sequencing of adjacent DNA using next generation analysers is likely to reveal further variants of interest, though these are likely to be of greater relevance to individual biology than populations.

The cost of genomic technologies is falling rapidly, however the costs of complete genome sequencing and genome-wide association studies (GWAS) are still prohibitive. Using GWAS identified variants in a priori studies does not require large sample sizes or cost, particularly when enriched or restricted phenotypes are used.(342) The use of preselection or enrichment has been proposed as a means of reducing the overall cost of clinical trials through the selection of high risk individuals or non-responders to contemporary treatments.(127)

It has been suggested that the use of electronic healthcare databases linked to genomic information will be of significant value in the implementation of personalised medicine.(343) Combined with electronic decision support (EDS) the use of genetic
Biomarkers may accelerate research and translation of genomics into clinical practice. (344) A recent study by Ritchie et al has shown that valuable phenotypic information is obtainable by using billing codes, patient encounters, laboratory data and natural language processing techniques on unstructured patient records. (345) Using this method has revealed novel relationships between response to antiarrhythmic therapy in patients with atrial fibrillation and 4q25 genetic variants. (346)

Theoretically, lifetime electronic health records linked to genomic data would yield a different perspective. This arrangement would allow the realisation of the prognostic value of genetic and other biomarkers over a prolonged period of time, potentially even over an individual’s lifetime. (343) Biobanks, such as the UK biobank, are designed to address this however these biobanks are often not linked to electronic healthcare databases and rely on their own infrastructure to collect longitudinal health outcome data. (254)

To realise the “predictive” and “preventive” vision of personalised medicine it will be necessary to perform extended longitudinal studies in an environment where high follow up rates can be assured. In countries such as the United States with fragmented providers and a highly mobile population this may be difficult and costly. In a less populous, relatively static population, this would be achievable with lower cost, greater uniformity in application and reduced barriers to adoption.

This study describes a population genomics study using a 9p21.3 SNP (rs10757278), associated with coronary disease risk in an enriched high risk population with severe three vessel coronary disease. (347) This was performed in a mixed population of Caucasian, Maori and Pacific Peoples. This was performed using a national electronic medical records database, which records health data on the 4.2 million inhabitants of New Zealand.
Methods

Study population

447 patients of mixed ethnicity, with severe surgical coronary artery disease, were enrolled into a prospective cohort study. Patients were enrolled after coronary angiography at Auckland City Hospital, New Zealand. Self-reported ancestry data was derived from the New Zealand Health Information Service (NZHIS) and classified according to New Zealand census criteria. Subjects consented to their DNA being sent overseas for testing. An opt-out clause was provided for DNA testing and transport.

DNA and follow up data was available for 376 (84 percent) patients who formed the study cohort. The study was approved by the Northern Regional Ethics Committee of New Zealand and all subjects gave written informed consent.

Study aim

The aim of the study was to identify novel biomarkers (including known cardiovascular biomarkers) that predicted risk in patients with coronary anatomy suitable for coronary artery bypass surgery.

Blood collection and analysis

Whole blood was drawn by venepuncture, using ethylene diamine tetra acetic acid (EDTA) vacutainer tubes. Buffy coats were separated from whole blood and stored without a preservation medium at minus 70° centigrade until DNA extraction. Plasma was separated by centrifugation at 3,500g for 10 minutes. Plasma was stored at minus 70° centigrade before analysis. Plasma biomarkers C-reactive protein (CRP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) were analysed using the Roche CRPLX immunoturbidimetric method on a Roche Modular analyser (Roche, Pleasanton, USA) from these samples.

DNA was extracted from Buffy coat using DNA mini extraction kits (Qiagen, Netherlands) and genotyped for the SNPs identified by deCODE, rs2383207 and rs10757278 using Sequenom MALDI-TOF mass spectrometry iPLEX technology (Australian Genome Research Facility, Brisbane, Australia). Both rs2383207 and
rs10757278 are in close linkage disequilibrium and therefore only rs10757278 is reported here.

**Clinical outcomes**

Clinical outcomes were obtained from the NZHIS, which tracks the healthcare outcomes of all New Zealanders with a National Health Index number. The NZHIS uses the Health Level Seven (HL7) standard for data management. Outcomes are coded using the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD10) system after a hospital admission. Outcomes were tracked from the enrolment of the first patient until four and a half years from enrolment of the last patient.

The pre-specified clinical outcome variables of interest were as follows: death of any cause, cardiovascular deaths, cancer deaths, admissions with acute coronary syndrome (ACS), admissions with stroke/transient ischemic attack (TIA), new diagnoses of cancer. Each was defined by the ICD10 code (Table 29).

**Table 29. Categories of disease and conditions and corresponding ICD-10-AM codes for the classification of clinical outcomes**

<table>
<thead>
<tr>
<th>Diagnoses and Conditions</th>
<th>Primary Diagnosis ICD-10-AM codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Coronary Syndromes</td>
<td>I20, I21, I22, I23, I24</td>
</tr>
<tr>
<td>Ischaemic TIA/CVA</td>
<td>G45 (except G453), G46, I63, I64, I66</td>
</tr>
<tr>
<td>Cancer</td>
<td>C00-D48</td>
</tr>
</tbody>
</table>

**Statistics**

The unadjusted survival was estimated for different outcomes using the Kaplan-Meier method, and the difference between strata was tested using the log-rank test. The association of the SNP rs10757278 with the risk of mortality was assessed using Cox proportional hazards regression analyses. Outcome events were only counted as the first event, and multiple events in the same individual were not included. Survival was measured from the date of consent and censored at the time of death or hospital
admissions for the primary outcomes. The Cochran-Armitage trend test was applied to assess for a linear trend in the baseline categorical risk factors across the genotypes. Kruskal-Wallis tests were applied to assess differences in the distribution of continuous risk factors across the genotypes. An Equilibrium test was applied to the prevalence of the at-risk SNPs in the cohort. The genotype distribution of the patient cohort was compared with the expected genotype proportion of the Caucasian population from Hapmap population database. Two sided tests were used and a P-value < 0.05 was considered statistically significant. Data was analysed using SAS statistical package, version 9.1.3 (SAS Institute, Cary, NC).

**Results**

**Baseline demographics**

Of the 376 patients in the study, 253 (67 percent) were New Zealand European, 47 (13 percent) were Maori, 21 (6 percent) were of Pacific Island ancestry, 33 (9 percent) were Asian and 22 (6 percent) were from other ethnic groups. Baseline demographics are shown in Table 30.

**Risk factors and plasma biomarkers**

Maori and Pacific patients were younger at presentation than NZ European (63 ± 11 versus 70 ± 9 years of age, p<0.0001) and had a higher prevalence of cardiovascular risk factors such as smoking and diabetes. The average body mass index (BMI) was also higher in Maori and Pacific peoples (Table 30). Maori and Pacific peoples were more likely to present with acute coronary syndrome, identifying them as having three vessel coronary disease.
Table 30. Baseline characteristics and outcomes during follow up for whole study population and by ethnicity

<table>
<thead>
<tr>
<th></th>
<th>Whole Cohort (N=376)</th>
<th>NZ European (n=253)</th>
<th>Maori and Pacific Peoples (n=68)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (SD)</td>
<td>67.8 (10.3)</td>
<td>69.8 (9.3)</td>
<td>62.9 (10.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>313 (83.2)</td>
<td>216 (85.4)</td>
<td>52 (76.5)</td>
<td>0.08</td>
</tr>
<tr>
<td>CV Risk Factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of smoking, n (%)</td>
<td>277 (73.7)</td>
<td>177 (70.0)</td>
<td>61 (89.7)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>196 (52.1)</td>
<td>123 (49.0)</td>
<td>40 (59.7)</td>
<td>0.12</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>102 (27.1)</td>
<td>54 (21.3)</td>
<td>25 (36.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>BMI, mean (SD)</td>
<td>28.3 (4.8)</td>
<td>28.1 (4.9)</td>
<td>30.4 (4.4)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Waist Circumference (cm), mean (SD)</td>
<td>101.6 (12.6)</td>
<td>101.7 (12.9)</td>
<td>104.2 (11.8)</td>
<td>0.10</td>
</tr>
<tr>
<td>CRP, median (IQR)</td>
<td>2.6 (1.2, 7.1)</td>
<td>2.8 (1.3, 8.0)</td>
<td>2.5 (0.5, 7.0)</td>
<td>0.36</td>
</tr>
<tr>
<td>Family History IHD, n (%)</td>
<td>178 (47.3)</td>
<td>122 (48.2)</td>
<td>27 (41.5)</td>
<td>0.34</td>
</tr>
<tr>
<td>Disease severity at baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left main stem &gt;70%, n (%)</td>
<td>42 (11.2)</td>
<td>29 (11.8)</td>
<td>9 (13.6)</td>
<td>0.69</td>
</tr>
<tr>
<td>Recent myocardial infarction (&lt;2 weeks), n (%)</td>
<td>234 (62.2)</td>
<td>148 (58.5)</td>
<td>51 (75.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Stroke, n (%)</td>
<td>27 (7.2)</td>
<td>17 (6.7)</td>
<td>4 (5.9)</td>
<td>0.80</td>
</tr>
<tr>
<td>Peripheral vascular disease, n (%)</td>
<td>29 (7.7)</td>
<td>21 (8.3)</td>
<td>5 (7.5)</td>
<td>0.82</td>
</tr>
<tr>
<td>NT-BNP, median (IQR)</td>
<td>44 (17, 117)</td>
<td>48 (19, 119)</td>
<td>42 (13, 108)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*NZ European vs Maori/Pacific
Table 31. Outcomes during follow up for whole study population and by ethnicity

<table>
<thead>
<tr>
<th></th>
<th>Whole Cohort</th>
<th>NZ European</th>
<th>Maori and Pacific Peoples</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Mortality, n (%)</td>
<td>34 (9.0%)</td>
<td>22 (8.7%)</td>
<td>8 (11.8%)</td>
<td>0.44</td>
</tr>
<tr>
<td>Cancer Death, n (%)</td>
<td>9 (2.4%)</td>
<td>7 (2.8%)</td>
<td>1 (1.5%)</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>CVD Death, n (%)</td>
<td>22 (5.9%)</td>
<td>13 (5.1%)</td>
<td>6 (8.8%)</td>
<td>0.25</td>
</tr>
<tr>
<td>Non-fatal MI, n (%)</td>
<td>167 (44.4%)</td>
<td>113 (44.7%)</td>
<td>29 (42.7%)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

*NZ European vs Maori/Pacific

**Outcomes**

Clinical outcomes are demonstrated in Table 31. During a median follow up of 38 months (IQR 33 to 45 months) there were 34 deaths (22 CVD deaths, nine Cancer deaths, three other deaths) 167 admissions with ACS, nine admissions with stroke/or transient ischaemic attack, and 27 admissions with cancer.

Of the total cohort 301 underwent coronary bypass surgery. In total five patients died within 30-days of surgery. Three deaths were cardiac related, one following stroke and one from infection.

**Genetics**

Four individuals chose not to have their DNA collected. Call rates for the 9p21.3 SNPs was 97 percent. Both rs2383207 and rs10757278 were in Hardy Weinberg equilibrium. The frequency of both the rs2383207 and rs10757278 SNPs were similar in Maori and Pacific peoples and therefore these groups were amalgamated. The rs2383207 G risk allele was 70 percent prevalent in the Maori and Pacific group compared to 54 percent in Europeans, p=0.003. The rs10757278 G allele was also present in higher frequency compared to European (68 percent versus 52 percent, p=0.003) (Genotype frequencies are presented in Table 32). This SNP did not correlate with other conventional risk factors.
Table 32. 9p21.3 genotype frequencies demonstrating higher prevalence of the at-risk GG genotype in Maori and Pacific Peoples

<table>
<thead>
<tr>
<th></th>
<th>rs2383207</th>
<th></th>
<th></th>
<th>rs10757278</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
<td>GG</td>
<td>P value</td>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>NZ European</td>
<td>50 (21%)</td>
<td>121 (51%)</td>
<td>68 (28%)</td>
<td>0.003*</td>
<td>55 (22%)</td>
<td>131 (52%)</td>
</tr>
<tr>
<td>Maori and</td>
<td>6 (9%)</td>
<td>28 (42%)</td>
<td>33 (49%)</td>
<td></td>
<td>7 (10%)</td>
<td>29 (43%)</td>
</tr>
<tr>
<td>Pacific peoples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hapmap data(162): Caucasian</td>
<td>18%</td>
<td>58%</td>
<td>23%</td>
<td>0.06***</td>
<td>24%</td>
<td>53%</td>
</tr>
</tbody>
</table>

Genotype frequencies for other ethnicities not included

* P=0.003, chi square test is applied to assess if the genotype distribution of rs2383207 is different between two ethnicity groups.

** P=0.003, chi square test is applied to assess if the genotype distribution of rs10757278 is different between two ethnicity groups.

*** Genotype distribution of Patient cohort against the expected proportion from HapMap

The rs10757278 G allele was significantly associated with an increase in all-cause death (Figure 29). GG carriers had a 15 percent (n=17) mortality at three years compared to 7 percent (n=14) for GA and 4 percent (n=3) survival for AA carriers (p=0.02). Most deaths were from CVD cause. There was a statistically significant difference in cardiovascular death by genotype (AA genotype: n=2 and GG genotype: n=11, p=0.04). GG carriers had a borderline significant higher incidence of cancer related death (AA genotype: n=1 and GG genotype: n=6, p=0.051). The hazard ratio for individuals carrying the at-risk GG genotype compared to the AA genotype was 3.83 (95% CI 1.12-13.08; P = 0.03).

No relationship was seen between the rs10757278 G allele and admissions with acute coronary syndrome, TIA or stroke, but the number of these events was small.
Discussion

In this small study the 9p21.3 SNP rs10757278 was not associated with subsequent acute coronary syndrome admissions over a four and a half year follow up period. However a relationship was seen between the rs10757278 G allele and cardiovascular and all-cause death over that time. This finding of borderline significance should not be concluded as causal and will require further larger studies with a greater understanding of the functional significance of this variant. This study demonstrates the possibility of linking genetic data to an electronic national health outcomes database, which follows hospital admissions, patient encounters, new diagnoses, cancer diagnoses, deaths etc. Moreover the tracking of outcomes reflects a real world perspective on the significance of this genetic variant as it applies to a population in a primary-care, community setting.

In this cohort Maori presented at a younger age and were more likely to present with acute coronary syndrome than Europeans. Men were overrepresented in this cohort but were more likely to be AA carriers than at-risk GG carriers. Although the 9p21.3 G variant was present in higher frequency in Maori with widespread coronary disease, the numbers in this study are too low for any conclusive statements on this
to be made. It is however notable that the 9p21.3 variant is associated with premature atherosclerosis(347) and may act in synergy with traditional risk factors.(349) In this cohort Maori and Pacific Peoples had a higher prevalence of traditional cardiovascular risk factors such as smoking and diabetes. A larger study would be required to assess gene-environment interactions. An age and sex matched healthy control group would have been ideal to assess the population frequency of the 9p21.3 rs10757278 variant in those without disease.

The relevance and function of the 9p21.3 variants have not been fully elucidated. Associations with the metabolic syndrome and systemic inflammation have been proposed.(349, 350) The SNPs are present within a region of non-coding DNA and are in proximity to two genes CDKN2A/2B that regulate cell cycling.(347) A recent mouse knockout study of the 9p21 region has shown altered expression of CDKN2A/2B and proliferation properties of vascular cells.(351) Mutations within these genes have also been associated with cancer.(352, 353)

A number of studies have found other vascular disease-related associations with the 9p21.3 SNPs. These include an association between the SNPs and abdominal aortic aneurysm(354, 355), intracranial aneurysm(354), arterial stiffness(356) and periodontitis.(357) The association between the SNPs and other forms of vascular disease such as stroke appears less well defined and published results are conflicting.(358-360) Other genome wide association studies have identified genomic markers associated with diabetes within the same relative region of the genome and the potential genomic link of a cardiovascular risk factor to coronary disease itself is intriguing.(361)

There are a number of potential applications for these genomic biomarkers. Their use, in addition to traditional risk factors, has been evaluated in risk prediction models. The 9p21.3 SNPs found in the Wellcome trust discovery cohort have been shown to enhance the Framingham risk score to predict myocardial infarction.(362) The ability of these variants to reclassify individuals into appropriate risk categories has also been demonstrated in two epidemiological studies including men(363, 364) and recently in a study of subjects in the GRACE registry.(365) The variant also added incremented value to carotid intima media thickness assessment in the Atherosclerosis Risk in the Community Study (ARIC).(366) However the variant provided no additional benefit to the Reynolds Risk Score, which takes into account CRP and family history, in the Women’s Genome Health study.(367)

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numbers in this study precluded us from making such an analysis but the possibility of improved risk prediction models using genetics is appealing.

Whilst personalised medicine appears to be just around the corner the application of the science is hindered by a perception of low clinical utility, lack of reimbursement, translational informatic issues and in some instances fragmented infrastructure in clinical laboratories. Recent concern has been drawn to inconsistent results between molecular laboratories.(368) Ideally a centralised laboratory utilising high quality, standardised methods would deliver this service to a population.(369) This project utilised existing infrastructure in a commercial laboratory, Linnaeus lab and established a small biorepository (Figure 30). DNA from subjects in this study has been also been used to assess the prevalence of functional variants in the CYP2C19, VKORC1 and CYP2C9, SLCO1B1 genes which are known to influence the response to commonly used cardiovascular medications.(212, 370, 371) This has allowed simulation of warfarin doses in a local population, described in more detail in Chapter Thirteen.(372) The frequency of the CYP2C19*2 variant in different ethnic groups has also been identified using this biorepository. This data was used to evaluate the cost-effectiveness of genetic testing for clopidogrel using pre-existing clinical trial data and data derived from the NZHIS (Appendix A). The biorepository used in this study has also been used to recall individuals and perform a genomic enrichment study. Validating pharmacogenomic markers, such as CYP2C19 and clopidogrel, has been performed in real-world settings using an Electronic Health Record.(373)
The system for reporting genetic data into the laboratory reporting systems in New Zealand uses the common standard HL7. The HL7 genomics standard may offer greater flexibility in linking genomic information with clinical data. (343) It may also be possible to link pharmacy dispensing, biochemistry and blood bank usage data, which passes through a single data provider in New Zealand. At present the electronic health records in New Zealand do not use computable disease definitions such as those defined in the SNOMED-CT nomenclature. (344) Natural language processing (NLP) algorithms within electronic medical records would be a logical alternative though this has not been investigated in New Zealand. In this study ICD10 codes derived from patient encounters proved to be useful and this method was also supported by the recent study by Ritchie et al. (345) Accurate quantitation of outcomes is reliant on the accuracy of coding however in the study by Ritchie et al algorithms using clinical notes adjudication were used to test the reliability of coding and showed it to be reliable. Large datasets combining information from these sources would allow for more sophisticated data mining activity and elucidate gene-environment, gene-drug and gene-disease interactions. This has successfully been
performed using NLP algorithms using the BioVu repository at Vanderbilt University.(374)

Although the cohort in this study is small, the methods demonstrate a proof of principle. Linking genetic data to a centralised, robust electronic medical database is cost efficient and scalable, particularly if DNA collection is part of clinical practice. New Zealand has particularly favourable attributes in this regard as it has a unique population of recent immigrants, spanning many ethnic groups, it has advanced and compatible EMRs and a centralised healthcare database in the New Zealand Health Information Service. Population movements are relatively stable and the foundations are set for the ability to perform high quality longitudinal studies over a lifetime. The largest longitudinal study using this database in New Zealand includes over 150,000 individuals and is designed to improve cardiovascular risk assessment and guidelines adherence through EDS. At present genomic DNA collection is not included in this study.(375) A more substantial resource is the stored two million neonatal screening cards, covering half of New Zealand’s population of four million. Chapter Ten described the successful retrieval of DNA from neonatal cards, up to 39 years old, for small scale clinical purposes.(376) This resource was not intended for population genetic studies and its use for this purpose is untested and would require further consent being obtained from the public. Ethical, cultural, spiritual and privacy issues clearly need addressing in all genetic studies of this nature, but the high level of acceptance in this clinical study reflects a positive attitude to the storage of biological samples with clearly defined informed consent.

**Limitations**

The small number of outcomes limited the power of this study to detect moderate associations between the 9p21 SNPs and recurrent cardiovascular events and cardiovascular death. Multivariate adjustment for known non-genetic risk factors to assess gene-risk factor interactions was not possible because of the small sample size. The higher frequency of the 9p21.3 variant may be the result of selection bias and a disease free control group to compare allelic frequencies was not available.
Conclusion

In this study a SNP at the 9p21.3 locus was present in higher frequency in Maori and Pacific peoples with severe coronary artery disease. In addition an increase in cardiovascular and all cause death was seen in carriers of these risk alleles. High quality health outcome databases such as the New Zealand Health Information Service (NZHIS) make population genomics studies simpler, inexpensive and allow for a reflection of real-world events. Integration of genomic data into electronic health records and decision support software will be essential for the implementation of personalised medicine in the future. Centralised biobanks linked to electronic health databases provide an invaluable resource, which can be used for a variety of purposes. Further population studies and functional studies will be required to understand the clinical relevance of genetic variants such as the 9p21.3 SNP.
Chapter 13. A simulation of warfarin maintenance dose requirement using a pharmacogenomic algorithm in an ethnically diverse cohort

Chapter Twelve showed it is possible to link genomic data to electronic medical records. In this Chapter the same genomic DNA and population data and was used to show that further information can be learned from the same dataset. The aim in this Chapter was to simulate the dose requirements of the drug warfarin in the New Zealand population. Warfarin is a hazardous drug with a high incidence of adverse bleeding events associated with its use. Although newer anticoagulants are becoming available, such as dabigatran, warfarin is likely to remain in use, since newer agents are significantly more expensive. As with the thienopyridines the argument for using pharmacogenetics to rationalise antithrombin agents is growing, particularly as the risk for bleeding with warfarin is in part genetically determined.(377)

An argument is raised that a centralised laboratory would have significant value in New Zealand for implementing personalised healthcare. Providing a clinical service alongside a research biorepository would be a unique and valuable asset for New Zealanders. Such a service is likely to have a significant impact not only on health but also New Zealand’s knowledge economy.

Introduction

The response to warfarin is highly variable and genetics accounts for up to 54 percent of the differing dose requirements between individuals.(378) Other clinical factors account for another 21 percent of the variability in warfarin requirements. Combining genetic and clinical factors into an algorithm to predict a warfarin dose is more accurate than current standard methods of optimising a warfarin dose, using paper-based nomograms.(379) The genetic variants that influence the response to warfarin have been discovered within a number of key genes (VKORC1, CYP2C9, CYP4F2 and GGCX).(380-383) and each variant (usually a single nucleotide polymorphism (SNP)) is given a star nomenclature (e.g. CYP2C9 *2). These SNPs are present in varying frequency within any given population.
Genetic variability within an ethnic group can be greater than between ethnic groups. Despite this, generalisations have been made about ethno-specific responses to drugs. It has long been appreciated that the warfarin requirements of Asian patients is 30 to 40 percent lower than that of Caucasian patients and this is in part due to differences in the CYP2C9 and VKORC1 genes.(384-386) The main reason for this difference is the high prevalence, in Asians, of the low-dose haplotype A, within the VKORC1 gene.(387) In African Americans this haplotype is present in low frequency(388) and this in part explains the higher doses required in this group, compared to Caucasians and Hispanics.(389) Although the frequency of the CYP2C9 *2 and *3 variants have been described in Maori(220) the frequency of the VKORC1 variants has not previously been examined in Maori or Pacific Peoples.

More accurate dosing using genetic testing may lead to improved clinical outcomes. This has been examined in small prospective studies and shown to have a small but positive clinical impact.(233) The cost-effectiveness of testing however is not yet proven at current pricing (~US$400).(251) It is possible that targeted testing, based on ethnicity, may be more cost-effective than testing a larger population. Alternatively knowledge about an ethnic group’s response to a drug may, without testing, aid in prescribing. This study demonstrates the frequency of the known important variants within the VKORC1 and CYP2C9 genes, in a New Zealand population of cardiac patients. This data was used to perform a simulation, based on genetic and personal factors, to estimate the mean dose of warfarin for local ethnic groups.

Methods

Study population

The study population consisted of 429 patients with severe coronary artery disease who were enrolled into a prospective cross-sectional study after coronary angiography. The aim of the project was to identify novel biomarkers that would aid in risk-stratification at the time of cardiac surgery; sub-analyses were made separate to the original study aim, with subsequent Ethics approval. The original study was approved by the Northern Regional Ethics Committee of New Zealand and all subjects gave written informed consent to have DNA stored and tested for research related to cardiac disease.
The population studied was of mixed ethnicity. Ancestry data was derived from the New Zealand Health Information Service (NZHIS) and classified according to New Zealand census criteria. An opt-out clause was provided during the consent process but only four individuals, chose not to have their DNA tested.

**Study objectives**

The objectives of this study were to assess the population allelic frequencies of the SNPs associated with warfarin and simulate the mean doses and distributions of doses required by different Ethnic groups in a relevant New Zealand cohort.

**Study design**

Cross-sectional simulation study

**DNA collection and Analysis**

Whole blood was drawn by venepuncture, using ethylene diamine tetra acetic acid (EDTA) vacutainer tubes. Buffy coats were separated from whole blood and stored without a preservation medium at -70 degrees centigrade until DNA extraction. DNA was extracted from whole blood using the QIAamp® DNA Blood Mini Kit (QIAGEN N.V., Netherlands). The following SNPs were tested; *CYP2C9*2, 3608C>T, (rs1799853), *CYP2C9*3, 42614A>C, (rs1057910) and *VKORC1*2, -1639 G>A, (rs9923231), using the Sequenom® mass spectrometer (Table 33).

**Table 33. Functional SNPs genotyped using mass spectrometry**

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>dbSNP</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYP2C19</em>2</td>
<td>19154G&gt;A</td>
<td>rs4244285</td>
<td>Reduced function</td>
</tr>
<tr>
<td><em>CYP2C9</em>2</td>
<td>3608C&gt;T</td>
<td>rs1799853</td>
<td>Reduced function</td>
</tr>
<tr>
<td><em>CYP2C9</em>3</td>
<td>42614A&gt;C</td>
<td>rs1057910</td>
<td>Reduced function</td>
</tr>
<tr>
<td><em>VKORC1</em>2</td>
<td>-1639 G&gt;A</td>
<td>rs9923231</td>
<td>AA Low dose Haplotype</td>
</tr>
</tbody>
</table>
**Modified pharmacogenetic algorithm**

Baseline weight, height, age and smoking status were available from the baseline case record file and were used in a modified warfarin maintenance dosing algorithm (Maintenance dose = Exp (0.9751 – 0.3238 x VKORC1 3673G>A + 0.4317 x body surface area (BSA) – 0.4008 x CYP2C9*3 – 0.00745 x age – 0.2066 x CYP2C9*2 + 0.2029 x target INR + 0.0922 x smoking). This formula was derived from an algorithm constructed by Gage et al (378) though with the omission of amiodarone usage (not available in the baseline case record files) and African American race (absent in this population). Body surface area was calculated using the formula; BSA (m²) = ((Height(cm) x Weight(kg))/3600)½.

**Statistical analysis and bootstrapping simulation**

Bootstrap analysis using the DecisionTools Suite software v5.0 (specifically @RISK for Excel and StatTools), (Palisade Co., NY) was performed to simulate and compare 1,000 sample means for each ethnic group. The original data (doses) for each ethnic group were randomly sampled using @RISK for Excel with replacement to create 1,000 data sets, each the same size as the original set. Two-sided Mann-Whitney Tests (General Version) were applied to perform population comparison using StatTools. A p-value < 0.05 was considered significant.

**Results**

Complete baseline demographics and paired DNA was available in 366 of the original cohort n=429 (85 percent). These data are presented in Table 34. The cohort contained New Zealand European (n=287), Maori (n=49), Pacific Peoples (n=21) and Chinese (n=9). Call rates on the SNPs analysed using the Sequenom® was 99 percent. Maori and Pacific peoples were significantly younger (65 +/- 9 and 59 +/- 12 years respectively) at the age of first presentation compared to Europeans (70 +/- 9 years, p<0.05) and this was factored into the algorithm. BSA was significantly lower in Chinese with an average BSA 1.83 +/- 0.17 versus 2.01 +/- 0.2 in both Maori and Pacific peoples and 1.98 +/- 0.2 m² (p<0.05) in Europeans. The current smoking rate was higher in Maori n=15 (31 percent) and Pacific peoples n=10 (48 percent) compared to Europeans n=55 (19 percent) however only reached statistical significance for Pacific peoples.
Table 34. Baseline demographics and allelic frequencies

<table>
<thead>
<tr>
<th>Demographics</th>
<th>European (n=287)</th>
<th>Maori (n=49)†</th>
<th>Pacific Peoples (n=21)†</th>
<th>Chinese (n=9)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) mean, SD</td>
<td>70 (9)</td>
<td>65 (9)†</td>
<td>59 (12)†</td>
<td>64 (12)†</td>
</tr>
<tr>
<td>BSA (m²) mean, SD</td>
<td>1.98 (0.21)</td>
<td>2.01 (0.2)</td>
<td>2.01 (0.19)</td>
<td>1.83 (0.17)†</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>19%</td>
<td>31%</td>
<td>48%‡</td>
<td>22%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allelic Frequencies</th>
<th>European</th>
<th>Maori</th>
<th>Pacific Peoples</th>
<th>Chinese</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19*2</td>
<td>22%</td>
<td>24%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYP2C9*2, T allele</td>
<td>13%</td>
<td>1%*</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>CYP2C9*3, C allele</td>
<td>6%</td>
<td>3%</td>
<td>0%</td>
<td>6%</td>
</tr>
<tr>
<td>VKORC1*2, A allele</td>
<td>40%</td>
<td>53%</td>
<td>31%</td>
<td>83%*</td>
</tr>
</tbody>
</table>

Statistically significant (p<0.05), compared to European using *Fisher exact test, †unpaired student-t test and ‡Chi squared test.

The allelic frequencies are reported in Table 34. The frequency of the CYP2C9*2 allele in Maori, Pacific peoples and Chinese was low; 1 percent, 5 percent and 0 percent respectively. Similarly the loss of function CYP2C9*3 allele was also in low frequency in these groups; 3 percent, 0 percent and 6 percent respectively. The VKORC1*2 allele was present in a very high frequency in the limited number of Chinese in this cohort; 83 percent compared to 53 percent in Maori, 31 percent in Pacific peoples and 40 percent in Europeans.

Simulated warfarin doses were lower in Chinese than NZ Europeans (Δ1.39mg, 95% CI 0.4 to 2.4, P = 0.006) due to the high prevalence of the VKORC1 *2 allele in Chinese (Figure 31). Doses were higher in Pacific Islanders compared to NZ Europeans (Δ1.26mg 95% CI = 0.6 to 1.9, P = 0.0002), due to the near absence of the CYP2C9 variant alleles. Simulated warfarin doses in Maori patients were similar.
to European patients (4.8mg versus 4.4mg) and were not statistically or considered clinically different.

**Figure 31. Mean warfarin maintenance dose simulation based on personal and genetic factors**

Warfarin mean dose is plotted along the x axis. Note that bootstrapping has led to a reduced range of doses particularly for the largest group, i.e. Europeans.

**Discussion**

This descriptive study has shown that genetic dosing can simulate dose requirements in different ethnic groups. The frequencies of the CYP2C9*2 and CYP2C9*3 alleles were low in Maori and Chinese as has been reported before.(220) The frequency of these alleles has not been well characterised in Pacific peoples. The CYP2C9*2 and *3 alleles are loss of function polymorphisms that reduce warfarin requirements by reducing the clearance of the more potent S-warfarin enantiomer.(381, 390) The risk of bleeding whilst on warfarin in carriers of these alleles is twice that of non-carriers.(377)
Of particular interest in this cohort is the frequency of the \textit{VKORC1}*2 allele, which encodes a promoter polymorphism -1639 G>A and alters the expression of the \textit{VKORC1} gene.\textsuperscript{(380)} This SNP, also known as low dose haplotype A, is known to reduce warfarin requirements by reducing mRNA expression of the \textit{VKORC1} gene. This study has shown a significant difference in the frequency of the \textit{VKORC1}*2 allele between Maori and Chinese. A lower frequency of the AA haplotype in part accounts for the higher dose requirement for Maori, which more approximates that of Europeans.

The difference in body surface area was not statistically significant except between Pacific peoples and Chinese. In the simulation genetics accounted for a greater influence on the dose requirements than did personal factors between Pacific peoples, Europeans and Chinese. Although Maori had a lower frequency of the \textit{CYP2C9}*2, T allele, than Europeans the weighting of this SNP in the algorithm, combined with the difference in frequency of the \textit{VKORC1}*2, A allele, and body surface area, may have accounted for the similarity in dose requirements between Maori and Europeans. Chinese had a lower mean simulated warfarin dose requirement based on both reduced body surface area and the high frequency of the \textit{VKORC1}*2 AA low dose haplotype, which has previously been described in this ethnic group.\textsuperscript{(385, 391, 392)}

Despite the high level of accuracy in estimating a warfarin dose, pharmacogenomics has yet to enter into clinical practice. Complexities in using algorithms and uncertainty about cost-effectiveness may be reasons for slow adoption. Studies have indicated that time to therapeutic INR is faster with genotype guided dosing.\textsuperscript{(233)} The risk of bleeding on warfarin can also be predicted by genotyping.\textsuperscript{(393)} An additional viewpoint is that identifying high risk individuals for bleeding while on warfarin by genotyping might allow the use of safer but expensive options such as dabigatran.\textsuperscript{(394)} The best use of available resources may be the most appropriate reason for using pharmacogenetics in the clinical setting.

In New Zealand funding for new patented medications is performed through an electronic decision support and authority process. Integrating genomics into this process would not be difficult or expensive. Integrating data from laboratory systems, and pharmaceutical dispensing databases with genomics could identify problems and inefficiencies with healthcare delivery. Simulated trials, such as the Monte Carlo
simulation in Appendix A, could evaluate the cost-effectiveness of new treatments. Providing a clinical service alongside a research biorepository would create a feedback loop for the discovery of new data which could be integrated into decision support processes and re-evaluated in realtime. With links to outcome databases this would enable adaptive clinical trials to be run from the service which would "evolve" to apply the best use of available resources in the local population. This would be a unique and valuable asset for New Zealanders since such a service would be hard to replicate elsewhere. Such a service is likely to have a significant impact on not only health but also New Zealand’s knowledge economy as new biomarkers, algorithms and informatics systems would be exportable.

Limitations

This study in this Chapter is descriptive and simulated the doses required by differing ethnic groups. A more powerful study design would involve genotyping individuals already on warfarin. The simulated doses do not account for the presence of rare genotypes that could also influence the maintenance dose of warfarin. Such examples include the $CYP2C9^*5$ variant, known to decrease the clearance of warfarin, and other $CYP2C9$ variants that have yet to be functionally characterised. Recent discoveries have also pointed to variants in the $GGCX$ and $CYP4F2$ genes as having an impact on warfarin doses. Rare variants within the $VKORC1$ gene have been shown to cause warfarin resistance, with carriers requiring doses of up to 90mg per week. The compound status of individuals for each of these different genotypes was not assessed. In the prospective genotype guided warfarin dosing study by Anderson et al only those carrying multiple genetic variants had an increased risk of an INR $\geq 4$. The algorithm used in this study has been used and validated in a cohort of over one thousand patients on warfarin. There are however many other algorithms in existence that use different personal factors or weightings for genetic variants.

Conclusion

This study has shown that the frequencies of the important genetic variants that dictate warfarin requirements differ between ethnic groups in New Zealand. This Chapter has demonstrated how this translates into simulated mean daily doses of warfarin in each group. It is possible that a clinician could generalise, using ethnicity as a surrogate, and approximate the starting dose close to the mean doses. Genotyping to predict treatment doses remains attractive, however, before
widespread adoption of this technology dosing algorithms would need to be tested in local populations. Simulating dose requirements such as in this Chapter and monitoring clinical outcomes such as in Chapter Twelve could be integrated into provide adaptive decision support for prescribing.

This Chapter summarises the state of current evidence for the clinical utility of clopidogrel pharmacogenetics. The purpose of this Chapter is to outline what is known, what parties are opposed to the idea of using genetic information and what steps are required for this field to progress.

On the 12th of March 2010 the US Food and Drug Administration released a Black Box warning on the antiplatelet drug clopidogrel. (397) This warning alerted clinicians of the heterogeneity of response to clopidogrel and advised pharmacogenetic testing and the consideration of alternative antiplatelet agents in non-responders. The response from the medical community was one of surprise and confusion, as no prospective randomised trial has shown genotype guided treatment strategy to improve clinical outcomes. Trials testing this hypothesis are underway but several questions remained to be answered.

**Barriers from industry**

It is common industry practice to screen new drug compounds at an early stage against cytochrome P450 (CYP) enzymes. This assesses whether a drug is metabolized by polymorphic CYP pathways i.e pathways that are known to vary in a population. Compounds that are metabolised by a single pathway are generally discontinued from further development. Eli Lilly and Company first reported a genetic basis to the response to clopidogrel in 2006 (398) and have continued to investigate this. Although this knowledge may not have been available for clopidogrel during its early development, it does not preclude laboratory work being done in Phase IV. As a result of these findings a biomarker program was built into the Phase I/II trials of Lilly’s drug development program for prasugrel. The subsequent TRITON trial (212) had a genomic component that showed that the CYP2C19*2 polymorphism conferred a risk to carriers taking clopidogrel but it did not to those receiving prasugrel. (399) This result supported the earlier scientific evidence, of in vivo and ex vivo studies, showing the importance of the CYP2C19 gene and enzyme in clopidogrel metabolism. (173, 400-403) Despite this finding, in TRITON, one of the largest pharmacogenetic trials performed, the message of using genetic testing has not been a strong component of Lilly’s marketing strategy.
**FDA decision**

The FDA decision overall is not completely unexpected. The FDA Critical Path Initiative has long supported the role of pharmacogenomics in drug development and views it as a pathway to improving patient responses to medication and reducing adverse drug events. The FDA’s Black box decision was based a number of clinical trials and meta-analyses but also a sponsor funded trial of 40 healthy volunteers (Table 35). These volunteers received 75mg and 150mg of clopidogrel in a crossover study, with platelet function as an outcome and showed that a marginal benefit was obtained in dose escalation. Although alternative antiplatelet treatments were recommended in the warning, specific agents were not mentioned. Guidelines on treatment strategies are available, provided by the Clinical Pharmacogenetics Implementation Consortium.

In making its decision the FDA is likely to have used this data and pharmacometrics to perform population modeling. From a population basis there is an argument that individualising treatment will not only benefit patients but also provide savings to healthcare providers.

**Cost-effectiveness of pharmacogenetic testing**

In countries like New Zealand and Germany, where clopidogrel is generic and inexpensive, there stands a clear advantage of targeting expensive agents to non-responders to achieve cost-savings. Using a two-part risk-benefit analysis consisting of a decision tree corresponding to the TRITON study, along with a patient lifetime extrapolation and Monte Carlo simulation the cost-effectiveness of using clopidogrel pharmacogenomics has been modeled (Appendix A). Although the method did not include clinical non-responder risk factors or platelet testing, genomics alone was sufficient to suggest the cost-effectiveness of personalizing treatment. The reason this is of importance to the providers of healthcare resources is that clopidogrel is the second most prescribed drug in the world, with global sales of over US$6 billion. There will soon be a generic version of clopidogrel in many countries. Substituting clopidogrel for a new patented medication on a 1:1 basis will lead to ongoing cost, for the duration of that medication’s patent life.

Of further interest in our cost-effectiveness study was the result that ethnic groups, with a higher frequency of the *CYP2C19* allele, benefited the most from a targeted
strategy. In the United States African Americans and Asians carry this SNP at a disproportionately higher rate than Caucasians. (212) The cost of testing in the simulation was modeled at $175 and needs to be performed once in a lifetime, something which makes it unattractive to industries that benefit from repeated diagnostic testing. (409)

Table 35. Clinical outcome studies evaluating CYP2C19*2 which influenced the FDA's warning regarding clopidogrel

<table>
<thead>
<tr>
<th>Study</th>
<th>No.</th>
<th>Clopidogrel dose</th>
<th>Clinical Outcome</th>
<th>Risk for CYP2C19*2 carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shuldiner et al. JAMA. 2009 Aug 26;302(8):849-57.</td>
<td>n = 429</td>
<td>300/75mg</td>
<td>CV event or death</td>
<td>HR 2.42; [95% CI, 1.18-4.99; P = 0.02]</td>
</tr>
<tr>
<td>Mega et al. N Engl J Med 2009;360:354-62</td>
<td>n = 1,477</td>
<td>300/75mg</td>
<td>MACE</td>
<td>AR between *2 carriers versus wildtype = 4.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ST</td>
<td>HR 1.5 [1.07-2.2; P = 0.01]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HR 3.09 [1.19-8.00, P = 0.02]</td>
</tr>
<tr>
<td>Simon et al. N Engl J Med 2009;360:363-75</td>
<td>n = 2,208</td>
<td>300/75mg</td>
<td>MACE</td>
<td>HR 1.98 [1.10-3.58, P = 0.05]</td>
</tr>
<tr>
<td>Collet et al. Lancet 2009; 373; 309–17</td>
<td>n = 259</td>
<td>75mg</td>
<td>MACE + Death</td>
<td>HR 3.69 [1.69-8.05, P = 0.0005]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ST</td>
<td>HR 6.02 [1.81-20.04, P = 0.0009]</td>
</tr>
<tr>
<td>Sibbing et al. European Heart</td>
<td>n = 2,485</td>
<td>600/75mg</td>
<td>MACE + Death</td>
<td>HR 3.81 [1.45-10.02, P = 0.007]</td>
</tr>
</tbody>
</table>
Role of platelet testing

Several recent studies have investigated the benefits of alternative treatments for non-responders but these have focused on platelet function to guide treatment strategies (172, 411-413). A fundamental problem with the field of functional platelet testing is the wide variety of functional tests available, each with their own purported advantages. Only a small handful of these have been shown in comparative outcome studies to actually predict events. Definitions of non-response with these functional tests are also lacking. Although recent studies have suggested that platelet function testing exceeds the positive predictive value of genotyping, the pharmacogenomics of clopidogrel is not yet fully elucidated (414, 415). Although the known CYP2C19 polymorphisms are thought to only contribute 12 to 20 percent of response variability.
it appears evident that other genes are involved.\textsuperscript{(416)} The result from a recent genome wide association study has shown that the response to clopidogrel is highly heritable (70 percent).\textsuperscript{(417)} Since $CYP2C19^*2$ only explains 12-20 percent of the variability it appears clear that other genes or rare variants within the $2C19$ gene will explain the heritable nature of the response to clopidogrel. There are a number of further factors to consider with the CYP2C19 gene. Firstly each individual carries two copies of the gene and $CYP2C19^*2$ heterozygotes still have one functional copy. This means that a higher dose of clopidogrel may be effective in heterozygotes, however saturable enzyme kinetics may limit this benefit.\textsuperscript{(411)} $CYP2C19^*2$ homozygotes, with two null alleles, may not respond to higher doses and may require an alternative antiplatelet drug.\textsuperscript{(418)}

Platelet testing may provide an objective measure of compliance however for patients who have been treated with an irreversible antiplatelet drug during a hospital stay, it will not be possible to identify clopidogrel responders using platelet testing. Genotyping in this circumstance is a logical option. In other examples of clinical pharmacogenomics the combination of testing genotype and phenotype testing has been proven to be valuable. Potentially testing both genetic factors and platelet aggregation will yield more information than testing either one alone.\textsuperscript{(215)}

**Multiple genes involved**

The field of pharmacogenomics is rich with examples where multiple interacting genes act in combination to influence drug response. Warfarin is such an example with the VKORC1, CYP2C9, GGCX and CYP4F2 genes being responsible for determining response.\textsuperscript{(382, 383, 419)} The basis for believing in multiple gene interactions is that each step in clopidogrel absorption and metabolism is coded for by biological bottlenecks, essentially efflux pumps and enzymes that have functional polymorphic genes. Biological bottlenecks are prone to significant influence from outside perturbations and are particularly effected by “multiple hits”. The genes for each step in clopidogrel absorption and metabolism have already been reasonably well characterised (Figure 32) and new evidence is emerging that the testing other SNPs, such as a SNP in the ABCB1 gene, in addition to $CYP2C19^*2$ is more predictive of non-response.\textsuperscript{(83)}
Figure 32. Pharmacokinetic/pharmacodynamic pathways for clopidogrel

Genes are shown in blue ovals. Permission to use this image has been given by PharmGKB and Stanford University. (420)

CYP2C19 is also involved with a number of responses to chemotherapeutics. The science in this field is more evolved and shows that the gene is highly inducible by disease states that are inflammatory and that the gene is influenced by master gene regulators, such as the human pregnane receptor gene. (421-423)

Clinician resistance

Clinicians are reserved about incorporating genotyping into clinical practice for a number of reasons. Lack of clinical trial evidence is perhaps the first issue which may soon be resolved. A therapeutic window for antiplatelet drugs is sorely needed and early demonstration of such a holy grail is alluring. (424) Integrating multiple factors involved in drug response is also necessary. An algorithm for clopidogrel pharmacogenomic non-response has already been formulated by a group in Germany, though this requires validation in independent cohorts and use in a prospective trial (Figure 33). (222)
Cost, reimbursement and availability of testing are often raised as concerns, however testing is available through a number of providers from both large laboratories and genotyping platform providers. Although costs of US$400 are quoted with turnaround times of a week, the cost of testing can be provided at substantially less than this, with turnaround times of two to eight hours.(411) The Nanosphere Verigene and Autogenomics INFINITI genotyping platforms are examples.

A final point worth making is that financial incentives need to be addressed, along with the directions in which they bias treatment. Personalised medicine is not popular with industries that benefit from a one-sizes-fit all business strategy. However, individualisation of drug treatment should be considered no different to choice of stent for a patient and clinicians should question comparative cost-effectiveness for all practices. What is the value of adopting a new patented stent over post-care preventative treatment? All of these questions need addressing in cost-effectiveness and comparative effectiveness studies. That includes weighing the benefit of strategies that are not obviously comparable, i.e. preventative versus reactive.
**Clinical factors assessed used in the PREDICT score**

i. DM 2  
ii. CRF 2  
iii. Age >65 1  
iv. ACS 1  
v. LVF 3

Authors suggested use of Clinical, Pharmacogenomic and Platelet testing.*

1) Calculate Score:

≥7 or CYP2C19*2 or *3 allele or ABCB1 TT genotype = prasugrel 60/10mg, others clopidogrel 600/75mg  
(Exclusions to prasugrel age ≥75 years, previous CVA or weight ≤ 60kg)

2) Platelet Testing:

Reassign treatment based on platelet response during chronic phase, to prevent misclassification based on genotype alone.

*Not based on clinical trial evidence or clinical guidelines consensus opinion. The authors take no responsibility for use of this treatment algorithm in clinical practice and recommend that this strategy be testing in a prospective randomised clinical trial. Integration of a bleeding risk score and further genetic markers will be required for optimization of this strategy.


A recent survey in Nature Drug Reviews reported that between 40 to 80 percent of all early stage pharmaceutical compounds have biomarker programmes running alongside drug development.(252) Many oncology drugs are now available with
companion diagnostics, which allow clinicians to target treatment. As we enter the age of genomic and personalised medicine it is highly probable that there will be more drugs entering the cardiovascular market with companion diagnostics, otherwise known as theranostics, to tailor treatment. Mass customisation has made its way into many aspects of our lives and proven itself to be an effective means of minimising cost and wastage. Clinicians will need to be prepared for this change in practice which is no longer just around the corner it is already here.
Chapter 15. Discussion and future directions

This thesis has focused on the use of individualised cardiovascular pharmacology and focused on antiplatelet and anticoagulant therapy. Chapter One provided an overview and definition of non-response to these medications. Chapters Two and Three examined drug-drug interactions between aspirin and commonly used non-steroidal and herbal medications. The results of these two studies showed that ibuprofen and indomethacin block the antiplatelet effect of aspirin, whereas most herbal medicines do not. The results of Chapter Four showed that common genetic variants are not associated with the response to aspirin. Chapters Five and Six showed that polymorphisms within the CYP2C19 gene influence the response to clopidogrel and that non-responders are more likely to be carriers of the CYP2C19*2 allele. Non-responders were more likely to have elevated biomarkers of myocardial necrosis after percutaneous coronary intervention, which may have clinical implications. Theoretically pharmacogenomics could be used to predict drug response prior to starting treatment. Chapter Seven demonstrated that increasing the dose of clopidogrel may overcome non-response identified by genetic testing. However doses above current clinical recommendations (75-150mg daily) may be required. Alternatively switching to another antiplatelet drug, as discussed in Chapter One, is an option. Since the genetic variant responsible for clopidogrel non-response is common in Maori and Pacific Peoples it is likely that pharmacogenomic testing will be particularly beneficial in these ethnic groups and may reduce ethnic disparities in clinical outcomes, as discussed in Chapter Eight; with reference to Appendix A.

The clinical trials, detailed in Chapters Two to Seven required the storage of clinical data, and paired biological samples. Chapter Nine discussed the methods through which these materials can be collected and stored in a biorepository. In Chapter Ten, a case series of patients with the genetic disorder, long QT syndrome, showed the value of storing DNA. In this case series DNA was recovered from neonatal screening cards stored for up to 39 years. Neonatal cards are currently used to screen newborns for inborn errors of metabolism, such as phenylketonuria. This screening process, in use for over a decade, is an example of clinically applied high throughput metabolomics. In Chapter Eleven metabolomic profiling, integrated with proteomic data, was used to elucidate the pathophysiological mechanisms involved in myocardial ischaemia due to percutaneous coronary intervention. This method
revealed a potentially important metabolite, myristic acid, which has previously been shown to protect myocytes against reperfusion injury.\(^{(331)}\) This result may have clinical value if it can be validated.

Chapters Twelve and Thirteen described population genomic and pharmacogenomic studies and proposed the value of a centralised diagnostics laboratory and biorepository for personalised medicine in New Zealand. This may have implications for a wide range of medical conditions beyond the scope of cardiovascular medicine, e.g. diabetes, neurodegenerative disease, cancer and other chronic illnesses. Development of this service would take considerable efforts in database linkages and informatics development. Government policy incentives will be required for progress to be seen in this field. The value of this service is likely to extend beyond just healthcare benefits to New Zealanders, as newly discovered biomarkers and informatics systems would build on our knowledge economy and could be exported overseas.

**Personalisation: marketing tool or inevitability?**

Over the last decade there has been an undeniable trend away from “one-size-fits all” to customized “markets of one” in everything from consumer products and education to medicine. Mass customization has been embraced as a means to increase efficiency and reduce costs, mostly by eliminating wastage in redundant work or ineffective products. Personalisation has become increasingly applied through internet-based technologies, such as behavioural profiling, and has engaged the use of data mining and pattern recognition for discriminatory analysis. These same methods are now being used in genomic research e.g. Chapter Eleven.

This thesis spans a broad range of science, from bench-top research to translational research, which is close to emergence in clinical practice. Although personalised medicine holds significant potential to enhance patient care, there are a number of barriers to widespread adoption of this technology. Primarily, an argument has to be won over whether individualised medicine has value in a system under resource constraint. Whilst some would believe that personalised medicine is the antithesis to population based healthcare, the work in this thesis would argue that they occur in parallel and must be integrated for the best application of cost-effective medicine, which principally benefits society and the individual. This final Chapter discusses the
barriers to implementation, an argument to support adoption and some future directions this field is likely to take.

**Definition**

A clear definition for personalised medicine is yet to emerge, though some basic elements seem clear. Personalised medicine is about applying healthcare on a patient-centric, individualised basis. It is a holistic approach that looks at global systems rather than being reductionistic or organ focused. It is also more generally inclusive of alternative models of care. For instance through the use of computation drug repositioning, molecular and systems approaches using non-Western medicine (e.g. Chinese herbal medicine) new therapies are discovered and new indications for old medicines are found. The underlying sciences to perform this use unbiased approaches and look for patterns within networks, searching for interconnectedness, rather than reductionstic single features to explain events. Genomics, proteomics, and metabolomics are some of the sciences involved in the field. The integration of these sciences with clinical tools such as imaging, to characterize phenotype, and electronic health records will be important in the future. The availability of ubiquitous supercomputing through Cloud based GPU-based servers provides the infrastructure necessary to perform this integration and pattern data mining. Some of these technologies have been demonstrated in the Chapters in this thesis.

**Making it work: Barriers to adoption**

Those who are in most need of healthcare are often the least likely to receive it. Due to poverty and access issues, most advances in medicine have remained a domain of the rich. Personalised medicine may be able to break that cycle through the use of low cost molecular diagnostics. Low-cost informatics combined with genomics, essentially only bytes of information, promises to be more widely accessible through the use of portable consumer electronics and usher in a greater role of prevention.

Achieving personalised medicine is likely to be slow; it will require incremental steps and some fundamental changes in thinking. Barriers to adoption are many and include a lack of prospective clinical trials utilising biomarkers, lack of reimbursement for molecular diagnostics, lack of knowledge amongst clinicians and need for more research into low cost, reliable, portable diagnostics systems. Advocates of
personalised medicine argue that stratifying populations into responder and non-responder groups allows for cost-efficient targeting of expensive treatments and invasive procedures. Those who object to this statement claim that the number of available treatments is limited and increasing the number of diagnostics will only confuse clinicians, create cost with an illusion of savings, and may limit access to proven treatment.

Despite considerable advances in medical science over the last century, population-health physicians are quick to assert that the greatest advances in improving population health have come from government policy on environmental health risks such as dietary salt intake and smoking. Some population health physicians have become advocates for a polypill, containing aspirin, an ACE inhibitor, diuretic and statin. This population-based approach to medicine is seemingly the antithesis to individualised medicine.(428) However is also appears that a wall has been hit with our current models of care.(127) For example the use of aspirin for primary prevention has come into question(429) and the use of potent statins has been shown to increase the risk of diabetes.(430, 431)

Arguably in the third world, where poverty is common and access to medical practitioners is limited, a polypill is the optimal public health solution. However low-cost and available solutions such low-cost supercomputing and cellular phone technology, is providing a method for democratizing medicine.(432) It may soon be possible for medicine to be delivered remotely, for high-risk individuals to self-identify and to access preventive treatment, without the need for an intermediary clinician (Figure 34). A once-in-a-lifetime genomic test, a noninvasive diagnostic possibly using a biosensor, with a single treatment matched to an individual profile may be more cost effective and beneficial to society, than blindly medicating a population. Research has shown that people are more receptive to personalised treatment plans than one-size-fits-all treatments that do not meet individual needs.(433)
Figure 34. Personalised Medicine framework in New Zealand

<table>
<thead>
<tr>
<th>Screening programs</th>
<th>Population Genomics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeted resource allocation</td>
<td>Modeling</td>
</tr>
<tr>
<td>Community oriented</td>
<td>Disease Simulation</td>
</tr>
<tr>
<td>Patient-centric</td>
<td>Cost-effectiveness Analysis</td>
</tr>
</tbody>
</table>

Diagram demonstrates the framework of Personalised Medicine. A population on the left is empowered with tools to self-identify disease risk. Through the use of sensors, cellular phone technology, and molecular diagnostics preclinical disease can be identified, using electronic databases. A digital avatar of an individual, existing of historic clinical records, laboratory and other data is used to predict outcomes and guide treatment for the individual. Population health may be monitored through the use of the existing database with modelling and simulations, evaluating the cost-effectiveness of new interventions, such as screening programmes and novel therapies.

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A lack of advocacy groups and disincentives in place to use personalised medicine in clinical practice are serious barriers to adoption. Pharmaceutical companies are averse to a perceived limiting of market size by stratifying patients. A plethora of “me too” products with questionable added benefits to traditional treatment have flooded the market over the last few decades, leaving clinicians confused. In the era of “comparative effectiveness”, the separation of effective from marginal or non-effective treatments should improve outcomes and reduce costs. (434) Genomics and other ‘omic sciences are likely to play a significant role in comparative effectiveness.
studies which will lead to an increase in the applicability of personalised medicine. (435)

At present financial incentives are acting against the adoption of personalised and preventive medicine. Contemporary medicine is focused on the management of end-stage disease, and clinicians learn to diagnose and treat that perception of disease. Financial incentives are in place for doctors-in-training to become specialists, rather than generalists, and to become proceduralists, rather than be involved in ambulatory care. There is little motivation for a doctor who gets a financial return from an expensive procedural diagnostic to advocate an automated, low cost diagnostic that does not require expert interpretation. In systems where reimbursement is not driven by fee-for-service, this becomes less of an issue; however, even in those circumstances, it is natural for the expert to want to see growth in his/her field. With emerging resource constraints the future focus will be on efficiency, doing more with less. Continued growth is no longer sustainable.

**Interdisciplinary teamwork, collaboration and sharing**

The breadth of this thesis has demonstrated the need for interdisciplinary teamwork, collaboration and sharing. The increasing subspecialisation of medicine and increased workload and density of information processing in medicine have multiplied the number of experts in the clinical workspace but decreased the interaction between experts. At the intersection of disciplines there is often the potential for significant innovation. The translation of genomic medicine into the clinic will require diverse interdisciplinary teams (Table 37). Rather than being an expert in a niche field, the willingness to approach fields outside of one’s expertise, and speak a common language should be valued.
Assessing value

Given the law of diminishing returns in many areas of clinical medicine, thorough cost-effectiveness analyses need to be performed that can model and simulate the application of a new diagnostic or treatment strategy prior to adoption. Chapters Twelve and Thirteen provide the basis for a method to perform this. Appendix A hypothesises the cost-effectiveness of implementing a companion diagnostic across a population for a commonly used drug, clopidogrel. This drug carries a high burden of cost to government managed healthcare systems and funding bodies. The result of this study showed that some disadvantaged ethnic groups may have the most to gain from a genomic, individualised approach to antiplatelet therapy.

Population Health and Government Policy

Government agencies will need to take a leading role in the assessment and implementation of personalised medicine.(436, 437) With the disincentives that are present and with resistance from industry, there needs to be an advocacy group which can map a path to adoption of these sciences into public health programs. Population genomics is a new field in public health which takes a global perspective and integrates the many layers of a population from community to the individual. This field is effectively placed to gauge the value of personalised healthcare to society.(438) Government incentives to create infrastructure, such as compatible
informatics systems and databases is the first step of recognition that the field is valued.

**Genomic medicine in practice**

The information available to make clinical decisions will inevitably become more rather than less. The large datasets and complex interactions demonstrated from omic research will require novel methods of visualization and interpretation. Clinicians are used to working in busy environments with high dataflow but the integration of omic data with clinical data will be another order of magnitude again. Chapter 14 discussed how this complex data may be integrated into clinical practice and discusses the potentials of electronic decision support systems and electronic prescribing.

With the widespread use of electronic medical records with linked clinical, laboratory and imaging data, an extremely valuable resource has been created for research. Clearly data mining of existing clinical records has significant ethical implications for non-consented patients who attend hospital. Proof that using these databases with laboratory data is possible comes from initiatives such as the BioVu project from Vanderbilt University, the Coriell Personalised Medicine Collaboration and the Marshfield Clinic's Personalised Medicine project. New Zealand is well placed to implement this initiative at low cost. New knowledge learned from this experience is likely to be beneficial not only to New Zealanders accessing the healthcare system but also to New Zealand’s knowledge economy.

**Conclusion**

The vision in this thesis offers a potential view of the healthcare delivery in the future, one that is based more closely around prevention, through the use of genomics. This will include screening programs that have a greater focus on empowering individuals with an ability to determine their own future. Predictive risk assessment paired with incentivized, personalised prevention strategies could reduce the financial burdens placed on hospital services. An evaluation of these strategies will need to be monitored and assessed on a population basis, whilst modeling cost-effectiveness.

At present too many new technologies in medicine are adopted without continued evaluation. Systems in which self-interest is the greatest driving factor are
unsustainable and will not work in an environment that has limited space and resources. There will need to be a shift in focus from the financially incentivized treatment of disease to prevention, through genomics and personalised medicine. As technologies in this field become miniaturised and portable the greatest impact is likely to occur in community medicine. Although improving healthcare through the use of technology has led to significant advances in the developed world this has largely ignored the greater unmet need of healthcare in the third world, where simple preventative strategies have already been proven to work. Advanced affordable molecular technologies should have a significant impact at either end of this spectrum. With reduced wastage at the top and enhanced diagnostic precision at the bottom. As the law of diminishing return on investment hits disease-driven healthcare in the developed world, a revolution is approaching which may extend molecular technologies to preventative health programs in both developed and third world countries.
Appendix A (in press). Personalised thienopyridine therapy: the cost effectiveness of genetic testing for CYP2C19 variants to guide treatment in patients with acute coronary syndromes

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Introduction: Previous studies suggest prasugrel may be cost effective when compared to clopidogrel for treating acute coronary syndrome patients (ACS). Recent research has shown that the reduced function allele CYP2C19*2 (*2 allele) is associated with an increased risk of adverse events for ACS patients taking clopidogrel and a decreased risk for patients taking prasugrel. The purpose of this paper is to test whether using generic clopidogrel for all patients is cost effective compared to a) prasugrel for *2 allele patients and clopidogrel for non *2 patients and b) to prasugrel only for New Zealand.

Methods: Effectiveness of clopidogrel and prasugrel from published TRITON-TIMI 38 (n=13,608) clinical trials was combined with rates of *2 occurrence in Maori, Pacific Islanders, Asian and NZ European and national hospital records on rates and costs of hospitalisations 15 months post ACS for stroke, MI, bleeding, stent thrombosis and cardiovascular death. The primary outcome was measured in incremental cost utility ratios (ICURs) based on QALYs. A outcome of less than NZ$50,000/QALY was considered cost effective. A decision tree model and Monte Carlo simulations examined the robustness of the results.

Results: Rates of the *2 allele differ significantly between NZ European (15 percent), Maori (24 percent), Asian (29 percent) and Pacific People (45 percent). Analysis of hospital records suggest that rates of MI, stroke, bleeding, stent thrombosis and cardiovascular death were much higher in the general New Zealand population than in the clinical trial population. The cost effectiveness analysis suggests that use of a genetic test to guide combined use of clopidogrel and prasugrel was cost effective for most age and ethnic groups, but particularly for Maori males (NZ$3184/QALY), Maori females (NZ$3687/QALY), Pacific men (NZ$4617/QALY) and Pacific women (NZ$7605/QALY). Prasugrel is more costly and less effective when used in isolation compared to genetically guided thienopyridine treatment.
**Discussion**: The results here suggest that the use of a genetic test to guide treatment decisions for ACS patients is cost effective, especially for Maori and Pacific peoples, and that prasugrel alone is not cost effective in New Zealand.
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