**A Study to Evaluate the Effects of Dual Anti-Platelet Therapy on the Platelet-Neutrophil Interaction**

**Study Investigators**

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**1. Introduction**

Background

*Epidemiology of Acute Myocardial Infarction (AMI).*

AMI, more commonly called a heart attack, is a leading cause of morbidity and mortality in the western world [1, 2]. In Aotearoa New Zealand, cardiovascular disease place a significant burden on our health system, accounting for over $500 million spent in the health sector [3]. In 2018, AMI was the primary diagnosis for 12,121 hospitalisations nationally, which is just less than 1% of all hospitalisations for this year [4]. Māori and Pacific peoples are disproportionately affected by AMI, with hospitalisation rates being 1.43 and mortality rates being 2.25 times higher for compared with other ethnicities [5, 6].

*Pathology of AMI.*

In most cases, AMI occurs through the erosion/rupture of a coronary atherosclerotic plaque, causing the formation of a blood clot (thrombus), subsequent blockage of the artery and eventual death of heart tissue downstream of this blocked artery [7]. Ischemia – lack of blood and oxygen flow – to myocardial (heart) tissue eventually causes a rapid depression of function in the heart and causes overall loss of blood flow and oxygen supply to all organs of the body. Prolonged myocardial ischemia leads to myocardial infarction (death); the adult heart has negligible regenerative capacity and healing at the infarct site fails to restore the heart to pre-infarct health [7].

AMI is the ultimate culmination of a complex and chronic process known as atherosclerosis. The underlying disease process is driven by a multitude of factors such as lipid accumulation in the arterial walls, vascular injury, and inflammation [8]. Over time, these factors form a plaque which, when ruptured or eroded, encounter the arterial endothelium and blood causing the formation of an occlusive thrombus [9].

*The role of platelets in AMI.*

Central players in thrombus formation during AMI are platelets: the contents of the ruptured atherosclerotic plaque activate and aggregate platelets. Platelets start a positive feedback loop to stabilize the thrombus within the coronary artery [10-12]. These platelets form and release thromboxane from arachidonic acid (AA), a prostanoid with vasoconstricting activity [13]. Vasoconstriction further slows blood flow and favours formation of the thrombus. Moreover, release of adenosine diphosphate (ADP) during this process recruits resting platelets from the circulation and induces them to aggregate with already adherent platelets [11].

*The role of neutrophils in AMI.*

In addition to platelets, neutrophils have recently acquired a distinct role in the pathophysiology of cardiovascular diseases [14-18]. Neutrophils are effector cells of the innate immune system with phagocytotic and antimicrobial activity [19]. Moreover, neutrophils have the ability to produce neutrophil extracellular traps (NETs), structures made up of chromatin and histones and decorated with proteases and granular proteins [20, 21].

NETosis is a last resort of neutrophils to control and capture microbial infections. During infection, NETs trap intruding pathogens, and subsequent platelet activation on NETs allows the immune system to control invaders efficiently [20]. However, during sterile inflammation such as AMI, neutrophils are activated to produce NETs, which can provide a scaffold for platelets to participate in coronary thrombus formation [16, 17, 22-24] An *in vivo* mouse study demonstrated that excessive NET formation aggravated AMI injury and that mice lacking NET-specific proteases showed overall lower atherosclerotic burden [25]. These proteases, such as neutrophil elastase (NE), can enhance coagulation and thrombus formation, suggesting a tight interplay between neutrophils, NETs and platelets.

This positive feedback loop of the neutrophil-NET-platelet axis is beneficial during infections; capturing and preventing the spread of microbes around the body reduces the risk of damage to the host. Yet, the positive feedback loop between these two effector cells is suspected to have detrimental effects during AMI; **NETs driving platelet activation and platelet activation driving NETs create an unwanted cycle of increased inflammation and thrombosis** and further blockage of the coronary arteries. Sustained inflammation and thrombosis leads to increased damage to the myocardium during AMI. **To this date, the extent to which NETs are induced in AMI has not been studied in detail.**

*Dual anti-platelet therapy (DAPT) for treating AMI.*

Inhibiting platelet activation and aggregation following an AMI through dual antiplatelet therapy (DAPT) is currently the gold standard treatment [26-28] because this treatment significantly reduces the risk of a patient experiencing a recurrent cardiovascular event [29]. Aspirin and Ticagrelor are the two anti-platelet agents administered following AMI. Aspirin irreversibly inhibits the cyclooxygenase 1, preventing the production of prostanoids such as thromboxane [30]. Thromboxane is a potent vasoconstrictor and facilitates platelet aggregation [13] – both functions which are undesirable following AMI. Ticagrelor blocks platelet activation by inhibiting the P2Y12-receptor, a receptor involved in responding to ADP which allows platelets to aggregate [31-33].

*Potential therapeutic effect of DAPT on neutrophils?*

Despite treatment with DAPT, some patients will suffer from a recurrent cardiovascular event and this occurrence has been connected to persistent platelet activation [34-36]. **It is possible that DAPT does not inhibit the complete reservoir of pathways available to platelets to cause activation.**

Further, indirect anti-inflammatory effects have been described for DAPT [37]. For example, cyclooxygenase is not exclusively found in platelets; a variety of human cells (i.e. endothelial cells, neutrophils, smooth muscle cells) also express cyclooxygenase [38-40]. Inhibiting P2Y12 prevents the formation of pro-inflammatory leukocyte-platelet aggregates [41, 42]. **It may be that DAPT can reduce the interaction between platelets and neutrophils, thus limiting their ability to participate in NETosis**.

Moreover, P2Y12 is not exclusively found on platelets and it is possible that P2Y12 inhibitors affect inflammation through direct effects on other cell types [43]. **The platelet-independent immune-modulating effects of DAPT remain to be elucidated** [44, 45].

Neutrophils can adapt rapidly to a diverse range of environments. Changes in gene expression occur immediately during activation, leading to a rapid regulation of neutrophil function [46-48] including initiating NETosis. **DAPT may act to modulate neutrophil-mediated processes during AMI, including NET formation, by influencing their gene expression.**

Overall, our study aims to investigate how DAPT influences the platelet-neutrophil axis and, subsequently, NET formation. Based on this literature, there are two outstanding questions that we aim to answer with this study:

1. Does DAPT affect neutrophils and their ability to undergo NETosis?
2. Is this by direct inhibition of neutrophils, or by indirect inhibition of platelets?

Gaining a better understanding of the effects of DAPT on the neutrophil-platelet axis and their role in NETosis may allow us to improve the pathway for care for AMI to significantly improve the overall health of these individuals [3].

**2. Aims of Study**

*Aim 1: To determine if DAPT has off-target effects on neutrophils and their ability to undergo NETosis.*

* *Sub-aim 1: To investigate whether DAPT changes the transcriptome of neutrophils.*

We hypothesize that DAPT dampens the immune function of neutrophils, which can be captured by measuring the neutrophil transcriptome. A transcriptome is the full range of messenger RNAs expressed by a cell, which indicates the particular functions of the cells that are activated or dampened at the time of sampling.

* *Sub-aim 2: To investigate how DAPT affects the ability of neutrophils to undergo NETosis.*

We hypothesize that DAPT will decrease the production of NETs by neutrophils.

*Aim 2: To determine if DAPT affects platelet-mediated NETosis.*

* We hypothesize that DAPT reduces the ability of platelets to amplify NETosis, compared to untreated platelets.

**3. Study Design**

The study design is demonstrated in Figure 1 and is explained in more detail below.

Recruitment of healthy individuals:

**Figure 1: Study design.** This study will involve recruitment of 10 healthy volunteers into a treatment intervention arm and recruitment of 3 healthy volunteers into a treatment-naïve arm. Treatment intervention is required to answer Aim 1 and Aim 2 whereas the treatment-naïve study arm is required to answer Aim 2. PRP = Platelet rich plasma, LTA = Light transmission aggregometry, MPO = Myeloperoxidase, ELISA = enzyme-linked immunosorbent assay.

We propose to recruit 13 healthy individuals into a *prospective interventional study.* Out of those 13 healthy individuals, 10 healthy individuals will be included in an intervention arm and 3 healthy individuals will be included in a treatment-naïve arm.

Inclusion criteria are self-reported healthy individuals aged between 45-70 years (for the intervention arm) or aged between 18-70 years (treatment-naïve arm). For the interventional arm, this age range has been set to reflect the average age of patients with AMI (approximately 63 years).

Exclusion criteria are:

* Known cardiovascular or inflammatory disease,
* Known disorders associated with platelet dysfunction or bleeding,
* Acute illness (including COVID-19) within 6 weeks prior to recruitment
* Ppregnancy
* Diabetes mellitus
* A history of adverse drug reaction
* Treatment with cardiovascular and/or immune-modulating drugs and/or antiplatelet agents within 7 days prior to recruitment
* Awaiting a COVID-19 test results at time of recruitment or at any point throughout the study.

Individuals will be invited to participate via flyer advertisement, advertisement in scientific seminars and by being approached by a Study Researcher. All individuals will be required to provide written informed consent. A Study Researcher will verbally explain the study, present the PIS/CF, allow each individual time to consider and will be available to assist in any way required. The study will be conducted in accordance with the Declaration of Helsinki.

Study visits.

Each individual (n=10) in the intervention arm will be required to attend two study visits on:

* Study visit 1
* Study visit 2 (24 hours after Study visit 1)

Each individual (n=3) in the treatment-naïve arm will be required to attend a maximum of 20 study visits. Where possible, these study visits will be coordinated with the study visits in the interventional study arm to minimize the amount of visits required by these treatment-naïve individuals.

Intervention: administration of DAPT in healthy individuals.

Healthy individuals in the intervention arm will receive dual antiplatelet therapy for two days, a combination of aspirin (300mg loading dose on day 1, 100mg maintenance dose on day 2) and ticagrelor (180mg loading dose on day 1, 90mg maintenance dose on day 1, 90mg maintenance dose on day 2). This mimics the routine of DAPT administration given to individuals with AMI in hospital in Aotearoa New Zealand.

The time frame of 2 days was chosen based on the half-life of circulating neutrophils (7-10 hours) [49, 50]: neutrophils are short-acting and short-lived cells that immediately react to changes in their environment. This time frame allows us to treat neutrophils already circulating at the time of drug administration as well as newly formed neutrophils. This timing is also suitable to capture changes in the neutrophil transcriptome due to DAPT administration: others have previously demonstrated that changes within the neutrophil transcriptome can occur within 30 minutes of treatment [51].

Further, platelet inhibition is seen within 3 - 6 hours of DAPT administration [31, 52, 53].

Data collection.

Baseline demographics (age, height, weight and ethnicity) will be recorded following informed consent from all individuals.

Questionnaire.

Individuals will be asked a series of questions at each study visit. These questions will assess: i) what other medication, apart from the study medication, have been taken by the participant, and ii) whether they have been unwell during the study period. A Study Researcher will verbally ask these questions and will provide support when required during completion of the questionnaire. Taking particular anti-inflammatory drugs, such as Nurofen, may have an effect on platelets and neutrophils. If taken regularly by the individual during the study, it may be necessary to exclude the individual from the study or delay their participation. The same precautions will be taken if the participant has been acutely unwell. This will be at the discretion of the Study Researchers.

Blood drawing.

For individuals enrolled in the intervention arm, blood (10 – 40 mL) will be taken immediately before they receive their first loading doses of DAPT (Study visit 1, pre-DAPT) and 4 hours after taking their second maintenance dose (Study visit 2, post-DAPT). The timeframe of 4 hours was chosen based on previously established data which showed maximum platelet inhibition is achieved 4 hours post drug induction [31]. Blood will be taken by a trained laboratory technician by venepuncture within the antecubital fossa. There is a total of 2x blood draws per healthy individual.

For individuals enrolled in the treatment-naïve arm, blood (10 mL) will be taken at each study visit by a trained study researcher.

Safety.

The risk of venepuncture, bleeding on anti-platelet medication and what to do if a participant develops new symptoms will be explained verbally to the patient and is contained within the PIS/CF.

Minor bruising and hematoma are common minor risks associated with venepuncture. Individuals may experience momentary discomfort when inserting the needle into the vein which can cause a vasovagal reaction. The risk of temporary clotting of the vein, infection or significant blood loss is extremely low due to operating in sterile conditions and being performed by a study researcher experienced in blood taking. The anti-platelet drugs used in this study (aspirin and ticagrelor) thin the blood. This means that individuals may be at an increased risk of experiencing both minor bleeding events, such as nose bleeds and bruising, and major bleeding events, such as intra-cranial bleeding. However, the risk of bleeding in healthy individuals exposed to these agents for a short period of time is extremely low. The risk of bleeding would only be for the period of treatment and platelet function will return to normal after 5 days of discontinuation. Each individual will receive a participant safety card after they have been enrolled into this study. This card contains instructions for what to do if an individual is worried about any new symptoms, a contact name and number for immediate medical advice, and information about which anti-platelet drugs the individuals are taking. In the event that any individual experiences any new symptoms that they are worried about or suspect any bleeding, this card has a number to call a Wellington-based Interventional Cardiologist (Dr. Scott Harding) involved with this study for immediate medical assistance. Participants will also be instructed to attend the nearest hospitals emergency department as a priority and present their patient safety card to the attending medical staff.

Laboratory techniques.

Volunteers enrolled into the intervention arm will help us to answer Aim 1 and Aim 2 whereas volunteers enrolled in the treatment-naïve arm will help us to answer Aim 2.

*Neutrophil isolation and* in vitro *NET stimulation*. To test the effects of DAPT on the ability of neutrophils to induce NETosis (Aim 1), neutrophils from treatment-naïve and DAPT-treated healthy volunteers (pre- and post DAPT administration in the intervention arm) will be isolated directly from EDTA-anticoagulated whole blood and resuspended in cell culture media to 2 x 106 neutrophils/mL. Neutrophils will be stimulated with various concentrations of PMA (4 nM to 500 nM), or Ionomycin (5 µM to 75 µM) (NET agonists) or FSL-1 (0.016 µg/mL to 1 µg/mL) (a neutrophil activator, but does not induce NETosis), or left unstimulated, for two hours at 37 ͦC/ 5% CO2. Supernatant from these conditions will be used in subsequent procedures (MPO assay and NET ELISAs, explained below).

*Light transmission aggregometry (LTA).* LTA will be used to confirm adequate platelet inhibition following DAPT treatment [54-56].

*Allogenic platelet-mediated NET stimulation.* To test the effects of DAPT on platelet-mediated NETosis (Aim 2), platelet rich plasma (PRP) from healthy volunteers in the intervention arm will be isolated pre-DAPT and post-DAPT treatment. PRP will then be co-cultured with neutrophils isolated from three treatment-naïve healthy volunteers. The use of neutrophils from the same three treatment-naïve volunteers for Aim 2 circumvents the issue of using neutrophils from volunteers in the intervention arm, which have been exposed to DAPT. Thus, allogenic co-culture has to be used to answer Aim 2. Platelet-neutrophil co-culture will be carried out as previously described [57]. Briefly, platelets (PRP) will be adjusted to 2.5 x 108 platelets/mL and PRP will be added to neutrophils (2 x 106 neutrophils/mL) in a ratio of 1:250 neutrophils: platelets. An equal amount of platelet-poor plasma (PPP) will be added to neutrophil-only cultures. *In vitro* NET stimulation will be induced as described above, and supernatant will be collected to assess the extent of NETosis. The extent of NETosis with treatment-naïve platelets will be compared to NETosis with DAPT-treated platelets.

*Myeloperoxidase (MPO) assay.* One way to quantitatively assay the release of NETs is to utilize the peroxidase activity of MPO, a peroxidase found within NETs, as described elsewhere [56]. Following neutrophil culture, free-floating MPO is removed by discarding the supernatant prior to vigorous resuspension of the neutrophil monolayer, which contains NET-appendant MPO. Briefly, MPO catalyses the hydrogen peroxidase-mediated oxidation of halide ions to hypochlorous acid which reacts with TMB and induces a colour change. The intensity of the colour change is proportional to the MPO enzyme activity. Colour intensity can be read at 450nm on a spectrophotometer and NET release can be inferred proportionally from MPO activity. We have established this technique in-house (Figure 2).

**Figure 2 Myeloperoxidase (MPO) assay**. NET release can be inferred proportionally from MPO activity. Absorbance at 450nm is graphed as a signal:noise ratio. PMA and Ionomycin induce NETosis in a dose dependent manner whereas FSL-1 does not induce neutrophils to produce NETs. Each data point is the mean ± SD of three biological replicates done in triplicate.

*NET Enzyme-linked immunosorbent assays (ELISAs).* NET ELISAs offer an alternative approach to quantitively assay the release of NETs. This technique is based on the interaction between NET antigens present in the supernatant (MPO-DNA complexes, neutrophil elastase-DNA complexes and citrullinated histone H3) and antibodies, and uses an HRP colorimetric detection system. NET-containing supernatants will be exposed to primary antibodies that bind to MPO, neutrophil-elastase and citrullinated histones. The quantity of NETs can then be inferred from the colorimetric intensity of the HRP-conjugated detection antibodies (anti-DNA and anti-histone). These assays have recently been validated in-house by our laboratory group [58].

*Transcriptomics*. Transcriptomics offer the possibility to capture changes in DNA expression levels, a technique suitable to investigate effects of DAPT on neutrophils. RNA will be isolated from neutrophils using a standard RNA extraction kit pre- and post-treatment with DAPT. RNA samples will be sent overseas to the Australian Genome Research Facility (AHRF) to be analysed.

**4. Study Setting/ Location**

This study will be conducted at two locations:

1. Victoria University Clinical Research Laboratory, Level 8, Clinical Services Block, Wellington Hospital. Healthy individuals will be screened and consented, and blood will be drawn, at this location.
2. The University of Otago, Level J, Ward Support Block, Wellington Hospital. All investigators operate from this location.

**5. Statistical Considerations and Data Analysis**

Sample size of healthy volunteer cohort:

With a sample size of 10 individuals in the intervention arm, we have the power to detect a 10% difference in platelet aggregation between healthy subjects with and without DAPT treatment with 85% power and 5% significance. These calculations were based on the mean (μ=93.67%) and standard deviation (σ=6.413%) of n=21 measurements of platelet aggregation from healthy individuals in response to 1000nM AA.

With a sample size of 10 individuals in the intervention arm, we have the power to detect a 28% difference in relative MPO signal between healthy subjects with and without DAPT treatment with 85% power and 5% significance. These calculations were based on the mean (μ=5.85 signal:noise) and standard deviation (σ=0.89 signal:noise) of n=6 measurements of normalized MPO absorbance values from healthy individuals in response to 500nM PMA.

**6. Ethical Considerations**

This study will be conducted in compliance with international standards, adhering to the code of ethical and responsible research, obliging to behave with honesty, integrity, professionalism, to claim competence within our expertise, act with cultural intelligence and intellectual rigour, to undertake the study with diligence and care, and to safeguard the health, safety, well-being, rights, and interests of people involved in or affected during the conduct of this study. Ethical approval by the Health and Disability Ethics Committee (HDEC) is sought before this study can be conducted.

Participation in this study is on volunteer base only. Participants have the choice to withdraw from the study at any time and are not required to give a reason. Information about the research will be comprehensively, properly, and appropriately provided to participants. Only after participants have been informed about these factors can informed, written consent by the participate be given. We will ensure that the results of this research and the methods employed are adequately and appropriately disseminated in a manner accessible to the participants, the scientific community and the public.

Only relevant participant data will be assessed, and we will keep those confidential. The privacy and confidentiality of all participants will be respected. Anonymity of participants is guaranteed by ensuring that blood samples and data collected is de-identified and precautions will be taken to safeguard the privacy and confidentiality of all participants.

As indicated above, cessation of DAPT restores platelet reactivity within 5 days [59]. This property is of clinical importance as it reduces the risk of adverse bleeding effects and highlights that the treatment is safe to be received by healthy individuals. In the event of any adverse effects, Dr. Scott Harding will be the point of contact for participants.

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