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Interactions between aspirin and $P2Y_{12}$ receptor antagonists in ischaemic heart disease

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‘It was not long before I had an opportunity of making a trial of it; but, being an entire stranger to its nature, I gave it in very small quantities’

An Account of the Success of the Bark of the Willow

Rev. Edward Stone, 1763

Abstract

A combination of aspirin 75 mg once daily (OD) and the $P2Y_{12}$ inhibitor ticagrelor represents the current standard antiplatelet treatment for acute coronary syndromes (ACS). Prolonged therapy is indicated in patients at high risk of ischaemic events but low risk of bleeding, though varying aspirin dose and frequency of administration may modulate its actions and consistency of effect. Daily doses of aspirin <75 mg may exert significant effect and twice-daily (BD) administration may improve consistency, but these regimens have not been studied in those receiving ticagrelor. Aspirin potentiates certain pathways of inflammation that may hypothetically drive atherogenesis and be counteractive in atherothrombosis. This project characterised a novel regimen of very-low-dose BD aspirin, when given alone and in combination with ticagrelor, compared to standard regimens of aspirin and DAPT.

In vitro studies of platelet aggregation confirmed a concentration-dependent inhibitory effect of aspirin, which was additive to potent $P2Y_{12}$ inhibition.

Twenty patients receiving aspirin 75 mg OD and ticagrelor 90 mg BD for ACS were randomised to aspirin 20 mg BD or 75 mg OD for 14 days then crossed over, continuing ticagrelor throughout. Compared to 75 mg OD, post-dose serum thromboxane B_2 (TXB_2) was higher when receiving 20 mg BD but pre-dose TXB_2, prostacyclin-metabolite and platelet aggregation to AA and ADP were similar. Significantly, bleeding time was shortened when receiving aspirin 20 mg BD compared to 75 mg OD. Supplementary analyses showed evidence of lower markers of inflammation when receiving aspirin 20 mg BD compared to 75 mg OD. This prompted further investigation of the dose-dependent effects of aspirin, with and without ticagrelor, using an established healthy volunteer experimental endotoxaemia model.

In the endotoxaemia study, participants are randomised to receive one of three regimens of aspirin (20 mg BD, 75 mg OD or 300 mg OD), or to receive no aspirin, during two 10-14 day treatment periods. At the end of each period, an intravenous injection of sterile bacterial endotoxin (2 ng/kg) is administered. 1 hour before endotoxin injection during one of the treatment periods, ticagrelor 180 mg is administered. At defined timepoints before and after endotoxin injection, assessment of markers of thrombosis, haemostasis, haemodynamics and inflammation is made. The trial was temporarily halted in March 2020 due to the circumstances of the coronavirus pandemic. An interim analysis of selected endpoints was performed. At peak aspirin effect, serum TXB_2 was inhibited in a dose-dependent manner but, when receiving ticagrelor, there was no significant difference between aspirin 20 mg BD and aspirin 75 mg OD groups. There was suppression of arachidonic acid-induced aggregation by all aspirin regimens. There was evidence of both a dose-dependent effect of aspirin and additive effect of aspirin and ticagrelor on collagen-induced platelet aggregation. Platelet P-selectin expression was reduced 6 hours after endotoxin injection compared to 1 hour before endotoxin, was unaffected by aspirin and was further suppressed by ticagrelor. At trough aspirin effect, bleeding time was not significantly prolonged by aspirin 20 mg BD, but was by 75 mg OD and 300 mg OD. Ticagrelor significantly prolonged bleeding time during endotoxaemia. Peak body temperature after endotoxaemia was greater when receiving aspirin 75 mg OD compared to other doses. Aspirin appeared to potentiate plasma TNF-α levels during endotoxemia. Ticagrelor significantly reduced peak plasma TNF-α levels when receiving aspirin 20 mg BD but not other aspirin doses.

In conclusion, during DAPT, compared to standard doses, a novel regimen of aspirin 20 mg BD offers improved haemostasis without detrimental effects on 24-hour levels of platelet inhibition, and may confer a more optimal inflammatory profile, hypothetically reducing atherothrombotic risk. As well as completing the endotoxaemia trial to gain further insights, it is planned to investigate the novel regimen in patients with chronic coronary syndromes receiving low-dose dual antithrombotic therapy.

List of publications relating to the work in the thesis and co-authored by the candidate

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Parker WAE, Orme RC, Hanson J, Stokes HM, Bridge CM, Shaw PA, Sumaya W, Thorneycroft K, Petrucci G, Porro B, Judge HM, Ajjan RA, Rocca B and Storey RF (2019). Very-Low-Dose Twice-Daily Aspirin Maintains Platelet Inhibition and Improves Haemostasis during Dual-Antiplatelet Therapy for Acute Coronary Syndrome. *Platelets*. 30:148-157.

Parker WAE (2020). Aspirin after PCI: in the twilight of its years? *Platelets*. 31:831-833.

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Presentations arising from this thesis

Parker WAE, Orme RC, Sumaya W, Hanson J, Stokes H, McMellon HC, Shaw PA, Judge HM, Storey RF. Ultra-Low-Dose Twice-Daily Aspirin Improves Haemostasis and Maintains Platelet Inhibition in Acute Coronary Syndrome Patients Receiving Ticagrelor. Oral presentation at the Acute Cardiovascular Care Association Meeting, Milan, Italy, March 2018.

Parker WAE, Orme RC, Sumaya W, Hanson J, Stokes H, McMellon HC, Shaw PA, Judge HM, Storey RF. Ultra-Low-Dose Twice-Daily Aspirin Improves Hemostasis and Maintains Platelet Inhibition in Acute Coronary Syndrome Patients Receiving Ticagrelor. Oral presentation of a moderated poster at the American College of Cardiology Annual Congress, Orlando, USA, March 2018.

Parker WAE, Orme RC, Hanson J, Stokes HM, Bridge CM, Shaw PA, Sumaya W, Petrucci G, Porro B, Judge HM, Ajjan RA, Rocca B, Storey RF. Aspirin-dose modification: a new frontier to optimise the pharmacodynamic profile of combination antiplatelet therapy in ischaemic heart disease. Poster presentation at Eurothrombosis 2018, Barcelona, Spain, October 2018.

Parker WAE, Thorneycroft K, Judge HM, Storey RF. Additive effects of aspirin and potent $P2Y_{12}$ inhibition on platelet macro- and microaggregation of relevance to acute thrombotic stroke. Poster presentation at the ESC Heart & Stroke Meeting, Barcelona, Spain, January 2020.

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Dedication

Looking to the past, this thesis is dedicated to my Grandmother, Marian Parker. As well as being a key influence in my early life, opening my eyes to science and the arts, her medical history is testament to many of the principles explored in this project. Being a heavy user for many years of prescribed non-steroidal anti-inflammatory drugs for inflammatory osteoarthritis and without classical risk factors for ischaemic heart disease, she suffered a non-ST elevation myocardial infarction that was treated by multivessel percutaneous coronary intervention and dual antiplatelet therapy. Some months later, she suffered a large duodenal bleed that would easily fall under the category of major in the Thrombolysis in Myocardial Infarction classification. Though recovering admirably from this, she was never quite the same again and died after a long decline in physical and cognitive health on the day I completed the writing of this thesis, December 4th 2020.

Looking to the future, this thesis is also dedicated to my two daughters, Florence and Charlotte. May you always have the desire, means and freedom to explore and express your own ideas. If you maintain determination, curiosity and integrity, at the least no one can say you deserved to fail.

Contents

List of abbreviations

%B	Percent of sample bound
5HT	5-hydroxytryptamine (serotonin)
AA	Arachidonic acid
ACS	Acute coronary syndrome
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AE	Adverse event
AF	Atrial fibrillation
ATP	Adenosine triphosphate
B_0	Maximum binding
BARC	Bleeding Academic Research Consortium
BD	Twice daily
BHF	British Heart Foundation
BMI	Body mass index
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CCS	Chronic coronary syndromes
CD	Cluster of differentiation
CI	Confidence interval
COVID-19	Coronavirus disease-2019
COX	Cyclo-oxygenase
CRF	Case report form
CRP	C-reactive protein
CV	Cardiovascular
CYP	Cytochrome P450
DAPT	Dual antiplatelet therapy

DBP	Diastolic blood pressure
DES	Drug-eluting stent
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ESC	European Society of Cardiology
FA	Final aggregation
GP	Glycoprotein
GUSTO	Global utilization of streptokinase and tissue plasminogen activator for occluded coronary arteries
HR	Hazard ratio
hs	High sensitivity
IHD	Ischaemic heart disease
IMP	Investigational medicinal product
INR	International normalised ratio
IV	Intravenous
LDL	Low-density lipoprotein
LOWESS	Locally weighted scatterplot smoothing
LT	Leukotriene
LTA	Light transmittance aggregometry
LX	Lipoxin
MA	Maximum aggregation
Mac-1	macrophage 1 antigen
MACE	Major adverse cardiovascular event
MAP	Mean arterial pressure
MFI	Median fluorescence intensity
MHRA	Medicines and Healthcare Products Regulatory Agency

MI	Myocardial infarction
mmHg	Millimetres of mercury
MPV	Mean platelet volume
NIMP	Non-investigational medicinal product
NO	Nitric oxide
NOAC	Non-vitamin K antagonist oral anticoagulant
NSAID	Non-steroidal anti-inflammatory drug
NSTE-ACS	Non-ST elevation acute coronary syndrome
NSTEMI	Non-ST elevation myocardial infarction
OAC	Oral anticoagulant
OD	Once daily
OR	Odds ratio
PAD	Peripheral artery disease
PAP	Platelet aggregation profiler
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PG	Prostaglandin
PGI-M	Prostacyclin metabolite
PGI2	Prostacyclin
PPP	Platelet poor plasma
PRP	Platelet rich plasma
QDS	Four times daily
rcf	Relative centrifugal force
RCT	Randomised controlled trial
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRR	Relative risk reduction

SAPT	Single antiplatelet therapy
SBP	Systolic blood pressure
SC	Subcutaneous
STEMI	ST elevation myocardial infarction
SUSAR	Suspected unexpected serious adverse reaction
TIA	Transient ischaemic attack
TIMI	Thrombolysis in myocardial infarction
TLR	Toll like receptor
TNF	Tumour necrosis factor
TX	Thromboxane
TxM	Thromboxane metabolite
UA	Unstable angina
UK	United Kingdom
US	United States
VASP	Vasodilator-stimulated phosphoprotein
VKA	Vitamin K antagonists
VWF	von Willebrand factor

Chapter 1: Introduction

A. Ischaemic heart disease

I. Definitions

Ischaemic heart disease (IHD) is a condition characterised by the presence of atheromatous lesions within the coronary arteries leading to a mismatch between myocardial oxygen supply and demand. IHD is associated with two main categories of clinical syndromes:

a) Acute coronary syndromes

Acute coronary syndromes (ACS) include myocardial infarction (MI) and unstable angina (UA). ACS events most commonly occur upon rupture or erosion of an atherosclerotic plaque, typically triggering thrombosis that results in distal ischaemia and/or infarction. As the name suggests, MI involves myocardial cell death, clinically detectable as a significant rise or fall in serum troponin (Thygesen et al. 2018). UA is not associated with myocardial damage. MI can be further classified into ST-elevation MI (STEMI) and non-ST elevation MI (NSTEMI). The former typically signifies acute total occlusion of a major epicardial coronary artery whilst the latter is usually associated with only transient or partial occlusion, or involvement of a minor arterial branch. NSTEMI and UA are sometimes referred grouped together as non-ST elevation ACS (NSTE-ACS).

b) Chronic coronary syndromes

Patients with chronic coronary syndromes (CCS) are a diverse group and include those with conditions such as stable angina, asymptomatic CAD and those with a history of an ACS >1 year ago (Knuuti et al. 2019).

II. Scale of the problem

IHD is currently the world's leading cause of death, leading to an estimated 9.4 million deaths in 2016. In the United Kingdom alone, there are over 100,000 hospital admissions with MI each year. Due to advances in diagnosis and treatment, the proportion of patients surviving an ACS event has increased from around 30% in the 1960s to 70% today, but this means there is an ever-growing number of patients with CCS (BHF 2020).

B. The thrombotic response

I. Overview

Haemostasis fulfils an important physiological role in the response to trauma, but its mechanisms may become pathologically activated, leading to thrombosis, the central pathological process responsible for most ACS events.

Remembering Virchow's triad, the broad triggers of thrombosis are factors relating to blood constituents, blood flow and, most importantly in ACS, the vessel wall (Bagot and Arya 2008).

Atherogenesis within the coronary arteries is a chronic inflammatory process driven by infiltration of oxidised low-density lipoprotein (LDL) and the interaction of monocytes/macrophages with the vessel wall, leading to plaque formation and endothelial injury. In health, the endothelium acts as a physical barrier between the blood constituents and the prothrombotic subendothelial matrix, as well as releasing antithrombotic substances such as prostacyclin (PGI_2) and nitric oxide (NO). On a background of endothelial damage, plaque rupture or erosion leads to local exposure of the bloodstream to factors that precipitate thrombosis (Libby 2000) (**Figure 1.1**).

II. Coagulation factors: formation of fibrin clot

Classically, there are two major pathways to activation of the acellular coagulation response that converge on a final common pathway, although this model may be an oversimplification of the in vivo state.

Loss of endothelium leads to exposure of subendothelial extracellular matrix and contact activation of factor XII, triggering the chain of clotting factor activation known as the intrinsic pathway (Renné et al. 2012).

Tissue factor, expressed on subendothelial cells and released in microparticles from atheromatous plaques, can activate factor IX when in a complex with factor VII: this is the extrinsic pathway (Mackman et al. 2007).

Initiation of either pathway can lead to activation of factor X, which associates with activated factor V, calcium (released from damaged tissue) and phospholipids to form the prothrombinase complex (Krishnaswamy et al. 1988). Prothrombin (II) is thus broken down to thrombin (IIa), which completes the process through cleavage of fibrinogen to fibrin, the latter being insoluble and forming strands. Tissue factor pathway inhibitor and antithrombin limit this response but, as recruitment of activated platelets contributes to higher levels of thrombin generation, this endogenous inhibition is quickly overwhelmed (Crawley and Lane 2008). Once fibrin is formed, factor XIIIa, activated by thrombin, stabilises the structure of clot by forming cross-links between fibrin strands (Ariens et al. 2002).

Endothelial injury results in exposure of the blood constituents to collagen, which leads to platelet adhesion to the vessel wall via the glycoprotein (GP) Ia/IIa receptor and platelet activation via the glycoprotein (GP) VI receptor (Moroi et al. 1989; Santoro et al. 1988). Von Willebrand Factor (VWF) strengthens adhesion by binding to the complex of GPIb with GPs IX and V (Handa et al. 1986). Thrombin, generated from activation of the coagulation system, also significantly contributes to platelet activation via action on protease-activated receptor (PAR) 1 and, at higher concentrations, PAR4 (Kahn et al. 1999).

III. Platelet activation: its central role in thrombosis

There are several important features of platelet activation. Shape change, brought about via rearrangement of the cytoskeleton with the formation of filopodia, increases surface area and facilitates mechanical adhesion to the vessel wall, other platelets and fibrin strands (Aslan et al. 2012). Platelet activation is also accompanied by the release of arachidonic acid (AA) from the cell membrane, which is locally converted to thromboxane (TX) A_2 by cyclo-oxygenase (COX) 1 and TXA_2 synthase. TXA_2, via the platelet TP-α receptor, contributes further to platelet activation (Patrono 1994).

Following activation, platelets also undergo degranulation. α-granules that contain procoagulant and proinflammatory factors are released and increase platelet surface P-selectin expression, mediating the formation of platelet-leukocyte aggregates and stimulating an associated inflammatory response. Dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP) and 5-hydroxytryptamine (5HT, also known as serotonin). ATP contributes to platelet activation through agonism of platelet $P2X_1$ and ADP via $P2Y_1$ and, most significantly, $P2Y_{12}$ receptors (Fagura et al. 1998; Mahaut-Smith et al. 2011). Activation of $P2Y_{12}$ amplifies the pro-aggregatory response to a range of agonists through downstream inhibition of vasodilator-stimulated phosphoprotein (VASP) phosphorylation, leading to activation of platelet cell membrane GPIIb/IIIa receptors, the final pathway of platelet aggregation (Storey et al. 2000). GPIIb/IIIa mediates platelet-platelet interactions via binding with other GPIIb/IIIa via VWF and fibrinogen bridges (Pytela et al. 1986). Finally, increased platelet scramblase activity leads to greater surface expression of phosphatidylserine, which supports the assembly of prothrombinase complex on the platelet surface and thereby potentiates the pathways of thrombin generation (Monroe et al. 2002).

IV. Summary

Activation of both coagulation and platelets by plaque rupture or erosion leads to thrombosis. Interplay between the two leads to amplification of the thrombotic response which overwhelms endogenous inhibitors. Propagation of thrombus within a coronary artery may result in total or subtotal coronary artery occlusion, resulting in an ACS. Knowledge of the mechanisms of thrombosis has enabled the development of antiplatelet and anticoagulant therapies that improve outcomes.

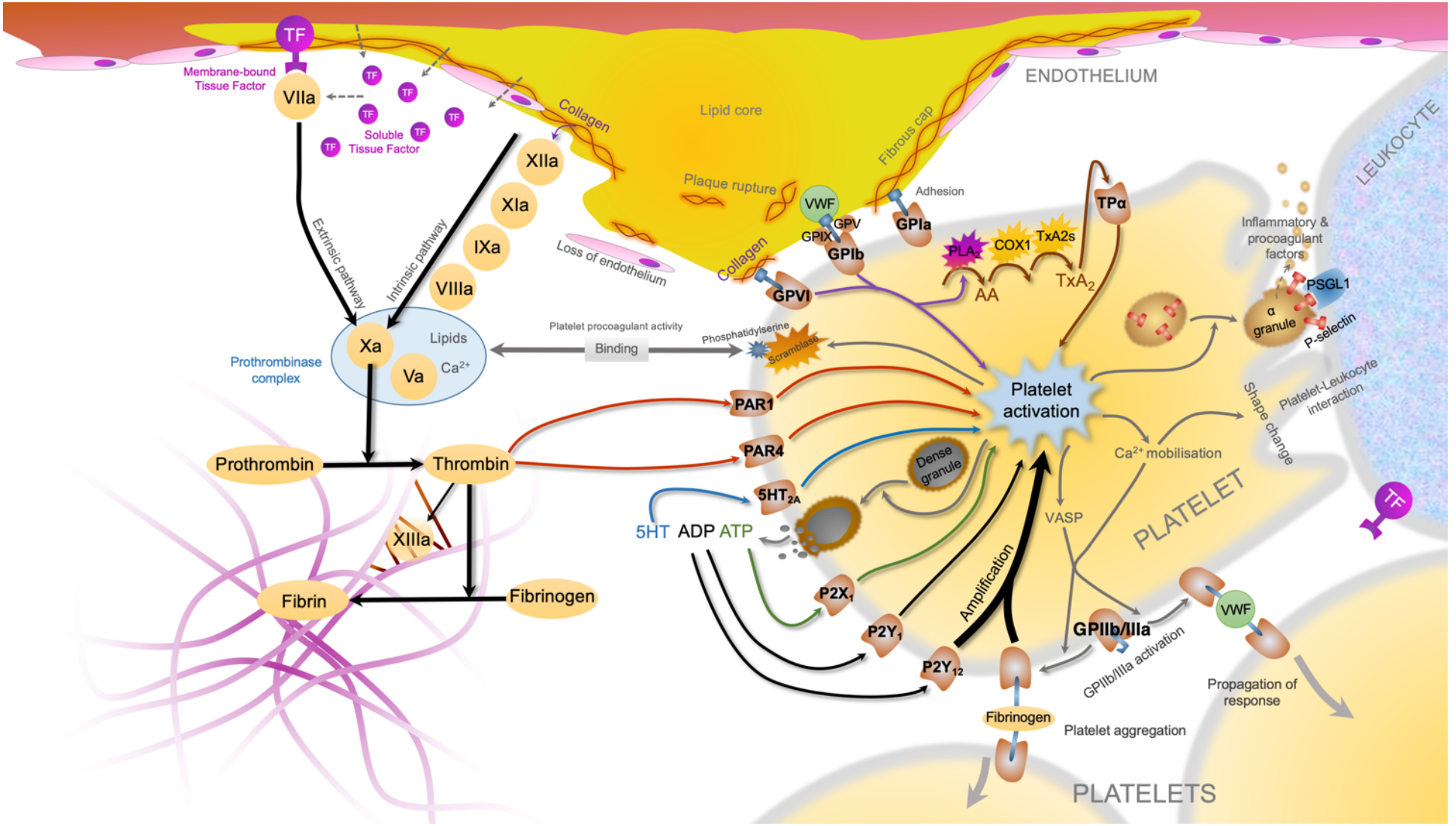

Figure 1.1 Mechanisms of thrombosis following coronary artery plaque rupture in ACS, illustrating the interaction between the coagulation system and platelet activation (Parker and Storey 2018). Reproduced by permission of Oxford University Press. 5HT, 5-hydroxytryptamine (serotonin); AA, arachidonic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Ca^{2+}, calcium; COX1, cyclo-oxygenase 1; GP, glycoprotein; IXa, activated factor IX; $P2X_1$, platelet ATP receptor; $P2Y_1/P2Y_{12}$, platelet ADP receptors; PAR, protease activated receptor; PLA_2, phospholipase A_2; PSGL1, P-selectin glycoprotein ligand 1; TF, tissue factor; TPα, thromboxane receptor α; TXA_2, thromboxane A_2; TXA_2S, thromboxane A_2 synthase; Va, activated factor V; VIIa, activated factor VII; VIIIa, activated factor VIII; VASP, vasodilator-stimulated phosphoprotein; VWF, von Willebrand factor; Xa, activated factor X; XIa, activated factor XI; XIIa, activated factor XII; XIIIa, activated factor XIII.

C. Percutaneous coronary intervention

If unstable or clinically significant, atherosclerotic coronary lesions are frequently treated with percutaneous coronary intervention (PCI), typically involving balloon angioplasty and implantation of metallic drug-eluting stents (DES) (Iqbal et al. 2013). Coronary stents have substantially evolved since their first use in the 1980s due to continuous work refining their design, structure and materials. A number of DES differing in their design and composition are currently available for clinical use. There are three major components of a drug-eluting stent, namely the metallic platform, polymer (if present) and anti-proliferative drug.

D. Antithrombotic drugs

A range of antiplatelet and anticoagulant drugs are currently used in the treatment of atherothrombotic cardiovascular disease (**Figure 1.2**).

I. Aspirin

a) A brief history of aspirin

Aspirin (acetylsalicylic acid) is a synthetic derivative of salicylic acid. The ability of plants containing salicylic acid (including those of the Salix and Spirea genera) to soothe pain and fever was known in ancient times. The Edwin Smith papyri (c.1500 BC) include references to willow bark and the Hippocratic texts include references to chewing willow bark or drinking it as a tea for this purpose. Similar is found in the Roman Age works of Galen (129-c.210 AD). Despite the persistence of Galenic medicine in the Dark Ages and then the renaissance of scientific investigation in the 15th-17th centuries, salicylic acid does not appear to have been a prominent remedy. Its modern rediscovery can generally be attributed to Revd. Edward Stone of Chipping Norton, Oxfordshire, who happened to notice, whilst idly chewing a twig in his churchyard, that bark of the white willow had a bitter taste rather like that of the Peruvian cinchona tree, which was all the rage for its anti-malarial properties and from which quinine was later isolated in 1820 (Desborough and Keeling 2017). He surmised that what was tasting bitter in the willow bark might be either quinine itself or something with similar properties. This was backed up by his assertion that willow typically thrived in wet soil conditions, which were thought to be the origin of ‘agues’, a term used to describe any periodic fever but generally referring to malaria, endemic in England at the time. A pervading idea in medicine of the time, still present within folk medicine, was that natural cures were to be found close to the source of the problem (dock leaves growing near nettles etc.).

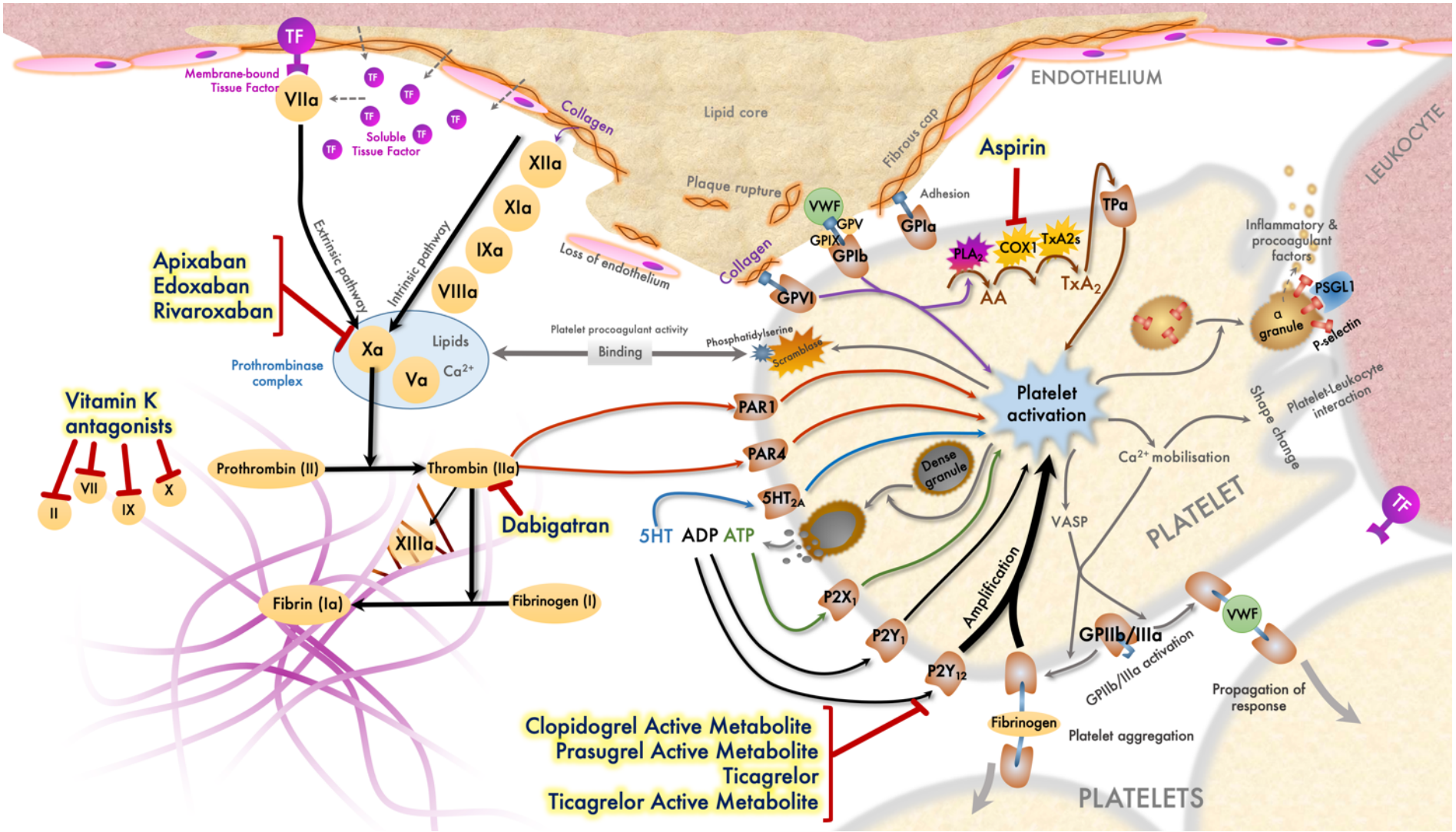

Figure 1.2 Pharmacology of commonly used antithrombotic drugs in ischaemic heart disease (Parker and Storey 2020b). Reproduced with permission from BMJ Publishing Group Ltd. 5HT, 5-hydroxytryptamine (serotonin); AA, arachidonic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; Ca^{2+}, calcium; COX1, cyclo-oxygenase 1; GP, glycoprotein; IXa, activated factor IX; $P2X_1$, platelet ATP receptor; $P2Y_1/P2Y_{12}$, platelet ADP receptors; PAR, protease activated receptor; PLA_2, phospholipase A_2; PSGL1, P-selectin glycoprotein ligand 1; TF, tissue factor; TPα, thromboxane receptor α; TXA_2, thromboxane A_2; TXA_2S, thromboxane A_2 synthase; Va, activated factor V; VIIa, activated factor VII; VIIIa, activated factor VIII; VASP, vasodilator-stimulated phosphoprotein; VWF, von Willebrand factor; Xa, activated factor X; XIa, activated factor XI; XIIa, activated factor XII; XIIIa, activated factor XIII

He administered dried willow bark to parishioners suffering from ague-related rigors, performing what might now be called a dose-escalation study, starting at 20 grains (equivalent to about 1.3 grams) every four hours, noting that increasing the dose to 1 drachm (approximately 2.6 grams) was sufficient to adequately abate symptoms in all but the most serious of 50 cases he tried it in over a 5-year period. He wrote of his findings to The Royal Society who duly read it aloud at one of their meetings and published 'An Account of the Success of the Bark of the Willow in the Cure of Agues' in their *Philosophical Transactions*, albeit getting his first name wrong (Wood 2015). Later, he took to combining willow bark with Peruvian bark, and found the effect of one appeared to potentiate the other.

The Munich pharmacologist Johann Buchner (1783–1852) isolated the active ingredient of willow bark, coming up with a yellowish substance he named salicin in 1828. A number of other chemists worked to refine the extraction, with Raffaele Piria (1814–1865) of Palmi, Italy arriving at a purified version: salicylic acid. Nevertheless, it was salicin that became the preparation investigated for clinical use. It was clearly an effective antipyretic and anti-inflammatory substance, though this was limited by its tendency to cause severe gastric irritation.

Whilst there is evidence several chemists investigated adding side chains, including acetyl groups, to salicin and salicylic acid in order to explore the properties of derivatives, there was no clinical goal to these studies and no reliable process was determined. However, the Bayer company, who originally had been established to invent and manufacture dyes but had ventured into pharmaceuticals, set about trying, perhaps ironically in hindsight, to find a less gastrotoxic form of salicylate (Jack 1997). Though there is some controversy over who exactly came upon acetylation as a way of achieving this objective, Felix Hoffmann (1868-1946), Arthur Eichengrün (1867–1949) and Heinrich Dreser (1860–1924) were in the team that reached this conclusion, developing a process for the reliable synthesis of acetylsalicylic acid in August 1897. The drug was trademarked Aspirin (for the salicylic acid-containing genus Spirea [meadowsweet]) and after little time became a runaway success for Bayer. It is remarkable that, using similar processes, Hoffman synthesised another blockbuster drug, diacetylmorphine (Heroin), within a couple of weeks of first producing aspirin. Both drugs remain in very frequent use in cardiology units today. Though aspirin was originally a trademark in the UK as well as Germany, on the outbreak of the First World War, the assets of the Bayer company in Britain were seized by the Government, its intellectual property and trademarks essentially moving into the public domain. Aspirin therefore became the generic name and has remained so since, in Britain at least.

Once aspirin use for pain, inflammation and fever became widespread, side effects began to be

noticed. Only recognised recently for his contribution was Dr Laurence Craven, a general medical and surgical practitioner in Mississippi (Miner and Hoffhines 2007). He was performing a lot of tonsillectomies and took to prescribing aspirin perioperatively to reduce pain and swelling. He noticed that when he did this, patients appeared to bleed more heavily. In a remarkably prescient deduction, he hypothesised that aspirin might reduce the risk of thrombotic events such as MI. Advocating long-term aspirin use in his own practice, he only reported his ideas in local literature and so there was little dissemination. Gradually, however, the notion that aspirin might be antithrombotic and hence of benefit after MI gained traction and began to be tested in clinical trials. At first these were small and individually inconclusive but, in an early use of meta-analysis, pooling the results suggested a relative risk reduction in ischaemic events of around 25%. Furthermore, the landmark International Study of Infarct Survival (ISIS)-2 (1988) showed that aspirin conferred a similar magnitude of benefit as thrombolysis (5-week vascular mortality odds reduction ± SD: thrombolysis versus placebo 25% ± 4%, aspirin versus placebo 23% ± 4%) and that, when the two treatments were combined this led to a much lower risk of vascular death than either alone (odds reduction ± SD for both treatments versus neither: 42% ± 5%).

b) Mechanism of action

Aspirin acts as an antiplatelet drug by irreversibly inhibiting platelet COX 1, also known as prostaglandin (PG) H_2 synthase, responsible for converting AA into PGH_2. In platelets, PGH_2 then undergoes transformation to the pro-thrombotic and vasoconstrictive eicosanoid TXA_2 (Patrono et al. 1985). As platelets are anucleic, they are unable to regenerate COX1 and therefore it remains inhibited for their lifespan, typically around 10 days (Leeksma and Cohen 1956). Aspirin also inhibits both endothelial COX1 and COX2. In this setting, COX1-derived PGH_2 is converted by PGI_2-synthase to the antithrombotic and vasorelaxant eicosanoid PGI_2 (Hanley and Bevan 1985; Kirkby et al. 2012). However, at least in the case of COX2, this requires greater levels of the drug and, as endothelial cells are nucleated, they are able to more readily overcome this inhibition by enzyme regeneration (Vane and Botting, 2003). Furthermore, because platelets circulate whereas endothelial cells typically do not, they may be exposed to higher drug concentrations whilst travelling through the portal system compared to endothelial cells lining the systemic circulation (Pedersen and FitzGerald 1984).

II. $P2Y_{12}$ receptor antagonists

A second group of antiplatelet agents, the platelet $P2Y_{12}$ receptor antagonists ('$P2Y_{12}$ inhibitors'), have subsequently been developed (**Figure 1.3**).

c) Orally administered $P2Y_{12}$ inhibitors

The thienopyridines ticlopidine, clopidogrel and prasugrel act via active metabolites that bind selectively and irreversibly to $P2Y_{12}$, a G-protein coupled receptor for ADP, found on the surface of platelets (Hollopeter et al. 2001; Abbracchio et al. 2015). This receptor plays a prominent role in amplifying platelet activation and sustaining platelet aggregation, leading to thrombus stabilisation and extension.

Ticlopidine is seldom used now but clopidogrel is still widely prescribed. Several features limit clopidogrel's effectiveness. Once administered, it has a relatively slow onset time, for example in comparison to the later-developed $P2Y_{12}$ inhibitor ticagrelor the mean time from oral loading to maximum platelet inhibition is 5.8 hours longer (Gurbel et al. 2009). This may be of particular concern in the context of urgent PCI when optimal platelet inhibition may not occur until after stent deployment, leaving a period of increased vulnerability to stent thrombosis, which can be catastrophic. Furthermore, there is established evidence of a sizeable subset of the population (approximately 30%) with clopidogrel 'resistance' related to impairment of its enzymatic conversion to the active metabolite (Matetzky et al. 2004). Finally, its irreversibility may cause problems if major bleeding occurs or if emergent coronary artery bypass grafting (CABG) is required, as there is no specific reversal agent and ongoing platelet inhibition in this context may be dangerous.

Prasugrel, another thienopyridine, has the advantage of less variable levels of platelet inhibition and shorter time to optimum effect. Like clopidogrel, it is a prodrug, requiring enzymatic conversion to an active metabolite before it can exert its effect. Unlike clopidogrel, however, the metabolism of prasugrel does not significantly limit the speed of onset and there is no evidence of a resistant phenotype in the population (Jakubowski et al. 2007), although dose-related interindividual variability in response occurs during maintenance therapy.

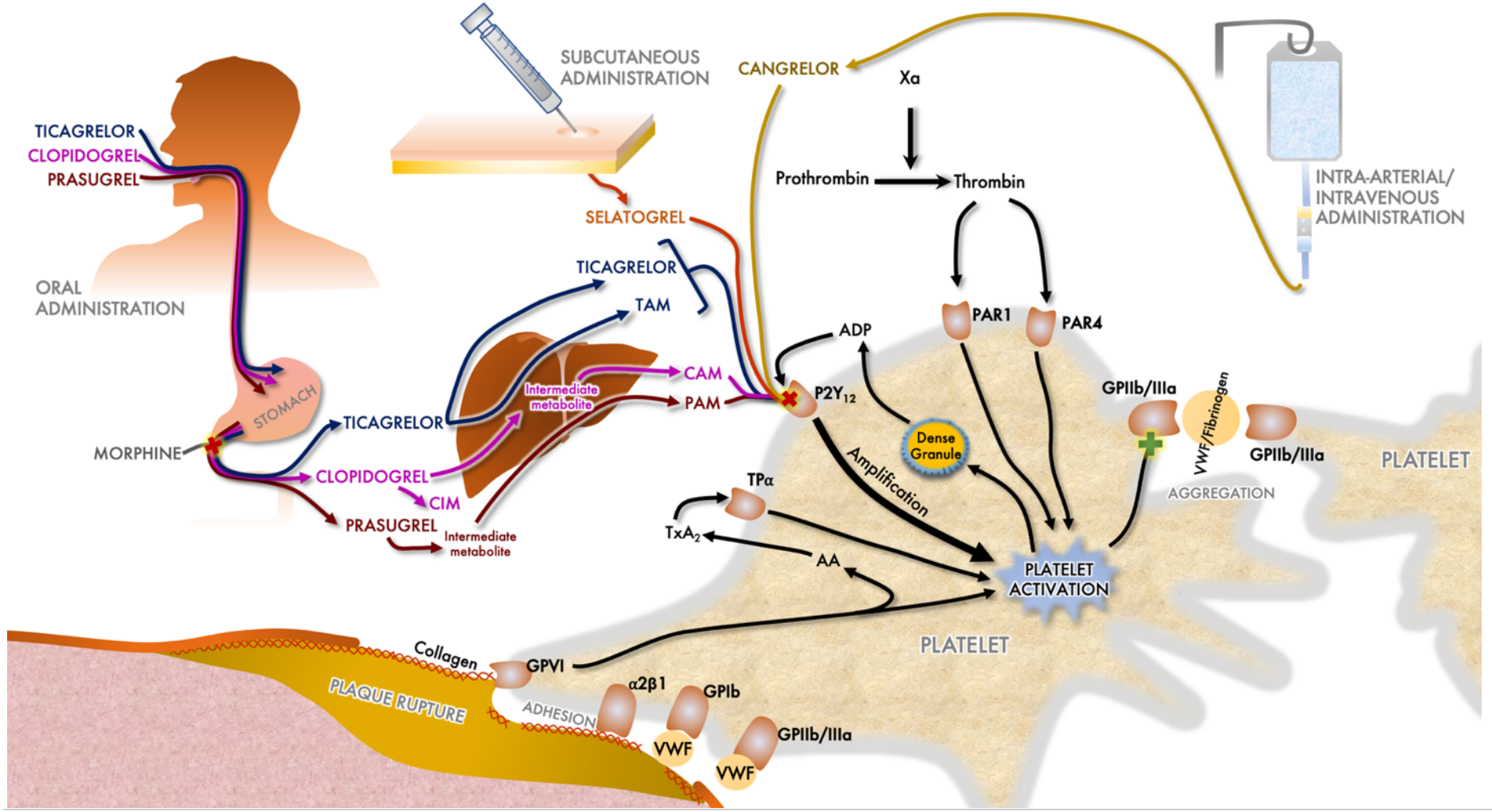

Figure 1.3 Administration, absorption, activation and action of $P2Y_{12}$ inhibitors cangrelor, clopidogrel, prasugrel, selatogrel and ticagrelor. Modified with permission from Elsevier Science & Technology Journals (Storey and Parker 2016); permission conveyed through Copyright Clearance Center, Inc. AA, arachidonic acid; ADP, adenosine diphosphate; CAM, clopidogrel active metabolite; CIM, clopidogrel inactive metabolite; GP, glycoprotein; PAM, prasugrel active metabolite; PAR, protease activated receptor; TAM, ticagrelor active metabolite; TP, thromboxane receptor; Tx, thromboxane; VWF, von Willebrand factor.

Reversibly-binding oral $P2Y_{12}$ inhibitors have also been developed, such as ticagrelor, which belongs to a novel chemical class: the cyclopentyl-triazolopyrimidines. Ticagrelor is an oral agent with an onset time of around 30 minutes in stable patients and offset of action over 2 to 5 days (Gurbel et al. 2009). Ticagrelor is an active drug that does not require metabolism to exert its effect. Unlike the thienopyridines, ticagrelor also inhibits the clearance of adenosine through inhibition of equilibrative nucleoside transporter-1 and this may provide an additional mechanism for inhibition of platelet aggregation by increasing activation of the platelet adenosine 2A receptor (Nylander et al. 2013; Aungraheeta et al. 2016) (**Figure 1.4**).

Several key randomised controlled trials (RCTs) have shown the benefits of $P2Y_{12}$ inhibitors in ACS when combined with aspirin (dual antiplatelet therapy, DAPT) (**Table 1.1**). The current standard therapy in our centre is a combination of aspirin 75 mg once daily (OD) continued lifelong and ticagrelor 90 mg twice daily (BD) continued for 1 year and then either stopped or, in selected individuals at high risk of atherothrombotic events and without high bleeding risk, continued long-term at a dose of 60 mg BD. This represents current recommended practice (Windecker et al., 2014, Roffi et al., 2015, Steg et al., 2012, Knuuti et al., 2019).

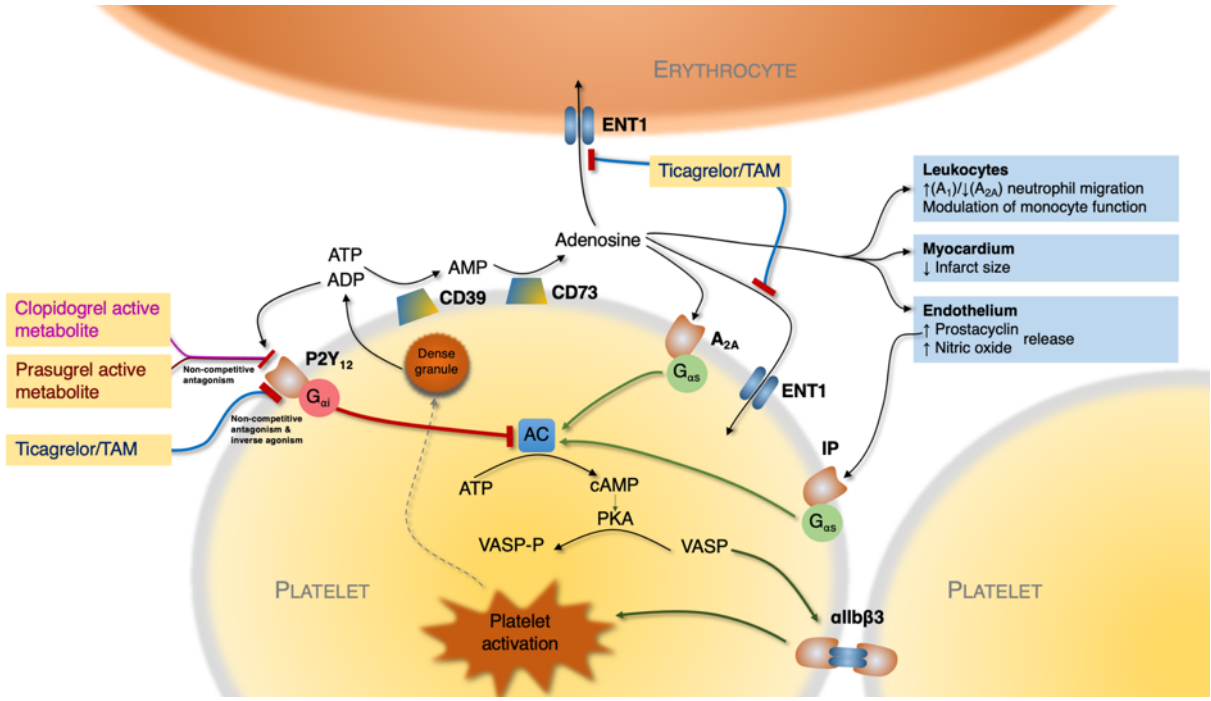

Figure 1.4 Intracellular signalling pathway of the $P2Y_{12}$ receptor, interaction with adenosine metabolism via the action of ticagrelor, and downstream effects of adenosine on leukocytes, myocardium and endothelium. Republished with permission of Elsevier Science & Technology Journals (Parker and Storey 2016b); permission conveyed through Copyright Clearance Center, Inc.

A1, adenosine A1 receptor; A2A, adenosine 2A receptor; αIIbβ3, glycoprotein IIb/IIIa complex; ADP, adenosine diphosphate; AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CD39, cluster of differentiation 39 (ectonucleoside triphosphate diphosphohydrolase-1); CD73, cluster of differentiation 73 (5'-nucleotidase); ENT1, equilibrative nucleoside transporter 1; $G_{\alpha i}$, inhibitory G protein α subunit, $G_{\alpha i}$, stimulatory G protein α subunit; IP, prostacyclin receptor; P2Y12, platelet ADP receptor; PKA, protein kinase A; TAM, ticagrelor active metabolite; VASP(-P), vasodilator-stimulated phosphoprotein (phosphorylated).

Table 1.1 Key randomised controlled trials of DAPT in ACS, showing that increasing intensity of antiplatelet therapy reduces ischaemic risk but increases bleeding risk (Parker and Storey 2016a). Reproduced with permission from BMJ Publishing Group Ltd. ACS, acute coronary syndrome; CABG, coronary artery bypass grafting; CV, cardiovascular; GUSTO, global strategies for opening occluded coronary arteries; HR, hazard ratio; MI, myocardial NSTE-ACS, non-ST elevation ACS; OR, odds ratio; PCI, percutaneous coronary intervention; PPCI, primary PCI; PPM, permanent pacemaker; RR, relative risk; STEMI, ST elevation MI; TIMI, thrombolysis in MI

Trial	n	ACS group included	Study medication group	Control group	Primary outcomes	Safety endpoints
CURE (Yusuf et al. 2001)	12,562	NSTE-ACS	Aspirin + Clopidogrel	Aspirin + Placebo	CV death/MI/stroke – RR 0.80 (95%CI 0.72-0.90, P<0.001) CV death/MI/stroke/refractory ischemia – RR 0.87 (0.79-0.94, P<0.001)	Major bleeding – RR 1.38 (1.13-1.67, p=0.001) No significant difference in life threatening bleeding, fatal bleeding or haemorrhagic stroke
CLARITY (Sabatine et al. 2005)	3491	STEMI	Aspirin + Clopidogrel	Aspirin + Placebo	Occluded infarct-related artery on angiography/death (all cause) or recurrent MI before angiography/death or recurrent MI before discharge or day 8 in those not undergoing angiography – Odds reduction 36% (95%CI 24-47%, P<0.001)	No significant difference in major bleeding at 24 hours post angiography, intracranial bleeding or bleeding post CABG
COMMIT (Chen et al. 2005)	45,852	STEMI	Aspirin + Clopidogrel	Aspirin + Placebo	Death/reinfarction/stroke OR 0.91 (0.86-0.97, p=0.002) Death (all cause) OR 0.93 (0.87-0.99, p=0.03)	No significant differences in fatal or non-fatal cerebral or non-cerebral bleeding

TRITON-TIMI 38 (Wiviott et al. 2007)	13,608	All ACS with scheduled PCI	Aspirin + Prasugrel	Aspirin + Clopidogrel	CV death/MI/stroke in both NSTE-ACS (HR 0.82 [0.73-0.93], p=0.002) and STEMI (HR 0.79 [0.65-0.97], p=0.02) groups	Major bleeding (HR 1.32 [1.03-1.68], p=0.03) Life threatening bleeding (HR 1.52 [1.08-2.13], p=0.01) CABG related major bleeding (HR 4.73 [1.90-11.82], P<0.001) No significant difference in cerebral haemorrhage or non-bleeding related serious adverse events
TRILOGY ACS (Roe et al. 2012)	7243	NSTE-ACS with medical management	Aspirin + Prasugrel	Aspirin + Clopidogrel	No significant difference in CV death/MI/stroke, although trend towards benefit with prasugrel after 12 months (p=0.07)	No significant differences in GUSTO or TIMI defined severe/life threatening/fatal bleeding TIMI major or minor bleeding (HR 1.54 [1.06-2.23], p=0.02) No significant difference in non-haemorrhagic serious adverse events
PLATO (Wallentin et al. 2009)	18,624	All ACS (STEMI patients included only if for PPCI)	Aspirin + Ticagrelor	Aspirin + Clopidogrel	CV death/MI/stroke (HR 0.84 [0.77-0.92]. P<0.001)	No significant differences in major, fatal, life threatening or CABG related bleeding Increased Non-CABG related major bleeding (p=0.03) Intracranial bleeding (p=0.06)/fatal intracranial bleeding (p=0.02). Increased non-intracranial fatal bleeding (p=0.03) Increased Dyspnoea (p<0.001) Increased ventricular pauses in 1st week (p=0.01) but not at 30 days and no significant difference in syncope or PPM requirement

d) Parenterally-administered $P2Y_{12}$ inhibitors

Cangrelor, a parenterally-administered, reversibly-binding $P2Y_{12}$ inhibitor, is a potent and rapidly acting antiplatelet drug. An analogue of ATP, it has an ultra-short half-life related to its rapid metabolism by dephosphorylation to a main metabolite that does not bind to $P2Y_{12}$ (Parker et al. 2017). The CHAMPION PHOENIX study was a randomised double-blind, placebo-controlled trial carried out in 11,145 patients undergoing urgent or elective PCI (Bhatt et al. 2013). Participants were randomised as initial therapy to either parenteral cangrelor or oral clopidogrel. The primary efficacy endpoint, a composite of all-cause mortality, MI, ischaemia-driven revascularisation and stent thrombosis at 48 hours, occurred significantly less frequently in the group receiving cangrelor than that receiving clopidogrel (4.7% vs. 5.9%, odds ratio [OR] 0.78 95% confidence interval [CI] 0.66-0.93, p=0.005). Overall, the rates of adverse effects were similar between the groups (20.2% in the cangrelor group, 19.1% in the clopidogrel group, p=0.13), including for those in whom study drug was discontinued (0.5% vs. 0.4% of adverse events, p=0.21)

Selatogrel is a novel, parenterally-active, reversibly-binding $P2Y_{12}$ inhibitor formulated for subcutaneous (SC) administration (Parker and Storey 2020a). Its molecular structure is derived from implementing the pyrimidine group of ticagrelor into a family of compounds previously investigated as $P2Y_{12}$ receptor antagonists (Caroff et al. 2014). Preclinical studies suggested that selatogrel was potent and selective, but also that it might have a wider therapeutic index when compared to clopidogrel or ticagrelor with regards to increase in bleeding risk whilst maintaining antithrombotic effect (Rey et al. 2017). Phase 1 studies of oral selatogrel or a prodrug were hindered by poor absorption and palatability (Baldoni et al. 2014). Subsequently, the SC preparation of selatogrel was tested and safety and tolerability demonstrated (Juif et al. 2019). The drug has rapid onset and a radiolabelled drug study suggested no significant plasma metabolites and that elimination was largely faecal, predicting no significant drug-drug interactions (Ufer et al. 2019). Phase 2 studies in both acute and chronic settings of ischaemic heart disease have now been reported with promising results. In the largest, 345 patients receiving maintenance antiplatelet therapy for coronary artery disease were randomized to receive SC selatogrel, at one of two doses, or placebo (Storey, Gurbel, et al. 2019). Selatogrel reliably and potently inhibited platelet reactivity by 30 minutes for around 8 hours, the effect wearing off by 24 hours. Importantly, selatogrel's effect appeared additive even in those already receiving oral $P2Y_{12}$ inhibitors, and there were no incidences of major bleeding. The drug's profile of effect was broadly similar when tested in 47 patients with acute MI (Sinnaeve et al. 2020). The clinical

setting(s) in which selatogrel may find a niche remains to be determined but, given that it provides potent, rapid and reversible $P2Y_{12}$ inhibition without the need for intravenous access nor an infusion, it seems rational that in particular early pre-hospital administration by caregivers or even self-administration by patients during a suspected ACS event may provide benefits over existing standard care, circumventing the issue of delayed absorption of oral $P2Y_{12}$ inhibitors by opioids (Thomas et al. 2016). Further study in phase 3 trials is imminent.

III. Oral anticoagulants

Oral anticoagulant (OAC) drugs target the coagulation cascade. Vitamin-K antagonists (VKA), such as warfarin, reduce vitamin-K-dependent factors. During chronic administration, prothrombotic factors (II, VII, IX, X) are inhibited more than antithrombotic factors (e.g. proteins C and S) (Hurlen et al. 2002). The non-VKA oral anticoagulants (NOACs) include the factor Xa inhibitors apixaban, edoxaban and rivaroxaban and the direct thrombin inhibitor dabigatran (Yeh et al. 2015).

E. Current recommendations for antithrombotic therapy in patients with acute coronary syndromes

Following the publication of the major trials of DAPT in ACS **(Table 1.1)**, the European Society of Cardiology (ESC) published guidelines on use of specific agents. In 2011, recommendations were published on the management of NSTE-ACS (Hamm et al. 2011) and these have been subsequently reinforced in the 2015 and 2020 NSTE-ACS guidelines (Roffi et al. 2015; Collet et al. 2020). A loading dose of aspirin (150-300 mg) followed by a daily maintenance dose of 75-100 mg and no higher (Class I recommendation, Level A evidence) is advised. An oral $P2Y_{12}$ inhibitor is recommended in addition and continued for 12 months unless contraindications such as excessive risk of bleeding are present. Ticagrelor given as a 180 mg loading dose followed by 90 mg BD is recommended for those patients at high risk of further ischaemic events, for example those with elevated cardiac biomarkers, regardless of whether or not a revascularisation strategy is planned (I, B). Prasugrel (at a loading dose of 60 mg followed by 10 mg OD) is recommended as an alternative only in patients in whom PCI is planned i.e. following coronary angiography (I,B). Clopidogrel (given as a 300-600 mg loading dose followed by 75 mg OD) should be reserved for those patients with contraindications to the newer agents

or who also require oral anticoagulation. If CABG is planned, withholding the $P2Y_{12}$ inhibitor is recommended for 5 days (ticagrelor/clopidogrel) or 7 days (prasugrel), although shorter durations may be guided by platelet function testing in those at lower bleeding risk.

With regards to patients with STEMI, the ESC published specific guidelines in 2012 and again in 2017 (Steg et al. 2012; Ibanez et al. 2017). Once again, clinicians are given the option of using aspirin (I,B) in combination with ticagrelor (I,C) or prasugrel (I,B), but the latter only an option in patients with no history of stroke or transient ischaemic attack (TIA) and age <75 years. Again, clopidogrel is only advised if the other agents are contraindicated (I,C). Aspirin monotherapy is recommended after this 1-year period. These recommendations were reinforced by the publication in 2014 of ESC guidelines on myocardial revascularisation (Windecker et al. 2014).

F. Current recommendations for antithrombotic therapy in patients with chronic coronary syndromes

A summary of the current ESC recommendations for antithrombotic therapy in patients with CCS is shown in **Figure 1.5** (Knuuti et al. 2019).

I. Long-term antithrombotic therapy in patients with CCS and in sinus rhythm

a) Single antiplatelet therapy

Robust evidence for versus against the use of antiplatelet therapy in patients with CCS comes, for example, from the Antithrombotic Trialists' Collaboration, who performed an individual-level meta-analysis including 135,000 patients with pre-existing cardiovascular disease (Antiplatelet Trialists' Collaboration 2002). They demonstrated clear benefit, mainly with aspirin as single antiplatelet therapy (SAPT), in reducing major adverse cardiovascular events (MACE, defined in this thesis as a composite of cardiovascular [CV] death, MI or stroke unless otherwise specified) by around a quarter, including a relative-risk-reduction (RRR) in those with prior MI of 21% ($p<0.0001$) and those with other CAD of 37% ($p<0.0001$). This analysis also showed the best protection from MACE when patients were receiving aspirin at a dose of 75 to 150 mg OD, when compared with higher doses.

Use of SAPT with antiplatelet agents other than aspirin has not been well tested against placebo;

however, there is evidence that clopidogrel monotherapy may offer advantages compared to aspirin in select groups. In the Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) study, treatment with clopidogrel 75 mg OD was compared with aspirin 325 mg OD (CAPRIE Steering Committee 1996). There was a slightly lower rate of the primary composite endpoint of MI, ischaemic stroke or CV death when receiving clopidogrel (5.32% vs. 5.83%, RRR 8.7% [0.3 to 16.5], p=0.043) as well as less gastrointestinal bleeding. Of note, the study group was heterogeneous and subgroup analysis suggested there was only a significant difference in the primary endpoint in those with peripheral artery disease (PAD) but not in those with CCS; there was also a trend towards a beneficial effect in those with prior stroke.

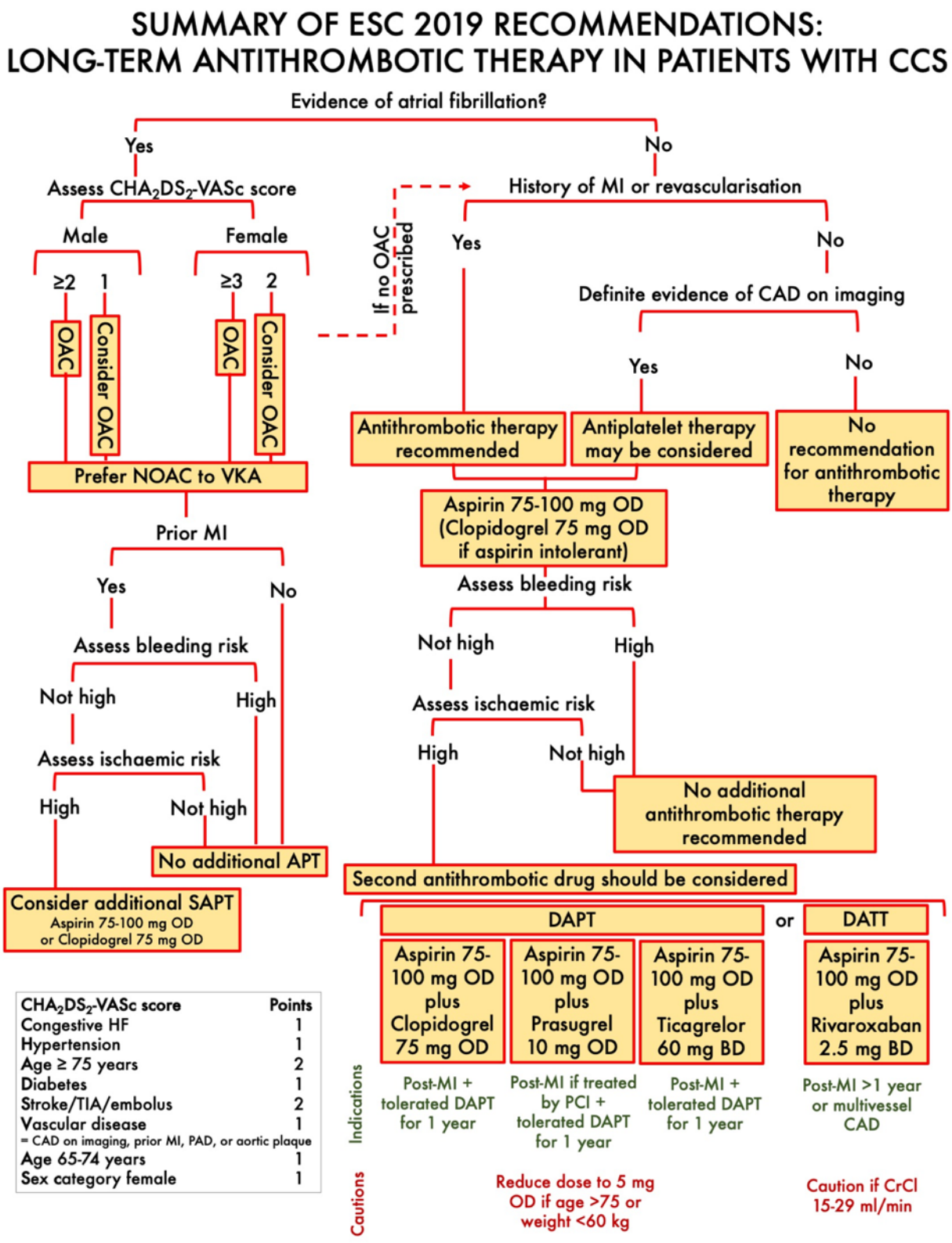

Figure 1.5 Decision algorithm summarising the approach to determining an optimum regimen of antithrombotic regimen suggested in the ESC 2019 CCS guidelines (Parker and Storey 2020b). Reproduced with permission from BMJ Publishing Group Ltd. APT, antiplatelet therapy; BD, twice daily; CAD, coronary artery disease; CrCl, creatinine clearance; DAPT, dual antiplatelet therapy; DATT low-dose dual antithrombotic therapy; HF, heart failure; MI, myocardial infarction; NOAC, non-vitamin-K-antagonist oral anticoagulant; OAC, oral anticoagulant; OD, once daily; PCI, percutaneous coronary intervention; SAPT, single antiplatelet therapy; TIA, transient ischaemic attack; VKA, vitamin K antagonist.

The ESC 2019 CCS guidelines recommend SAPT with aspirin 75-100 mg OD in patients with a history of MI or revascularisation (class I, level A) and state that aspirin may also be considered in those without prior MI or revascularisation but with definitive imaging evidence of CAD (IIb, C) (Knuuti et al. 2019). Clopidogrel 75 mg OD is recommended in cases of aspirin intolerance (I, B), or as an alternative to be considered in preference to aspirin in those CCS patients with PAD or prior cerebrovascular events (IIb, B).

b) Dual antiplatelet therapy

Whilst there is clear evidence that DAPT is beneficial across the spectrum of ACS, it has been less definitive for patients with CCS, who are a more heterogeneous group with a wide range of ischaemic risk. Nevertheless, a growing body of work has shown the benefits of long-term DAPT in selected CCS patients (**Table 1.2**).

The Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management, and Avoidance (CHARISMA) study included 19,185 stable aspirin-treated patients who had established atherothrombotic disease or had multiple risk factors for it, randomised to receive either clopidogrel or placebo (Bhatt et al. 2006). In the study group as a whole, there was a numerical reduction in the primary efficacy endpoint of MACE, but this did not reach significance (hazard ratio [HR] 0.93, 95 % CI 0.83 to 1.05, p=0.22). This was likely because of the inclusion of relatively low-risk as well as high-risk groups. However, in the subgroup of patients with prior MI, prior stroke or PAD, there was some evidence of benefit (0.77, 0.61 to 0.98, p=0.031) (Bhatt et al. 2007).

Similarly, the DAPT study showed that 30 vs. 12 months of thienopyridine treatment after PCI (65% clopidogrel, 35% prasugrel), alongside aspirin, significantly reduced death, MI or stroke in patients with prior MI (HR 0.56, 95% CI 0.42 to 0.76, p<0.001) (Mauri et al. 2016).

Table 1.2 Summary of key trials providing evidence for addition of a second antithrombotic agent during long-term treatment of high-risk CCS patients (Parker and Storey 2020b). Reproduced with permission from BMJ Publishing Group Ltd. CCS, chronic coronary syndromes; CV, cardiovascular; GUSTO, Global strategies for opening occluded coronary arteries; HR, hazard ratio [95 % confidence interval]; ISTH, international society on thrombosis and haemostasis; MI, myocardial infarction; PAD, peripheral artery disease; TIMI, thrombolysis in myocardial infarction.

Short name (year published)	Population	Experimental group(s)	Comparator	Primary endpoint	Relevant CCS subgroup primary endpoint analysis	Key safety endpoint
CHARISMA (Bhatt et al. 2006)	15,603 patients with clinically-evident CV disease or multiple risk factors (48% with CCS)	Clopidogrel 75 mg OD + aspirin 75 to 162 mg OD	Aspirin 75 to 162 mg OD	CV death, MI or stroke: 6.8% vs. 7.3%, HR 0.93 [0.83 to 1.05], p=0.22	Prior MI: 6.6% vs. 8.3%, HR 0.77 [0.61 to 0.98], p=0.031	GUSTO severe bleeding (intention-to-treat analysis): 1.7% vs. 1.3%, HR 1.25 [0.97 to 1.61], p=0.09
DAPT (Mauri et al. 2016)	9961 patients 12 months post-PCI (26% for MI) followed up for a further 18 months	Aspirin 75 to 162 mg OD + continued thienopyridine (65% clopidogrel 75 mg OD, 35% prasugrel 5 or 10 mg OD adjusted to weight)	Aspirin 75 to 162 mg OD	Stent thrombosis: 0.4% vs. 1.4%, HR 0.29 [0.17 to 0.48], p<0.001; CV death, MI or stroke: 4.3% vs. 5.9%, HR 0.71 [0.59 to 0.85], p<0.001	Prior MI: CV death, MI or stroke: 3.9% vs. 6.8%, HR 0.56 [0.42 to 0.76], p<0.001	GUSTO moderate or severe bleeding (intention-to-treat analysis): 2.5% vs. 1.6%, HR 1.61 [1.21 to 2.16], p=0.001
PEGASUS TIMI 54 (Bonaca et al. 2015)	21,162 patients aged ≥50 years with a history of spontaneous MI 1–3 years prior to enrolment and at least one additional atherothrombosis risk factor	Ticagrelor 60 mg BD plus aspirin 75-150 mg OD	Aspirin 75-150 mg OD	CV death, MI or stroke: 7.77% vs. 9.04%, HR 0.84 [0.74 to 0.95], p=0.008	N/A	TIMI major bleeding (on-treatment analysis with 3-year Kaplan-Meier rates): 2.3% vs. 1.1%, HR 2.32 [1.68 to 3.21], p<0.001
COMPASS (Eikelboom et al. 2017)	27,395 with CCS (91%) + additional risk factors if <65 years old) or symptomatic PAD (27%)	Aspirin 100 mg OD + rivaroxaban 2.5 mg BD	Aspirin 100 mg OD	CV death, MI or stroke: 4.1% vs. 5.4%, HR 0.76 [0.66 to 0.86], p<0.001	CCS: 4.2% vs. 5.6%, HR 0.74 [0.65 to 0.86]	Modified ISTH major bleeding (intention-to-treat analysis): 3.1% vs. 1.9%, HR 1.70 [1.40 to 2.05], p<0.001

There is more robust, primary endpoint-derived evidence for long-term use of ticagrelor in high-risk CCS patients. The Prevention of Cardiovascular Events in Patients with Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin–TIMI 54 (PEGASUS-TIMI 54) study showed a reduction in MACE when receiving DAPT with aspirin and ticagrelor, either 60 mg or 90 mg BD, vs. aspirin alone (e.g. 60 mg BD vs. placebo: HR 0.84 [0.74 to 0.95], p=0.008) in patients with prior MI (>1 year ago) and an additional risk factor (age ≥65 years, diabetes mellitus [DM], recurrent MI, multivessel CAD or chronic non-endstage renal disease) (Bonaca et al. 2015). Although TIMI-major bleeding was significantly more frequent in ticagrelor-treated patients, events such as intracranial haemorrhage, haemorrhagic stroke or fatal bleeding were not.

Ticagrelor-based DAPT has also been tested in those with CCS and type 2 DM but without prior MI in THE effect of ticagrelor on health outcomes in diabetes Mellitus patients Intervention Study (THEMIS), which included 19,220 aspirin-treated patients randomised to receive ticagrelor (90 mg BD, reduced to 60 mg during the course of the trial) or placebo (Steg et al. 2019). After an average follow-up of 40 months, there was a modestly lower incidence of MACE in those receiving ticagrelor vs. placebo (HR 0.90, 95% CI 0.81 to 0.99, p=0.04); however, there was a greater converse relative increase in Thrombolysis In Myocardial Infarction (TIMI)-major bleeding (2.32, 1.82 to 2.94, p<0.001). Whilst meeting its primary endpoint, the net clinical benefit has not supported adoption in practice, although subgroup analysis has suggested this may have been more favourable in those trial patients with a history of PCI (Bhatt et al. 2019).

ESC guidelines state that long-term addition of a second antiplatelet agent (i.e. a $P2Y_{12}$ inhibitor if already receiving aspirin, or aspirin if already receiving a $P2Y_{12}$ inhibitor) should be considered in those with CCS and a high risk of ischaemic events (**Table 1.3**) but without conditions associated with high bleeding risk (IIa, A) and may be considered in those with moderate ischaemic risk but without high bleeding risk (IIb, A) (Knuuti et al. 2019). Indications for use of clopidogrel or ticagrelor in this situation include those post-MI patients who have tolerated DAPT for at least a year, whereas prasugrel is an additional possibility if the MI was treated by PCI. The use of long-term prasugrel is not recommended in those age 75 years and over.

Table 1.3 Definitions, in the ESC 2019 CCS guidelines, of high or moderate ischaemic risk, or high bleeding risk for the purposes of determining optimal treatment strategies in maintenance antithrombotic therapy for CCS patients (Parker and Storey 2020b). Reproduced with permission from BMJ Publishing Group Ltd .CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; GI, gastrointestinal; MI, myocardial infarction.

<table>
<tr><th>High risk of ischaemic events</th><th>High risk of bleeding events</th></tr>
<tr><td>Diffuse multivessel CAD with at least one of:

• Drug-treated diabetes mellitus
• Recurrent MI
• Peripheral artery disease
• eGFR 15-59 ml/min/1.73m^2</td><td rowspan="2">At least one of:

• History of intracerebral haemorrhage or ischaemic stroke
• History of other intracranial pathology
• Recent GI bleeding
• Anaemia with possible GI cause
• GI pathology associated with increased bleeding risk
• Liver failure
• Bleeding diathesis/coagulopathy
• Extreme old age or frailty
• Dialysis-dependent or eGFR <15 ml/min/1.73m^2</td></tr>
<tr><th>Moderate risk of ischaemic events</th></tr>
<tr><td>At least one of:

• Mutivessel/diffuse CAD
• Drug-treated diabetes mellitus
• Recurrent MI
• Heart failure
• Peripheral artery disease
• eGFR 15-59 ml/min/1.73m^2</td><td></td></tr>
</table>

c) Low-dose dual antithrombotic therapy

The WArfarin Re-Infarction Study (WARIS) provided evidence that a full-dose OAC, with or without concurrent aspirin, provides protection against death, nonfatal reinfarction, or thromboembolic cerebral stroke, for CCS patients in sinus rhythm, but at the expense of excessive bleeding (Hurlen et al. 2002).

In more recent times, a regimen of low-dose dual antithrombotic therapy (DATT), comprising aspirin 75-100 mg once-daily and rivaroxaban 2.5 mg twice-daily, has been investigated in patients with CCS. The Cardiovascular OutcoMes for People using Anticoagulation StrategieS (COMPASS) study randomised 27,395 patients with high-risk CCS (91%) or symptomatic PAD to treatment with aspirin 100 mg once-daily, low-dose DATT or rivaroxaban 5 mg twice-daily as monotherapy (Table 1) (Eikelboom et al. 2017). To be eligible for the study, if CCS was the qualifying diagnosis, participants under the age of 65 were required to have documented atherosclerosis involving at least two vascular beds or at least two additional risk factors (current smoking, DM, eGFR <60 ml/min, heart failure, or non-lacunar ischaemic stroke ≥1 month

earlier). When compared to aspirin alone, low-dose DATT, but not rivaroxaban alone, led to a significant reduction in MACE after a mean follow-up of 23 months (HR 0.76, 95% CI 0.66 to 0.86, p<0.001).

Low-dose DATT is recommended as an alternative option to adding a second antiplatelet agent in patients with high ischaemic risk but not high bleeding risk (IIa, A) or moderate ischaemic risk but not high bleeding risk (IIb, A), in patients with a history of MI (at least one year ago) or multivessel CAD (Knuuti et al. 2019). Caution should be exercised if creatinine clearance is 15-29 ml/min.

II. Antithrombotic therapy in CCS patients undergoing PCI

d) Patients in sinus rhythm

Coronary stenting has an attendant risk of stent thrombosis, highest in the immediate post-PCI period and diminishing upon device endothelialisation (van Werkum et al. 2009). The risk is lower after PCI for CCS than for ACS, in which there is typically ongoing plaque disruption, thrombosis, and a systemic inflammatory and catecholaminergic response that may increase thrombotic risk (Claessen et al. 2014). After early concerns regarding stent thrombosis risk, in particular associated with drug-eluting devices, design has improved significantly. Stent strut size, which is a strong predictor of stent thrombosis, has reduced notably as newer generations of devices have become available (Iantorno et al. 2018). Nevertheless, the post-PCI setting in CCS patients represents a higher-than-baseline risk state typically mandating an intensification of antithrombotic therapy.

A period of DAPT reduces the risk of stent thrombosis after elective PCI when compared to aspirin alone. Using current-generation drug-eluting stents, there is evidence that a default duration of 6 months is as efficacious as 12 months. For example, in the largest study, Intracoronary Stenting and Antithrombotic Regimen: Safety and Efficacy of 6 Months Dual Antiplatelet Therapy After Drug-Eluting Stenting (ISAR-SAFE), 4005 patients undergoing PCI (approximately 50% electively) receiving aspirin were randomised to either duration of clopidogrel therapy (Schulz-Schupke et al. 2015). At 9 months after randomisation (i.e. 15 months after PCI), there was a non-inferior rate of death, MI, definite or probable stent thrombosis, stroke or TIMI major bleeding (1.5%, 95% CI 0.9 to 2.0 vs. 1.6%, 1.1 to 2.2, p <0.001), although the study was somewhat undermined by poor recruitment and a lower-than-

expected event rate. However, this is also supported by other smaller studies that together represent a body of evidence for this approach (Valgimigli et al. 2018). Whilst ticagrelor and prasugrel offer pharmacodynamic advantages over clopidogrel, including in patients with CCS undergoing elective PCI, there is no evidence this leads to improved clinical outcomes when used in the setting of DAPT (Orme et al. 2018; Parker et al. 2020; Silvain et al. 2020). Two large RCTs have recently investigated ticagrelor monotherapy as an alternative to DAPT after PCI, including in high-risk CCS patients. In particular, the Ticagrelor With Aspirin or Alone in High-Risk Patients after Coronary Intervention (TWILIGHT) study demonstrated significantly less bleeding, defined according to the Bleeding Academic Research Consortium (BARC) type 2, 3 or 5 classification, with 12 months of ticagrelor monotherapy compared with aspirin and ticagrelor, in patients who had already received and tolerated 3 months of DAPT (HR 0.56, 95% CI 0.45 to 0.68, $p<0.001$) (Mehran et al. 2019). The overall thrombotic event rate appeared non-inferior (0.99, 0.78 to 1.25, non-inferiority-$p<0.001$), although this was only a secondary endpoint and the non-inferiority margin was relatively broad.

Long-term antiplatelet therapy with at least aspirin is recommended after any PCI (I, A). In addition to aspirin, clopidogrel 75 mg OD is recommended, after loading, for six months (I, A). In those with a high risk of life-threatening bleeding, this can be considered for shortening to either 3 months (IIa, A) or, in those with very high bleeding risk, 1 month (IIb, C). Prasugrel or ticagrelor may be considered as an alternative to clopidogrel in cases of high-risk elective PCI, such as where there is stent under-deployment or when complex left main or multivessel stenting is performed, or if aspirin cannot be given in combination because of intolerance (IIb, C) (Storey, Valgimigli, et al. 2019).

In the setting of CAD, $P2Y_{12}$ inhibitors have generally been given alongside aspirin as DAPT and, when single antiplatelet therapy has been recommended, this has typically been with aspirin except in cases of sensitivity or intolerance (Knuuti et al. 2019; Roffi et al. 2015; Steg et al. 2012). However, $P2Y_{12}$ inhibitor monotherapy with clopidogrel is regarded as a standard-of-care treatment in the chronic phases of both PAD and cerebrovascular disease (Aboyans et al. 2018). The recommendation for these strategies originates from, for example, the findings of the Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) study that demonstrated modest benefits of single antiplatelet therapy with clopidogrel over aspirin in the general study population that were, however, more pronounced in these two subgroups (CAPRIE Steering Committee 1996). It is now clear from pharmacodynamic studies in a range of populations that ticagrelor provides more potent and reliable $P2Y_{12}$ inhibition than clopidogrel (Storey et al. 2010; Orme et al. 2018).

The novel strategy of ticagrelor monotherapy has now been tested against standard-of-care comparators in a number of large RCTs (**Table 1.4**). In the acute StrOke or Transient IsChaemic attack tReated with Aspirin or TicagrElor and patient outcomeS (SOCRATES) study, ticagrelor monotherapy was compared with aspirin monotherapy in the setting of acute (<24 hours since symptom onset) non-severe ischaemic stroke or high-risk transient ischaemic attack, excluding those treated by thrombolysis and those thought to have had a cardioembolic event (Johnston et al. 2016).

Assessed after a follow-up period of 90 days, the trial narrowly missed its primary objective of showing a significant difference in time to stroke, MI or death (6.7% [ticagrelor] vs. 7.5% [aspirin]; HR 0.89; 95% CI 0.78 to 1.01; p=0.07) between the groups. Safety endpoints such as major bleeding, fatal bleeding, intracranial haemorrhage or major and minor bleeding occurred at similar rates in both groups. On subgroup analysis, there was a trend towards greater benefit of ticagrelor in those who had received aspirin in the week before randomisation compared to those who had not (HR 0.76; 95% CI 0.61 to 0.95; p=0.02; vs. 0.96; 0.82 to 1.12; p=0.59; interaction-p=0.10). This generated the hypothesis that, in this population, DAPT with aspirin and ticagrelor may be superior to aspirin alone, and this was tested in THe Acute stroke or transient ischaemic attack treated with ticagreLor and aspirin for prEvention of Stroke and death (THALES) trial, which demonstrated a significant reduction in the primary composite endpoint of stroke or death at 30 days (5.5% vs. 6.6%, HR 0.83 [0.71-0.96, p=0.02), but at the expense of more frequent Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (GUSTO)-defined severe bleeding (0.5% vs. 0.1%, HR 3.99 [1.74-9.14], p=0.001 (Johnston et al. 2020).

Table 1.4 Randomised clinical trials of ticagrelor monotherapy for secondary prevention in patients with atherosclerotic disease (Parker and Storey 2020c). Reproduced with permission from Taylor & Francis Group. ACS, acute coronary syndrome; BARC, bleeding academic research consortium; BD, twice-daily; CAD, coronary artery disease; CI, confidence interval; HR, hazard ratio; LD, loading dose; MI, myocardial infarction; OD, once-daily; PAD, peripheral artery disease; PCI, percutaneous coronary intervention; PLATO, PLATelet inhibition and patient outcomes; TIA, transient ischaemic attack; TIMI, thrombolysis in myocardial infarction.

Study short title	Year published	n	Randomised population	Experimental regimen	Comparator	Primary endpoint	Primary safety endpoint*
SOCRATES (Johnston et al. 2016)	2016	13,199	Patients with non-severe ischaemic stroke or high-risk TIA, within 24 hours of symptom onset	Ticagrelor 180 mg LD on day 1 then 90 mg BD from day 2-90	Aspirin 300 mg LD on day 1 then 100 mg OD from day 2-90	Stroke, MI or death at 90 days: 6.7% vs. 7.5% (HR 0.89; 95%CI 0.78 to 1.00; p=0.07)	PLATO-defined major bleeding at 90 days: 0.5% vs. 0.6% (HR 0.83; 95% CI 0.52 to 1.34; p=0.45)
EUCLID (Hiatt WR 2017)	2017	13,885	Patients with symptomatic PAD	Ticagrelor 90 mg BD for 36 months	Clopidogrel 75 mg OD for 36 months	CV death, MI or ischaemic stroke: 10.8% vs. 10.6% (HR 1.02; 95% CI 0.92 to 1.13; p=0.65)	TIMI major bleeding: 1.6% vs. 1.6% (HR 1.10; 95% CI 0.84 to 1.43; p=0.49)
GLOBAL LEADERS (Vranckx et al. 2018)	2018	15,968	Patients undergoing PCI for stable CAD (53%) or ACS (47%), between angiography and PCI	Aspirin 75-100 mg OD and ticagrelor 90 mg BD for one month, then ticagrelor 90 mg BD for 23 months	Aspirin 75-100 mg OD plus either clopidogrel 75 mg OD (stable CAD) or ticagrelor 90 mg BD (ACS) for 12 months, then aspirin 75-100 mg OD for 12 months	All-cause death or new Q-wave MI at 730 days: 3.81% vs. 4.37% (RR 0.87; 95% CI 0.75 to 1.01; p=0.073)	BARC grade 3 to 5 bleeding: 2.04% vs 2.12% (RR 0.97; 95%CI 0.78 to 1.20; p=0.77)
TWILIGHT (Mehran et al. 2019)	2019	7119	'High-risk' patients undergoing PCI for stable CAD (35%) or Non-ST elevation ACS (65%), after 3 event-free months of DAPT with aspirin 75 mg OD and ticagrelor 90 mg BD	Ticagrelor 90 mg BD plus placebo for 3 years	Aspirin 81-100 mg OD plus ticagrelor 90 mg BD for 3 years	BARC grade 2, 3 or 5 bleeding: 4.0% vs. 7.1% (HR 0.56; 95% CI 0.45 to 0.68; p<0.001)	All-cause death, non-fatal MI or non-fatal stroke: 3.9% vs. 3.9% (HR 0.99; 95% CI 0.78 to 1.25; p(non-inferiority)<0.001

*or key secondary safety endpoint if no primary safety endpoint defined

Whereas SOCRATES compared monotherapy with ticagrelor vs. aspirin, in the Examining Use of tiCagreLor In peripheral artery Disease (EUCLID) trial, ticagrelor was compared with clopidogrel in 13,885 patients with symptomatic PAD (Hiatt WR 2017). After a follow-up period of 3 years, there was no appreciable difference between the groups in rates of the primary endpoint of cardiovascular death, MI or ischaemic stroke (10.8% vs. 10.6%; HR 1.02; 95% CI 0.92 to 1.13; p=0.65), nor in TIMI major bleeding (1.6% vs. 1.6%; 1.10; 0.84 to 1.43; p=0.49). The lack of any benefit of a $P2Y_{12}$ inhibitor with proven greater strength and consistency than clopidogrel to reduce ischaemic events was unexpected. Potential explanations for this finding include the fact that ticagrelor may mediate some of its benefits over clopidogrel by improving endothelial function (Vlachopoulos et al. 2019), which, hypothetically, in patients with such extensive atheromatous deposits might be less relevant. Alternatively, there is some evidence that clopidogrel has off-target effects, such as the observation that it reduces leukocyte count and other inflammatory markers, that might compensate for its unreliable antiplatelet effect in a population with a high baseline level of inflammation (Storey et al. 2014; Brevetti et al. 2010).

Most recently, two RCTs have tested ticagrelor monotherapy in the post-PCI setting. In the GLOBAL LEADERS trial, 15,968 patients with CAD undergoing PCI were randomised (Vranckx et al. 2018). Half were allocated to receive one month of DAPT with aspirin and ticagrelor, followed by 23 months of ticagrelor alone. The other half received one year of DAPT (aspirin plus ticagrelor if ACS or clopidogrel if stable CAD) followed by a year of aspirin monotherapy. After 730 days of follow up, the study failed to show a significant difference, between the groups, in its ambitious primary composite endpoint of all-cause death or new Q-wave MI (3.81% vs. 4.37%; relative risk [RR] 0.87; 95% CI 0.75 to 1.01; p=0.073), nor in its key safety endpoint of Bleeding Academic Research Consortium (BARC) grade 3 to 5 bleeding (2.04% vs 2.12%; 0.97; 0.78 to 1.20; p=0.77). A sub-analysis of the study has recently demonstrated that, in those participants in the ACS cohort, the difference in BARC grade 3 or 5 bleeding at 1 year (i.e. a ticagrelor monotherapy vs. aspirin and ticagrelor comparison) was in fact nominally significant (0.8% vs. 1.5%, HR 0.52; 95% CI 0.33 to 0.81; p=0.004) (Tomaniak et al. 2019). Moreover, there was no evidence of an increased rate of ischaemic events (1.5% vs. 2.0%; 0.73; 0.51 to 1.03; p = 0.07). The interpretation made was that ticagrelor monotherapy, compared with aspirin and ticagrelor, led to less bleeding but no increase in ischaemic events in this group. As this analysis was not pre-specified, its findings can only be regarded as hypothesis-generating. However, subsequently the Ticagrelor With Aspirin or Alone in High-Risk Patients After Coronary Intervention (TWILIGHT) trial has been reported. This included patients determined to be high-risk for ischaemic events undergoing PCI for stable CAD (35%) or non-ST-elevation ACS (65%). After 3 event-free months of DAPT with aspirin 75 mg OD and

ticagrelor 90 mg BD, 7119 patients were randomised to receive a further 12 months of either ticagrelor 90 mg BD plus placebo OD or aspirin and ticagrelor. At the end of follow-up, the primary endpoint of BARC grade 2, 3 or 5 bleeding occurred significantly less frequently in the ticagrelor monotherapy group (4.0% vs. 7.1%; HR 0.56; 95% CI 0.45 to 0.68; p<0.001), whilst demonstrating non-inferiority for the primary safety endpoint of all-cause death, non-fatal MI or non-fatal stroke (3.9% vs. 3.9%; 0.99; 0.78 to 1.25; p(non-inferiority)<0.001). It seems clear, therefore, that the addition of standard doses of aspirin to ticagrelor increases bleeding risk whilst not apparently offering superior protection against major adverse cardiovascular events during long-term treatment in a broad population treated with PCI. Whether lower-than-standard doses of aspirin in the context of DAPT might prove superior to standard doses remains to be explored. These might provide benefits of enhanced antithrombotic effect when compared to ticagrelor monotherapy whilst reducing harmful effects compared to the current standard aspirin doses. Other outcomes of interest are more difficult to study: whilst current-generation drug-eluting stents with thin struts and biocompatible or absent polymer confer a very small risk of stent thrombosis meaning that ticagrelor monotherapy is sufficient to adequately prevent this after an initial period of stent endothelialisation, the low frequency of events makes this very difficult to definitively determine in clinical trials.

It is also established that ticagrelor maintenance therapy provides more potent mean platelet inhibition than prasugrel maintenance therapy in aspirin-treated ACS patients (in contrast to the similar mean platelet inhibition levels achieved after loading doses) (Joshi et al. 2014). The recent results of ISAR REACT 5 challenge the assumption that more potent $P2Y_{12}$ inhibition in the setting of DAPT leads to reduced cardiovascular events. 4018 ACS patients with planned invasive management were randomised to DAPT with aspirin and either prasugrel or ticagrelor. It is worth noting, however, that this was an open-label study with a relatively high proportion of femoral-access PCI procedures and short hospital stays that may not allow early detection of intolerance to ticagrelor. Furthermore, an off-target favourable effect of prasugrel unrelated to strength of platelet inhibition cannot be excluded: notably, clopidogrel, another thienopyridine, has been associated with lower levels of inflammatory markers than ticagrelor (Storey et al. 2014). Nevertheless, this may support the de-escalation of antiplatelet therapy as a strategy. Studies of ticagrelor monotherapy compared with aspirin plus prasugrel might offer further insights into the optimal strategy in this group.

e) Patients with an indication for full-dose oral anticoagulation

Patients undergoing PCI who also have an indication for a therapeutic dose of an OAC for AF present a particular challenge because of the need to prevent both stent thrombosis, which is primarily platelet-dependent, and cardioembolism, primarily mediated by activation of the coagulation cascade. Whilst there is growing evidence that many CCS patients with AF obtain satisfactory anti-ischaemic protection in the stable phase of treatment, this has not been well tested in the peri-PCI setting. Combining antiplatelet therapy with full-dose OAC increases the risk of bleeding significantly, therefore it is important to balance risks and benefits when considering the optimal regimen.

Until recently, there was a paucity of evidence to guide recommendations in this situation, complicated by the numerous possible permutations of treatment combinations and durations that feasibly exist. Initial data from the small-scale What is the Optimal antiplatElet and anticoagulant therapy in patients with oral anticoagulation and coronary StenTing? (WOEST) study suggested that combining clopidogrel and an OAC reduced bleeding following PCI compared to triple therapy with aspirin, clopidogrel and an OAC (Dewilde et al. 2013). The study was underpowered to detect ischaemic endpoints but showed no clear penalty in de-escalation of therapy. Subsequently, several larger RCTs have provided further evidence to support this approach. The largest of these, the Open-label, 2 x 2 Factorial, Randomized Controlled, Clinical Trial to Evaluate the Safety of Apixaban vs. Vitamin K Antagonist and Aspirin vs. Aspirin Placebo in Patients with Atrial Fibrillation and Acute Coronary Syndrome or Percutaneous Coronary Intervention (AUGUSTUS) randomised 4614 patients with AF and ACS and/or recent PCI (39% elective), due to receive a $P2Y_{12}$ inhibitor for 6 months, to receive aspirin or placebo plus apixaban (5 mg BD, reduced to 2.5 mg BD where clinically indicated) or warfarin with a target international normalised ratio (INR) of 2.0 to 3.0 (Lopes et al. 2019). The two main conclusions of the study were that receiving aspirin increased the risk of the primary endpoint of ISTH major or clinically-relevant non-major bleeding (HR 1.89, 95% CI 1.59 to 2.24, $p<0.001$) without significantly reducing the incidence of the secondary endpoint of death or ischaemic events (0.89, 0.71–1.11), and that receiving a NOAC led to significantly less bleeding than a VKA (HR 0.69, 0.58–0.81, $p<0.001$) without increasing the incidence of death or ischaemic events (0.89, 0.71–1.11). However, there were numerically fewer stent thrombosis events in those treated with aspirin versus placebo, which raised concerns about the requirement for aspirin to prevent this particular outcome.

ESC guidelines place significant weight on judging the balance between risk of stent thrombosis, risk of ischaemic stroke (CHA_2DS_2-VASc score) and risk of life-threatening bleeding (Table 2). Both risk of stent thrombosis occurring and the potential gravity of the consequences depend on numerous factors, including clinical features, such as DM, renal disease or a history of on-treatment stent thrombosis, and procedural aspects, such as stent underdeployment, stent length >60 mm, double stenting of a bifurcation, or stenting of the left main stem, left anterior descending, last remaining patent artery or chronic total occlusion. It is recommended in all cases to continue OAC after PCI, but, where possible, a NOAC is strongly preferred to a VKA (I, A). Where a VKA is used, the target INR should be 2.0-2.5 with an aim of >70% of the time in the therapeutic range (IIa, B). Similarly, it is advised, on the basis of expert opinion, to load with and maintain aspirin and clopidogrel, as well as continue OAC (triple therapy) at the time of PCI (I, C). If stent thrombosis risk is deemed low, or deemed to be outweighed by bleeding risk, aspirin can then be discontinued within a week after PCI, continuing clopidogrel and OAC (IIa, B). In cases where risk of stent thrombosis is believed to outweigh that of bleeding, a longer period of triple therapy (1 to 6 months) may be considered (IIa, C). Ticagrelor or prasugrel as part of triple therapy is not recommended (III, C) but in combination with OAC may be considered as an alternative to triple therapy in those with at least moderate risk of stent thrombosis.

G. Interactions between thrombosis and inflammation

I. The inflammatory response

The inflammatory response includes a complex network of factors, triggered by insults such as infection, trauma or toxicity. Broadly, this is initiated by damage pattern recognition receptors found on a range of leukocytes (Kawai and Akira 2010). This leads to recruitment and translocation of other leukocytes, release of cytokines and other inflammatory mediators, activation of complement and physical attack of pathogens or infected cells (Chaplin 2010).

II. Role of platelets during inflammation

Platelets too play an important role in the regulation and enactment of the inflammatory response. Acute inflammation, for example during endotoxaemia, results in increased in numbers of platelet-monocyte and platelet-neutrophil aggregates, mediated via, for example, enhanced P-selectin expression (Thomas and Storey 2015).

Platelet reactivity, measured by numerous assays, is increased during acute inflammatory states, for example, sepsis (Akinosoglou et al. 2017). Mechanisms may include, for example, the direct stimulation of the GP VI pathway by interleukin (IL)-6 (Houck et al. 2019). Platelets themselves are acute phase reactants and inflammation induces thrombocytosis, mediated by an increase in thrombopoetin levels, an effect potentiated by IL-6 (Kaser et al. 2001). Furthermore, platelet turnover is accelerated in proinflammatory states (Grove et al. 2011). Raised circulating levels during inflammation of other platelet agonists such as epinephrine, via α_2 receptors, and serotonin (5-HT), via 5-HT_{2A} receptors, may also contribute to enhanced reactivity (Bevan and Heptinstall 1985; Keularts et al. 2000).

III. Role of acellular coagulation during inflammation

Acute inflammation is also associated with pro-thrombotic changes in fibrin clot dynamics, including increased fibrin strand density and clot turbidity (Thomas et al. 2015). Similarly, levels of markers such as D-dimer and fibrinogen may be elevated. Thrombin generation, which is increased during inflammation (Petros et al. 2012), drives not only coagulation and platelet activation, but also inflammation more directly through promotion of leukocyte recruitment (Chen and Dorling 2009).

Conversely, severe inflammation can lead to such an increase in prothrombotic tendency that widespread microvascular thrombosis occurs, known as disseminated intravascular coagulation. This results in a fall in detectable clotting factors as these are consumed rapidly, and can paradoxically lead to reduced haemostatic function and therefore increased bleeding risk (Levi 2007).

IV. Effects of inflammation on the endothelium

Endothelial function is adversely affected during systemic inflammation. In particular, there is increased release of VWF, which facilitates platelet-endothelium and platelet-platelet binding, and reductions in the activity of the anti-thrombotic factors tissue factor pathway inhibitor and protein C (Ince et al. 2016).

In the arterial circulation, inflammation can also drive atheromatous plaque progression, impacting on local haemodynamics and also plaque stability, making plaque rupture or erosion events, which can trigger thrombosis, more likely (Libby et al. 2019).

V. Therapeutic targeting of inflammation in IHD

Inflammation drives atherogenesis and thrombosis, so is an obvious target for therapies to reduce MACE, including in patients with IHD (Libby et al. 2019).

a) Drug therapy to target inflammation

Several approaches have now been explored in outcome-driven RCTs of patients with IHD. For example, in the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), canakinumab, a monoclonal antibody against IL-1ß significantly reduced the incidence of MACE in patients with prior MI and evidence of ongoing inflammation (e.g. 150 mg canakinumab vs. placebo HR 0.85, 95% CI 0.74 to 0.98, p=0.021) (Ridker et al. 2017). Benefits were offset, however, by an increased risk of fatal infections (pooled incidence rate [canakinumab vs. placebo] 0.31 vs. 0.18 per 100 person-years, p=0.02), which reflects the potential balance of benefit and risks of this approach that limits its overall efficacy. Nevertheless, CANTOS affirmed the value of targeting inflammation in patients with IHD.

Similarly, the recent results of the COLchicine Cardiovascular Outcomes Trial (COLCOT) have demonstrated the value of anti-inflammatory therapy with colchicine if commenced within 30 days of an ACS event and continued for 42 months (Tardif et al. 2019). Particularly impressive was the reduction in incidence of stroke: even though the numbers were small, the upper limit of the 95% confidence interval for the HR was well below 1 (0.2% vs. 0.8%, HR 0.26 [0.10 to 0.70]). As well as the notorious gastrointestinal side effects of colchicine, there was a significantly increased risk of pneumonia. The body of evidence relating to colchicine in IHD has most recently been expanded by the results of the Low-Dose Colchicine (LoDoCo) 2 and the Colchicine in Patients with Acute Coronary Syndromes (COPS) trials (Tong et al. 2020; Nidorf et al. 2020). In both of these studies, there was evidence that colchicine reduced the incidence of ischaemic events compared to placebo; however, they also both showed increased non-cardiovascular mortality when receiving active drug. The mechanism for this latter finding was not clear from the breakdown of causes, though it has been suggested this may be due to chance alone. It remains to be seen whether colchicine, an already widely available drug, will be recommended for routine use in this population, but further insights are likely to come from the ongoing 2x2 factorial RCT of colchicine and spironolactone in patients with acute ST-elevation

MI (Colchicine and Spironolactone in Patients with STEMI / SYNERGY Stent Registry, CLEAR-SYNERGY, NCT03048825) that aims to enrol around 4000 patients and has plans to increase its scope and size further in the near future.

Not all therapies targeting inflammation may offer vascular protection, however. Notably, the use of the anti-folate drug methotrexate in a high-risk CAD population offered no reduction in MACE vs. placebo in the Cardiovascular Inflammation Reduction Trial (CIRT) (Ridker et al. 2019). As there were no reductions in inflammatory markers relevant to atherothrombosis, such as C-reactive protein (CRP), IL-1ß or IL-6, it may be that more pathway-specific strategies are required.

b) Treatment of periodontitis

Any source of chronic inflammation that leads to increased circulating levels of pro-inflammatory cytokines may hypothetically accelerate atherothrombosis. In particular, a large burden of chronic inflammation may arise from periodontitis, which is estimated to affect around half of adults in Western countries, around two-thirds of cases being moderate or severe in those over the age of 65 (Eke et al. 2012). Periodontitis has been linked not only with detectable increases in parameters such as platelet activation (Papapanagiotou et al. 2009), circulating IL-6 and high-sensitivity (hs) CRP (Marcaccini et al. 2009), but also, as an independent risk factor, with CAD (Humphrey et al. 2008) and stroke (Grau et al. 2004). Intensive treatment is associated with, for example, a reduction in circulating IL-6 and hsCRP (Marcaccini et al. 2009), and improvements in endothelial function (Tonetti et al. 2007). There is also limited retrospective evidence that treatment also reduces the incidence of MACE in high-risk groups such as those with previous stroke or type 2 DM (Peng et al. 2017; Lin et al. 2019).

H. Aspirin: posological considerations

I. Effects of varying dose

Aspirin was originally developed as an analgesic and antipyretic. The suggested doses for these purposes were equivalent to around 600-900 mg four times daily (QDS). It was quickly realised that even higher doses of 1.2 g QDS and above also have anti-inflammatory effects of use in

conditions such as rheumatoid arthritis and rheumatic fever. Aspirin remains in the plasma for only a relatively short period of time after administration, being readily hydrolysed to salicylate which persists much longer in the circulation. Salicylic acid, used as a drug in its own right since at least the time of the Ancient Egyptians, also possesses anti-inflammatory properties, but has no effect on platelet activation (Rosenkranz et al. 1986). Several studies have measured peak plasma concentrations of aspirin after administration of various doses of different formulations, and these are summarised in **Figure 1.6**. Whilst limited by non-standardisation of assays and heterogeneity of sampling times, this provides a representation of expected pharmacokinetics when considering dose modification, and can guide in vitro studies.

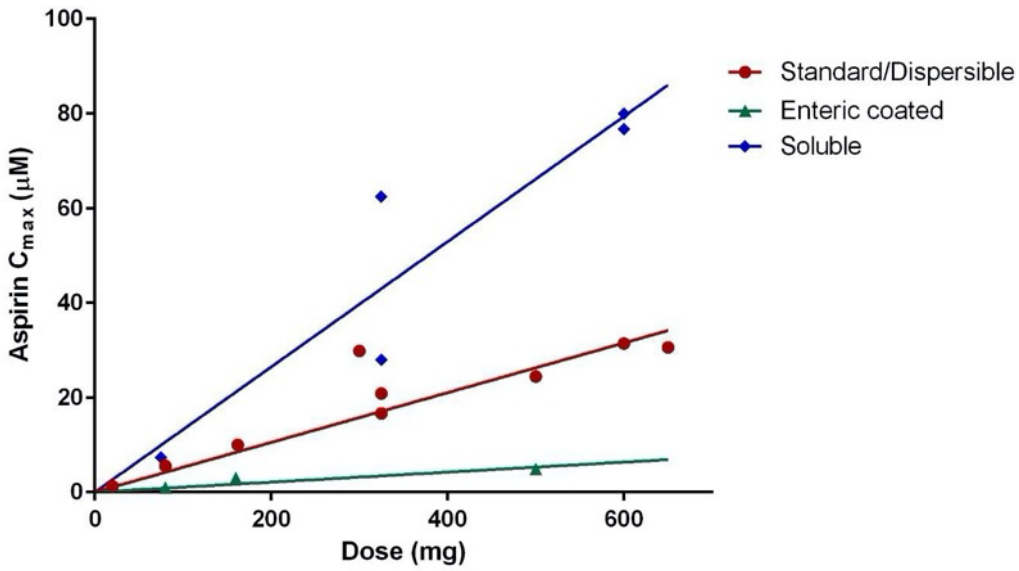

Figure 1.6 Estimated mean maximal venous plasma concentrations of aspirin from 8 pharmacokinetic healthy volunteer studies administering single doses of 75 - 650 mg standard/dispersible, soluble or enteric-coated formulations (Seymour and Rawlins 1982; Pedersen and FitzGerald 1984; Charman et al. 1993; Benedek et al. 1995; Muir et al. 1997; Sagar and Smyth 1999; Cerletti et al. 2003; Hobl et al. 2015).

This is also complicated by the fact that peripheral plasma levels of the drug may underestimate the peak concentration which platelets are exposed to in the portal venous system, where there is a high level of presystemic acetylation (Pedersen and FitzGerald 1984). Peripheral levels, however, *are* likely to represent the aspirin concentration adjacent to the systemic endothelium and hence are particularly relevant when considering effects on PGI_2 release.

A number of studies have compared the effects of different doses of aspirin (as SAPT) on arachidonic acid metabolites, platelet function and bleeding time. Those that included doses less than the current standard of 75 mg are summarised in **Table 1.5**. In summary, single and repeated doses of as low as 10-12 mg have been shown to significantly reduce serum levels of the principal TXA_2 metabolite, TXB_2, whilst such doses do not inhibit PGI_2 release, which appears to begin to occur at doses of around 40 mg and above.

In the setting of secondary prevention of ischaemic heart disease (IHD), there is now a general consensus that the lowest currently available doses of 75-100 mg daily are as effective as higher doses and may result in fewer complications. The most significant evidence for this comes from a subanalysis by the Antithrombotic Trialists' Collaboration (2002).

II. Effects of varying frequency of administration

Current guidelines suggest aspirin is given OD for treatment and secondary prevention of ACS. This is based on the assumption that, because of its irreversible action on platelet COX1, there is sufficient duration of effect to maintain inhibition of circulating platelets, which have a lifespan of around 10 days in health (Leeksma and Cohen 1956). However, in a significant proportion of patients, platelet turnover is increased, meaning that, at trough (pre-dose) effect, a greater proportion of circulating platelets are uninhibited than in those with normal platelet turnover. In vitro studies have suggested that even a small number of uninhibited platelets can initiate thrombus formation (Hoefer et al. 2015). Conditions that have been associated with increased platelet turnover include diabetes, smoking, obesity and procedural intervention – all common in patients with ACS (Henry et al. 2011). Several studies of multiple daily aspirin dosing have been performed, including in patients with IHD. These are summarised in **Table 1.6**.

Table 1.5 Studies of the effects of aspirin dosing on arachidonic acid metabolites, bleeding time and platelet function, showing that lower-than-standard doses of aspirin inhibit thromboxane generation and platelet aggregation whilst sparing prostacyclin release and without prolongation of bleeding time. 6-keto PGF1α, 6-keto prostaglandin F1α ; ADP, adenosine diphosphate; NA, not assessed;; PGI-M, prostacyclin metabolite; TXB_2, thromboxane B_2

Study details					**Reported effects**			
Study	**n**	**Population**	**Doses of aspirin studied**	**Samples obtained**	**Thromboxane A_2**	**Prostacyclin**	**Bleeding time**	**Platelet function**
(Hoogendijk and ten Cate 1980)	8	Healthy volunteers	40 mg daily for up to 1 month	Venous blood	Significant and cumulative inhibition by 40 mg OD, 95% inhibition by day 10	NA	NA	NA
(Hanley et al. 1981)	68	Patients undergoing varicose vein surgery	40, 81, 300 mg - single doses	Peripheral vein tissue	TXA_2 metabolites inhibited at all doses for at least 96 hours	Inhibited by 81 and 300 but not 40 mg	NA	NA
(Patrignani et al. 1982)	46	Healthy volunteers	6, 12, 25, 50, 100 mg - single doses. 0.45 kg/day (20-40mg) - for 7 days.	Venous blood, urine	Single doses as low as 12 mg significantly inhibited serum TXB_2 in a dose dependent manner	20-40 mg/day did not significantly reduce urinary 6-keto PGF1α	NA	NA
(Weksler et al. 1983)	70	Patients undergoing CABG	0, 40, 80, 325 mg - single doses	Venous blood, Samples of vein and artery	Reduced at all doses, dose dependent	Venous production only reduced at 325 mg, Arterial reduced at all doses (dose dependent)	NA	NA
(Davi et al. 1983)	8	Healthy volunteers	20, 100 mg - single doses	Venous blood	Significant inhibition of serum TXB_2 by both doses	Significant inhibition of serum 6- keto $PGF_{1\alpha}$ by 100 mg but not 20 mg	NA	NA

(FitzGerald et al. 1983)	10	Healthy volunteers	20, 2600 mg daily for 7 days in successive weeks; 20, 40, 80, 160, 325, 650, 1300, 2600 mg each for 7 days (dose escalation)	Venous blood, urine	Dose dependent inhibition, with significant reduction of TXB2 at doses greater than 40 mg	Dose dependent, with significant inhibition of urinary PGI-M at doses greater than 80mg	Bleeding time significantly prolonged by 2600 mg but not 20 mg	Response to ADP and epinephrine significantly inhibited at both 20 mg and 2600 mg, response to collagen significantly inhibited by 2600 mg but not 20 mg
(Hanley and Bevan 1985)	62	Patients undergoing bowel resection	0, 40, 75, 300 mg - single doses	Mesenteric artery and vein tissue	NA	Arterial and venous 6-keto PGF1α production reduced by all doses compared to baseline, dose dependent	NA	NA
(Chetty et al. 1985)	18	Healthy volunteers	20, 162 mg daily for 4 weeks	Venous blood	Both doses significantly inhibited serum TXB2 at 2 and 4 weeks	NA	Significantly prolonged by both doses	Significantly inhibited by both doses
(Kallmann et al. 1987)	19	Healthy volunteers	10, 30 mg daily for 3 weeks	Venous blood, urine	Significant inhibition by both doses	Neither dose affected urinary 6- keto PGF1α	Significantly prolonged by 30 mg but not 10 mg	NA
(Tohgi et al. 1992)	19	Patients with a history of stroke	40, 320, 1280 mg daily for 7 days	Venous blood	Significantly inhibited at all doses, but magnitude of effect dose dependent	Significant inhibition only at 320 and 1280mg	NA	Significant inhibition all doses to 5μM, 10μM ADP and 2μg/ml Collagen
(Boger et al. 1993)	10	Healthy volunteers	50, 100 mg intravenously - single doses	Venous blood, urine	Urinary 2,3-dinor-TXB2 reduced by both doses in equal magnitude	Urinary PGI-M reduced by both doses, but significantly less by 50 vs. 100 mg	NA	NA

Table 1.6 Studies of more than once daily aspirin dosing regimens and their antiplatelet effects, showing that increasing the dosing frequency leads to greater consistency of effect. BD, twice daily; CABG, coronary artery bypass grafting; CAD, coronary artery disease; OD, once daily; PGI-M, prostacyclin metabolite; QDS, four times daily; TXB_2, thromboxane B_2.

Study	n	Population	Dosing regimens studied	Summary of outcomes
(Addad et al. 2010)	25	Patients with diabetes and stable CAD	100 mg OD for 10 days then 100 mg BD for those with persistent high platelet reactivity	Persistent high platelet reactivity significantly less frequent when taking aspirin BD.
(Spectre et al. 2011)	25	Patients with diabetes and macro/microvascular complications	75 mg OD vs. 75 mg BD vs. 320 mg BD >14 days (randomised crossover)	Whole blood platelet aggregation to arachidonic acid significantly reduced by BD dosing compared to OD doses, urinary thromboxane reduced by high dose but not low dose OD or BD aspirin.
(Dillinger et al. 2012)	92	Patients with diabetes and IHD at high risk of aspirin resistance	150 mg OD vs. 75 mg BD for 7-14 days (crossover). 52% were receiving clopidogrel.	Pre-dose platelet aggregation to arachidonic acid significantly less in BD group. Biological aspirin resistance significantly less frequent when receiving BD aspirin.
(Rocca et al. 2012)	173	Patients with (n=100) and without (n=73) diabetes	100 mg OD vs. 200 mg OD vs. 100 mg BD for 28 days	BD dosing significantly reduced thromboxane recovery over 12 hours compared to OD doses.
(Paikin et al. 2015)	110	Patients undergoing CABG surgery	325 mg OD vs. 81 mg QDS for around 7 days	Serum TXB_2 and platelet aggregation to arachidonic acid significantly less in QDS dosing compared to OD regimen.
(Cavalca et al. 2017)	37	Patients undergoing on-pump cardiac surgery receiving 100 mg OD at baseline	100 mg OD vs. 200 mg OD vs. 100 mg BD for 7 days	100 mg BD, but not OD regimens, reduced TXB_2 compared to baseline. PGI-M was reduced by 200 mg OD but not 100 mg OD or BD.

I. Relationship between ticagrelor dosing regimen and its effects

Husted et al performed comparative pharmacodynamic and pharmacokinetic (PK) studies of ticagrelor (known as AZD6140 at that time) and clopidogrel in stable patients with known atherosclerotic disease already receiving aspirin (Husted et al. 2006). In this study (later known as the DISPERSE trial), ticagrelor at a dose of 100 mg BD, 200 mg BD and 400 mg OD inhibited ADP-induced platelet aggregation significantly more than clopidogrel 75 mg OD. Ticagrelor 50 mg BD provided slightly greater mean inhibition than clopidogrel but similar interindividual variability (Storey 2008). Bleeding times after 28 days of treatment were increased compared both to baseline and to the patients on clopidogrel. Subsequent to the DISPERSE study, ticagrelor was administered in a new formulation for which 45mg provided a similar PK profile to 50 mg of the original formulation used in the DISPERSE study. DISPERSE2 was a phase 2 trial of ticagrelor compared with clopidogrel in the setting of NSTE-ACS, in 990 patients (Cannon et al. 2007). It suggested that ticagrelor (at loading doses of 90 - 270 mg and maintenance doses of 90 mg and 180 mg BD) had similar safety and tolerability profiles to clopidogrel despite providing higher and more consistent levels of $P2Y_{12}$ inhibition. In view of lower numerical rates of minor bleeding, dyspnoea and asymptomatic ventricular pauses, a dose of 90 mg BD was chosen over 180 mg BD for the phase 3 study of ticagrelor (PLATO). The PLATO platelet substudy confirmed that, in ACS patients, the current recommended doses of 180 mg loading followed by 90 mg BD achieve greater and more rapid inhibition of ADP-induced platelet aggregation compared to standard doses of clopidogrel.

Effects on platelet function of the 60 mg BD regimen of ticagrelor have been compared to 90 mg BD in the PEGASUS platelet and STEEL PCI studies, in patients with a history of prior MI and undergoing elective PCI respectively (Storey et al. 2016; Orme et al. 2018), showing broadly similar pharmacodynamic profiles.

J. Interactions between aspirin and ticagrelor

I. Studies of clinical outcomes

Using pre-specified interaction analyses, data from the PLATO study suggested that participants from the United States (US) appeared to have reduced benefit of aspirin and ticagrelor over aspirin and clopidogrel when compared to those from the rest of the world (interaction-p for geographic region = 0.045), an observation subsequently termed the 'North American Paradox' (Wallentin et al. 2009). The PLATO investigators performed a post-hoc analysis of the data to explore potential explanations for this. Study conduct, drug assignment and data quality errors were ruled out. Whilst there were other potential explanations including that adherence to study treatment was less in US patients compared to the rest of the world (64.0 vs. 84.7%) and the possibility of statistical chance, it was noted that US participants were on average receiving higher doses of aspirin during the study compared to the rest of the world, and there appeared to be a negative linear correlation between aspirin dose and benefit of ticagrelor over clopidogrel towards benefit with clopidogrel when aspirin maintenance doses ≥300 mg were used (HR 1.45 [95% CI 1.01-2.09); and towards benefit with ticagrelor when aspirin dose ≤100 mg (HR 0.77 [0.69-0.86]; interaction-p=0.00006). Adjustment for factors such as revascularisation and other therapy affecting ischaemic risk maintained the statistically significant nature of the interaction. Furthermore, rates of major bleeding did not appear to explain the difference (Mahaffey et al. 2011). This finding led to the Food and Drug Administration implementing an advisory notice that ticagrelor should not be used with maintenance doses of aspirin >100 mg.

There are no published studies that have prospectively sought to evaluate different doses of aspirin in combination with ticagrelor. Only 1 large RCT has assessed the effect of aspirin dose when given with a $P2Y_{12}$ inhibitor. In CURRENT-OASIS 7, which had a 2 x 2 factorial design, 25,086 patients with acute MI were randomised to receive either high (300-325 mg) or low (75-100 mg) OD doses of aspirin and high (150 mg) or low (75 mg) OD doses of clopidogrel. At 30 days, there were no significant differences in the primary composite endpoint of cardiovascular death, MI or stroke (high dose aspirin 4.2%, low dose 4.4%, HR 0.97 [95% CI 0.6-1.09], p=0.61) or major bleeding between the aspirin doses. There was a slightly higher risk of gastrointestinal haemorrhage in the higher-dose aspirin group which was of nominal significance (Mehta et al. 2010). This relationship has also been noted in patients receiving aspirin monotherapy (Valkhoff et al. 2012).

The European Stroke Prevention Study 2 investigated a very-low-dose, BD aspirin regimen (25 mg BD) alone or in combination with another antiplatelet drug (dipyridamole) in 6,602 stroke patients, showing a significant benefit of BD aspirin, alone or in combination, vs. placebo in preventing recurrent cerebrovascular events (Diener et al. 1996). Both all-site and gastrointestinal bleeding were significantly higher in those receiving aspirin compared to placebo.

The findings relating to the potential adverse interaction between high-dose aspirin and the efficacy of ticagrelor have prompted further investigation of its wider effects and how these may be modulated by aspirin.

II. Pharmacokinetic interaction

During the development of the drug, Teng and colleagues administered ticagrelor with or without aspirin to healthy volunteers and measured plasma concentrations of ticagrelor and its active metabolite AR-C124910XX. Aspirin had no effect on levels of ticagrelor or AR-C12490XX (Teng et al. 2013).

III. Platelet aggregation

The ability of aspirin to exert an additive antiplatelet effect in the presence of $P2Y_{12}$ inhibition remains an issue of some contention. The PEGASUS platelet substudy included patients with a history of prior MI randomised to ticagrelor 90 mg BD, 60 mg BD or placebo BD. All of the 180 patients were receiving aspirin at a dose of 75-100 mg OD. When assessed by light transmittance aggregometry (LTA) and the VerifyNow aspirin assay, there were no differences between the treatment groups in arachidonic acid-induced platelet aggregation, and no differences in serum TXB_2 suggesting no additive effect of ticagrelor over aspirin on this pathway at the studied doses (Storey et al. 2016).

The Warner group performed studies of platelet function and TXA_2/PGI_2 generation using samples from healthy volunteers either in vitro or ex vivo. Their conclusions were that, in the presence of potent $P2Y_{12}$ antagonism with prasugrel (in vitro and ex vivo) and ticagrelor (in vitro), aspirin at therapeutically relevant doses/concentrations did not significantly add to the inhibition

of platelet aggregation and TXA_2 generation (Armstrong et al. 2011; Leadbeater et al. 2011). These studies used a 96-well plate aggregometry technique as well as more conventional LTA but with low stirring speeds. These methods may not adequately simulate the high shear stress forces applied to platelets in the human circulation, which contribute to platelet activation, and therefore might underestimate the antiplatelet effect needed to inhibit in vivo aggregation. This is also a limitation of another healthy volunteer study which suggested that inhibition of haemostatic activation is similar with aspirin SAPT and DAPT (Traby et al. 2016).

The Cattaneo group studied 3 patients with $P2Y_{12}$ receptor deficiency and showed no difference in TXB_2 production compared to healthy controls. Studies of platelet function carried out in appropriate shear stress conditions showed that the effects of cangrelor (achieving potent $P2Y_{12}$ inhibition) and aspirin were additive compared to that of either single agent. This was in contrast to the effects seen in unstirred conditions (Scavone et al. 2016). Under physiological conditions, $P2Y_{12}$ antagonists potently inhibit platelet aggregation to a range of agonists, reflecting the receptor's central role in amplification of platelet activation. Whilst this inhibition of platelet aggregation reduces release of platelet-derived prothrombotic factors, it does not abolish the intrinsic ability of platelets to produce TXA_2, including in response to mechanical stimulation, which *is* effectively inhibited by the addition of aspirin. In highly thrombogenic conditions, such as those encountered in the coronary arteries during and after native plaque rupture, $P2Y_{12}$ inhibitor monotherapy is therefore unlikely to be able to fully inhibit platelet activation and the effect of aspirin is therefore likely to be additive in these circumstances.

Until recently, this has not been well studied specifically with ticagrelor, which is a more potent $P2Y_{12}$ inhibitor than the thienopyridines (Joshi et al. 2014). However, the results of the TWILIGHT platelet function substudy have lately been published (Baber et al. 2020). The investigators enrolled 51 participants from a single centre taking part in the main trial, obtaining baseline and follow-up perfusion chamber and platelet aggregation measurements from 41. Assessed using multiple electrode aggregometry, there were significantly greater arachidonic acid- and collagen-induced aggregation responses in those receiving ticagrelor monotherapy compared to DAPT, whilst there was no difference in responses to ADP or thrombin receptor activating peptide. However, determining whether the pharmacodynamic effects of combining antiplatelet drugs that act on different pathways are additive can be difficult to interpret. The investigators therefore further explored this using the Badimon perfusion chamber, which incorporates de-endothelialised porcine aorta to stimulate atherothrombosis whilst maintaining physiological levels of shear stress. They found no difference in the area of the resulting thrombi between those receiving ticagrelor monotherapy and those receiving DAPT. It would therefore

appear that the differential effects seen on aggregometry do not affect ex vivo thrombosis, at least when assessed in this way, and this supports the fact there appeared to be no significant increase in ischaemic events with ticagrelor monotherapy in the main study.

IV. Acellular coagulation

As well as inhibiting platelet aggregation, both aspirin and ticagrelor may also affect the acellular arm of coagulation in distinct ways. Platelet activation leads to thrombin generation and therefore any drug that inhibits this also reduces activation of the coagulation cascade during thrombosis. More specifically, aspirin directly acetylates fibrinogen, increasing clot porosity and tendency for lysis, and decreasing rate of polymerisation (Ajjan et al. 2009; He et al. 2001). Factor XIII activation is also reduced by aspirin (Undas et al. 2003; Ajjan and Grant 2006). An observation that lower doses of aspirin resulted in more favourable clot structure compared to higher doses and controls has been noted in several studies from the same group both in vitro (He et al. 2009) and in a human study that suggested an optimal dose of aspirin of 37.5 mg BD (Antovic et al. 2005). The investigators hypothesised that this effect might be due to the fact that higher levels of the aspirin metabolite salicylic acid might be inhibiting the acetylation of fibrinogen. This relationship was not seen, however, in another study of patients with type 1 diabetes, in whom 325 mg OD was more effective at reducing clot density than 75 mg OD (Tehrani et al. 2012).

Regarding ticagrelor's effects on acellular coagulation, in a human endotoxaemia model both ticagrelor and clopidogrel inhibited the procoagulant effect of inflammation. Turbidimetric assays showed that clot density and lysis time were reduced by $P2Y_{12}$ inhibitor pretreatment. Scanning electron microscopy confirmed a looser clot structure with thinner fibrin strands compared to those not receiving a $P2Y_{12}$ inhibitor (Thomas et al. 2015).

As a global marker of haemostasis, a study carried out in healthy volunteers showed that the combination of aspirin and ticagrelor significantly prolonged the bleeding time, when measured by a standard lancet method, compared to either agent alone (Teng et al. 2013).

V. Vessel wall effects

In addition to the effects on blood constituents, there is evidence that both aspirin and ticagrelor can modulate endothelial function. The endothelium acts as a physical, chemical and negatively charged barrier between circulating platelets and prothrombotic subendothelial tissue, and thus has a key role in preventing inappropriate thrombosis. COX1, COX2, endothelial NO synthase (eNOS) and $P2Y_{12}$ receptors are all present in the vessel wall (Schonbeck et al. 1999; Fulton et al. 2002; West et al. 2014). eNOS produces NO, a potent vasodilatory compound that also inhibits platelet aggregation via stimulation of guanylate cyclase (Kirkby et al. 2013).

Aspirin has been shown to increase endothelial NO availability. Aspirin dose-dependently increased production of NO by vascular smooth muscle cells in a rat study. Inducible NOS expression, whilst not increased by aspirin in the baseline state, was increased by interleukin-1β stimulation and this effect was potentiated by sodium salicylate (Shimpo et al. 2000). Hetzel and colleagues randomised 37 patients receiving SAPT with aspirin 81 mg OD for stable coronary artery disease (CAD) to receive OD doses of aspirin 81, 162.5, 325, 650, or 1300 mg for 12 weeks (Hetzel et al. 2013). When the higher dose groups were pooled, there was an increase from baseline in serum homocysteine and reduced levels of the endogenous eNOS inhibitor asymmetric dimethylarginine (ADMA). There were no significant differences between the individual dosing groups, however the studies were unlikely to have been powered to detect these. A study using a low-density lipoprotein-induced rat model of endothelial injury confirmed that levels of ADMA were reduced by aspirin with a corresponding increase in the activity of dimethylaminohydrolase, the enzyme responsible for ADMA inactivation. Interestingly, this effect was present at lower (30 mg/kg) but not higher (100 mg/kg) aspirin doses (Deng et al. 2004).

A second mechanism increasing aspirin-related endothelial NO availability might be an upregulation in the NOS-stimulating eicosanoid 15-epi lipoxin A_4 (LXA_4) that can be related to COX2 activity (Paul-Clark et al. 2004; Gilroy 2005). Thirdly, NOS can be directly acetylated by aspirin, increasing the enzyme's activity. This has been shown both in platelets (O'Kane et al. 2009) and the endothelium (Jung et al. 2010).

In vivo endothelial function can be assessed with tonometry studies. In a human model of inflammation using Salmonella typhi vaccination, endothelial function, assessed by mercury-in-silastic strain gauge plethysmography, was impaired compared to baseline. Pretreatment with 1.2 g oral aspirin significantly inhibited this effect (Kharbanda et al. 2002). In another study, aspirin

81 mg OD improved endothelial function measured as peripheral arterial augmentation index but not as reactive hyperaemia index in individuals exposed to exertional heat stress (Olafiranye et al. 2015).

Ticagrelor has also been shown to have effects on the endothelium. An in vitro study of cultured human aortic endothelial cells stimulated with tumour necrosis factor (TNF) α (published in abstract form only) showed that ticagrelor, but not clopidogrel, increased eNOS activity and increased both COX2 activity and expression (Reiner et al. 2014). This effect may therefore increase endothelial production of both NO and PGI_2, the former potentially increased by aspirin and the latter inhibited by it, particularly at higher doses. The balance of the effect of aspirin on these actions in the context of ticagrelor therapy remains to be explored. Both NO and PGI_2 potentiate the antiplatelet effect of $P2Y_{12}$ inhibition and therefore if ticagrelor (but not thienopyridines) potentiates release of these factors, this may prove advantageous (Kirkby et al. 2013; Cattaneo and Lecchi 2007; Chan et al. 2015).

ADP-induced vasoconstriction was also inhibited by ticagrelor in an animal model. Grzesk and colleagues administered ticagrelor or placebo and aspirin or placebo to 28 rats and measured ADP- and phenylephrine-induced constriction of tail arteries (Grzesk et al. 2013). Aspirin itself sensitised vascular smooth muscle cells to these stimuli at high but not low doses, and this effect was endothelium-dependent. Ticagrelor had an anticontractile effect on reactivity to ADP that was attenuated by high but not low doses of aspirin and was again dependent on the presence of endothelium. In a separate study by the same group, in contrast to ticagrelor, the thienopyridines clopidogrel or prasugrel did not exert this effect (Grzesk et al. 2012).

There is also evidence from human in vivo studies that ticagrelor improves endothelial function. Torngren and colleagues performed peripheral arterial tonometry on patients with a history of ACS receiving aspirin either as SAPT or in combination with ticagrelor, prasugrel or clopidogrel (Torngren et al. 2013). Ticagrelor-treated patients had significantly better markers of endothelial function compared to the other groups. However, in another study of 30 patients with a history of stable CAD receiving ticagrelor 90 mg BD, on discontinuation of the drug there was no evidence of deterioration in endothelial function when assessed using the same method (Xanthopoulou et al. 2016).

VI. Myocardium

Pretreatment with ticagrelor reduced the size of myocardial infarction compared to clopidogrel in a rat model of acute coronary occlusion. The protective effect of ticagrelor was inhibited by a selective adenosine receptor (A_1/A_{2A}) antagonist indicating the mechanism may be related to ticagrelor's pleiotropic actions. Inhibition of COX2 (but not COX1) by a specific inhibitor and by high-dose but not low-dose aspirin also attenuated this effect. Ticagrelor, but not clopidogrel, increased myocardial COX2 expression and NOS and COX2 activity, and also increased levels of the NOS-stimulating eicosanoid 15 epi-lipoxin (LX) A_4 (Nanhwan et al. 2014; Ye et al. 2015).

In the Complete vs Lesion-only PRIimary PCI Trial cardiac Magnetic Resonance (CvLPRIT cMR) substudy, which randomised patients presenting with STEMI and found to have multivessel CAD to either complete or culprit-only revascularisation, cMR imaging was used to assess infarct size (Khan et al. 2016). Preferred antiplatelet therapy was not dictated in the protocol and therefore patients receiving aspirin and either clopidogrel, prasugrel or ticagrelor entered the study. The cMR substudy was therefore observational rather than interventional in terms of antithrombotic therapy, but interestingly showed significantly reduced infarct size in those receiving prasugrel or ticagrelor compared to clopidogrel, possibly related to potency and consistency of $P2Y_{12}$ inhibition. Sample size was too small to compare between the two newer agents and patients were also receiving aspirin at doses that might inhibit myocardial COX2. Evolution towards more potent $P2Y_{12}$ inhibitors during the study period might have been accompanied by other significant advances in management of ACS and therefore confounded the findings.

A summary of the reported and hypothesised interactions of aspirin and ticagrelor with regards to effects on platelets, endothelium, cardiac myocytes and erythrocytes is represented in **Figure 1.7**.

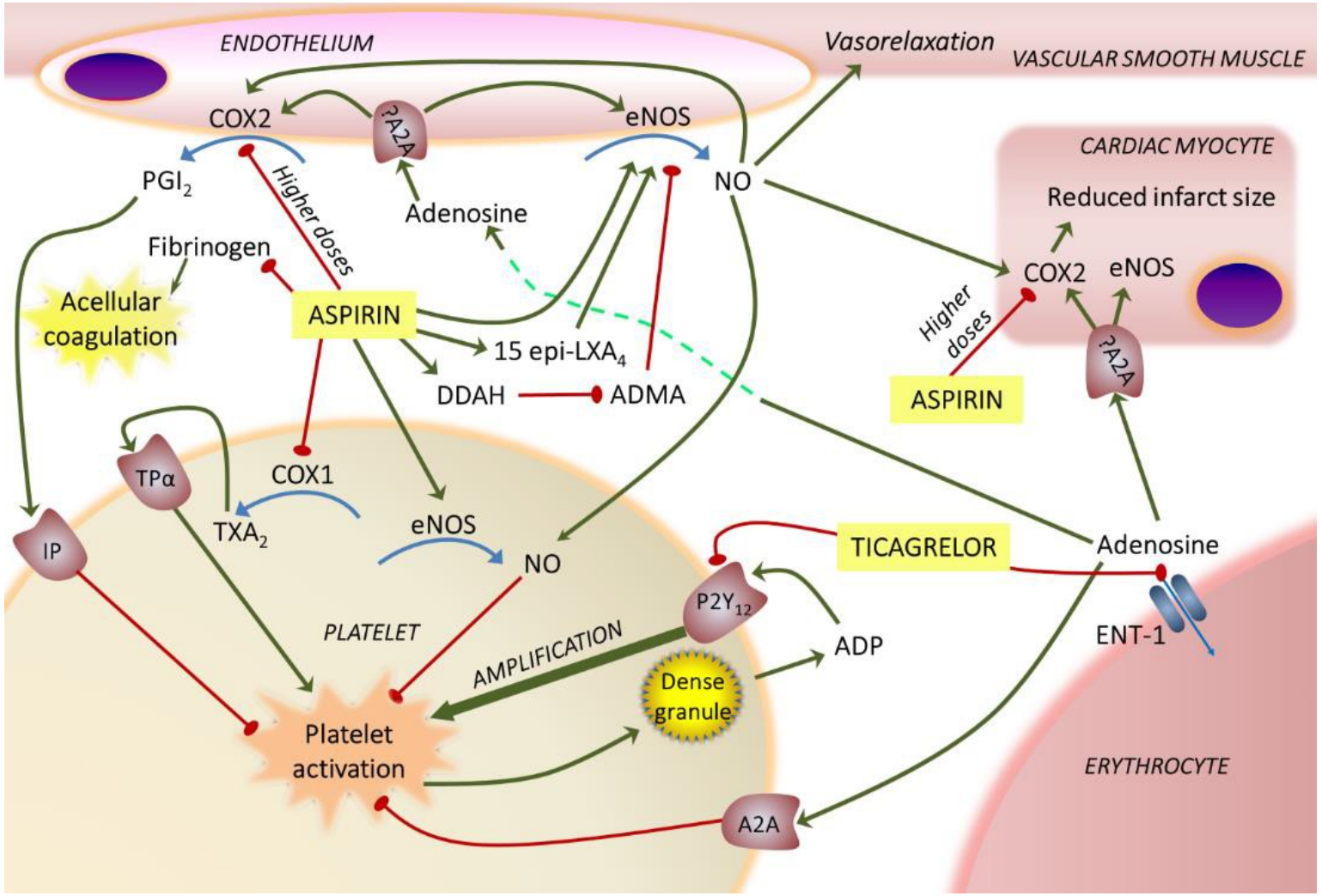

Figure 1.7 Key reported and hypothesised interactions of aspirin and ticagrelor (Parker 2020a). Reproduced with permission from Taylor & Francis Group. A2A, adenosine 2A receptor; COX, cyclo-oxygenase; ADP, adenosine diphosphate; ENT-1, equilibrative nucleoside transporter 1; TXA_2, thromboxane A_2; IP, prostacyclin receptor; TPα, thromboxane receptor; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; DDAH, dimethyldiarginine hydrolase; ADMA, asymmetric dimethylarginine; LXA_4, lipoxin A_4; PGI_2, prostacyclin; $P2Y_{12}$, platelet ADP receptor

VII. Inflammation

In addition to its antiplatelet effects, both aspirin and ticagrelor have immunomodulatory effects. On the one hand, aspirin, including at a dose of 75 mg OD, may have anti-inflammatory effects mediated via generation of 15-epi-LXA_4 (Paul-Clark et al. 2004), but, on the other hand, there is growing evidence that aspirin may increase certain factors key to the progression of atherothrombosis in other situations. As well as observed effects in vitro and ex vivo, aspirin 80 mg OD potentiated IL-6 and TNF-α release in an experimental endotoxaemia study (Kiers et al. 2017). Although a standard regimen of ticagrelor appeared to counteract these effects to an extent, supporting an earlier study of ticagrelor's effects during endotoxaemia (**Table 1.7**), significant augmentation of inflammatory response by aspirin persisted despite this (**Figure 1.8**) (Kiers et al. 2017; Thomas et al. 2015). The effects of aspirin regimens other than 80 mg OD on the response to endotoxin have not been characterised thus far.

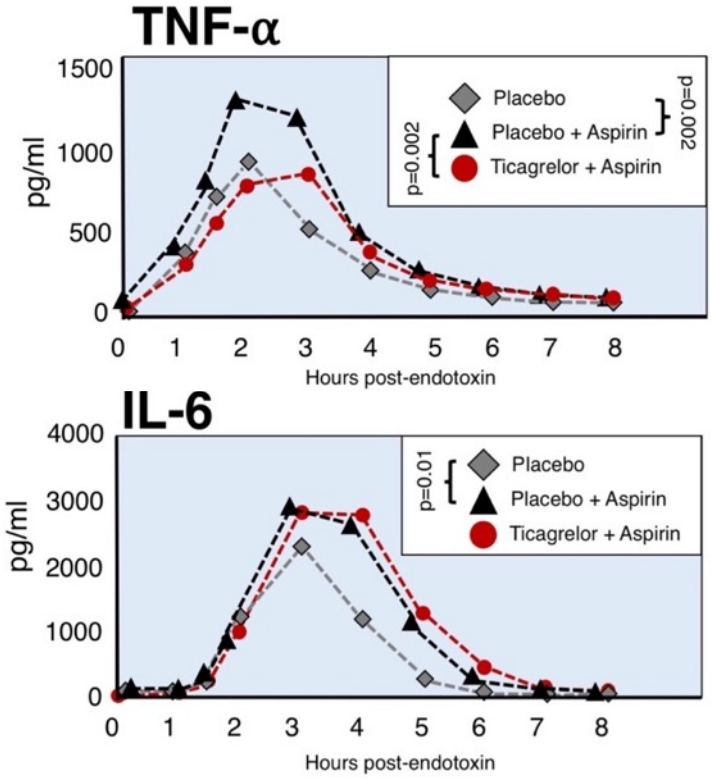

Figure 1.8 Response to endotoxaemia in healthy individuals receiving placebo (◆), placebo & aspirin 80mg OD (▲), or ticagrelor 90mg BD & aspirin 80mg OD(●), redrawn with permission from Georg Thieme Verlag KG (Kiers et al. 2017).

Table 1.7 Divergent effects of aspirin and ticagrelor on cytokine responses during experimental human endotoxaemia (Kiers et al. 2017; Thomas et al. 2015). G-CSF, granulocyte colony stimulating factor; CCL, C-C motif ligand; IL, interleukin, MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory proteinl RA, receptor antagonist; TNF, tumour necrosis factor.

Factor	Effect of aspirin vs. control	Effect of ticagrelor vs. control
G-CSF	Unknown	Significantly reduced
IL-1RA	No significant effect	No significant effect
IL-6	Significantly increased	Significantly reduced
IL-8	Significantly increased	Significantly reduced
IL-10	No significant effect	Significantly increased
MCP-1/CCL2	Increased (p=0.07)	Significantly reduced
MIP-1α	Significantly increased	Unknown
MIP-1β	No significant effect	No significant effect
TNF-α	Significantly increased	Significantly reduced

The mechanism for aspirin's effects during endotoxaemia has not been fully elucidated, but may be related to inhibition of platelet-derived PGE_2, another eicosanoid synthesised by platelets and leukocytes in response to endotoxin (Kiers et al. 2017; McAdam et al. 2000) that can inhibit the synthesis and release of pro-inflammatory cytokines from monocytes via its action on EP2 and EP4 receptors (Birrell et al. 2015; Na et al. 2015) (**Figure 1.9**). Endotoxin binds to toll like receptor (TLR)4 on the surface of monocytes, resulting in signalling including upregulation of nuclear factor kappa B, with an increase in synthesis and release of pro-inflammatory cytokines, including IL-6 and TNF-α, mediating vascular inflammation (Chow et al. 1999; Brasier 2010). The action of PGE_2 on EP2 can also reduce the translation and surface expression of TLR4 (Degraaf, Zaslona, et al. 2014). In addition to binding endotoxin, TLR4 also functions as a damage receptor, activated by a range of factors released during atherogenesis, endothelial injury and thrombosis, including fibrin degradation products and damage-associated molecular patterns (Lee and Seong 2009; Smiley et al. 2001). An established link also exists between bacterial infection, for example pneumonia, which results in inflammation via the endotoxin-TLR4 pathway, and elevated risk of major adverse

cardiovascular events (Smeeth et al. 2004). This makes endotoxaemia a highly relevant model of inflammation to use in the study of atheroinflammation, in contrast to other models that may not be so pathophysiologically pertinent to cardiovascular disease and may not be potentiated by aspirin (Morris et al. 2009; Layne et al. 2016).

Aspirin, a COX inhibitor, may therefore reduce release of PGE_2, potentiating the pro-inflammatory response to endotoxin. COX inhibition may also hypothetically result in increases in other non-COX derived eicosanoids such as leukotriene (LT) B_4, which antagonises the effect of PGE_2 via action on the BLT1 receptor (Serezani et al. 2011).

There are additional interactions with the endothelium to consider. As well as directly acting on endothelial cells, endotoxin stimulation of monocyte TLR4 leads to endothelial activation via release of soluble cluster of differentiation (CD)14, resulting in leukocyte adhesion which is key to atherogenesis (Pugin et al. 1993; Golenbock et al. 1995). Platelets also have a role in this process. They too possess functional TLR4 (Andonegui et al. 2005) and can modulate monocyte-endothelial interactions, including forming bridges between the two cell types (Barry et al. 1998; Kuckleburg et al. 2011). Eicosanoids are also regulatory: PGE_2, along with PGI_2, enhances endothelial barrier function through a protein kinase A-dependent mechanism (Birukova et al. 2007), whereas LTB_4 promotes monocyte adhesion to the endothelium via increasing monocyte macrophage 1 antigen (Mac-1, consisting of CD11b and CD18) expression (Lee et al. 2013). Downstream, one particular pathway of interest is that involving IL-1 and its endogenous inhibitor IL1-receptor antagonist, an axis now known to be open to clinically significant therapeutic targeting (Ridker et al. 2017).

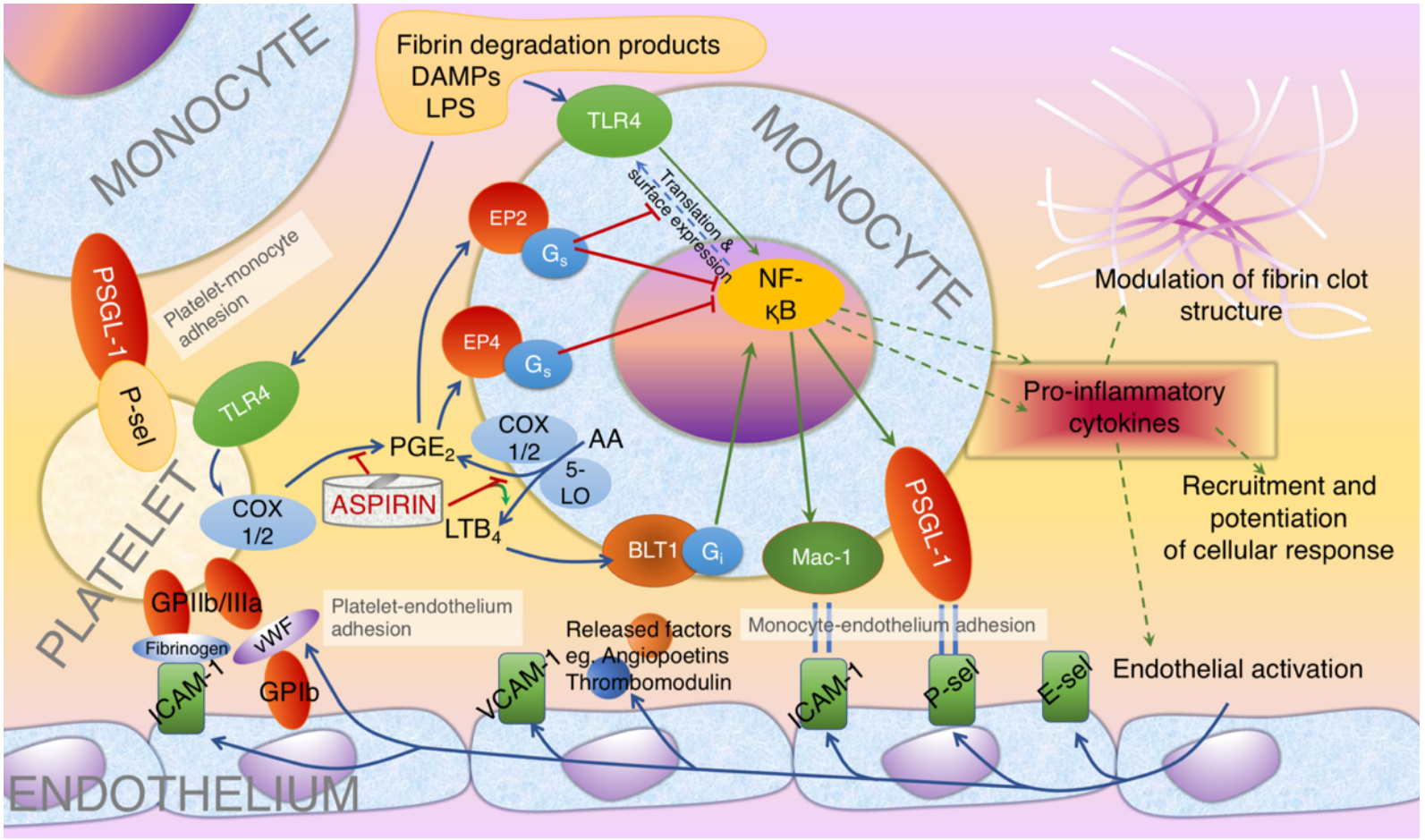

Figure 1.9 Hypothetical mechanisms by which aspirin might potentiate the inflammatory response to endotoxin.
AA, arachidonic acid; BLT1 leukotriene B1 receptor; CAM, cell adhesion molecule; COX, cyclo-oxygenase; DAMP, damage associated molecular pattern; EP, PGE_2 receptor; G_i, inhibitory G protein; GP, glycoprotein; G_s, stimulatory G protein; ICAM, intercellular CAM; LO, lipoxygenase; LPS, lipopolysaccharide (endotoxin); LT, leukotriene; Mac-1, macrophage-1 antigen (CD11b/CD18); NF, nuclear factor; PG, prostaglandin; PSGL, P-selectin glycoprotein ligand; sel, selectin; TLR, toll-like receptor; VCAM, vascular CAM; vWF, von Willebrand Factor.

K. Summary of existing literature

Aspirin 75-100 mg OD and ticagrelor 90 mg BD represents one of the current recommended combinations of oral antiplatelet drugs in ACS. Whilst they inhibit platelet aggregation by targeting different pathways, there is significant overlap of their effects on platelets, acellular coagulation and endothelium.

Despite potent dual antiplatelet therapy, the risk of a MACE remains significant, particularly in those within high-risk groups. Further prolongation of ticagrelor-based DAPT in high-risk groups significantly improves ischaemic outcomes but leads to an increase in bleeding events, which can dissuade clinicians and patients alike from continuation of therapy (Bonaca, Bhatt, Oude Ophuis, et al. 2016).

Hence, there are multiple interactions between aspirin and ticagrelor therapy, many of which require further investigation in the ACS patient population. Looking towards optimising the current regimen of aspirin and ticagrelor, the ideal antithrombotic strategy would be one that maintains the anti-ischaemic benefit of DAPT through effective inhibition of platelet COX1 and $P2Y_{12}$, ensures consistency of effect across the dosing period, avoids inhibition of $COX2/PGI_2$ and has optimised (reduced) effects on haemostasis and any potentiation of inflammation (**Figure 1.10**). Based on the evidence reviewed here, ticagrelor administered with a lower-than-standard total daily dose of aspirin divided into more than once-daily administration might hypothetically match this profile more effectively compared to the current recommended regimen and warrants further investigation.

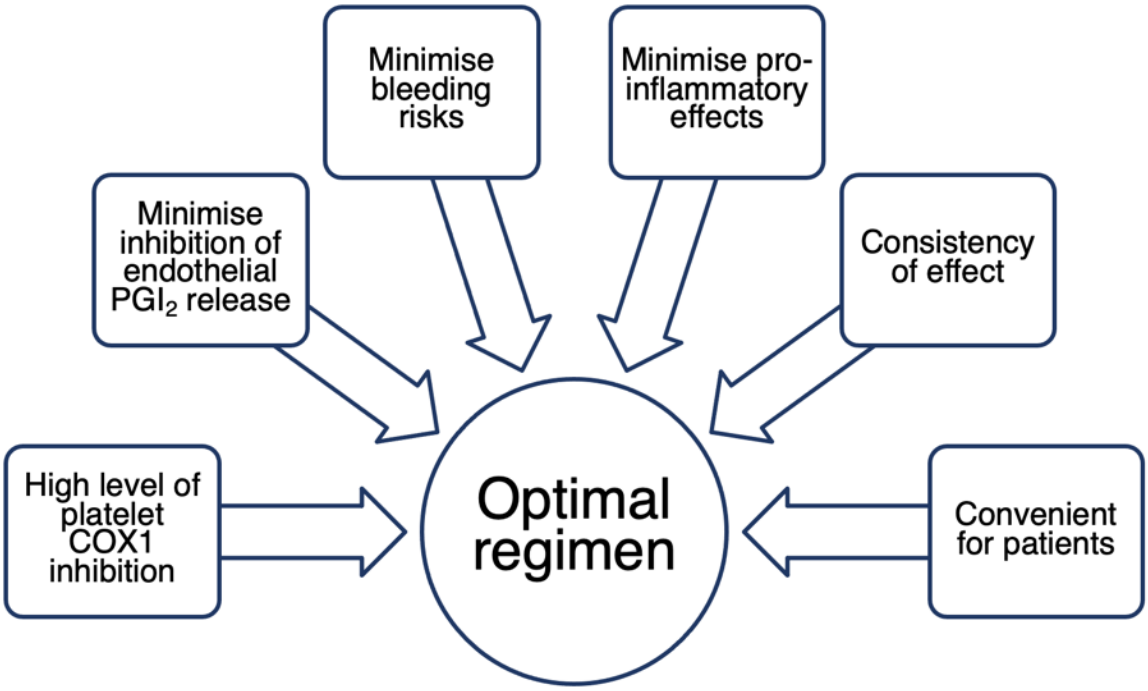

Figure 1.10 Some optimal characteristics of an aspirin regimen for patients with IHD.

Chapter 2: Objectives and hypotheses

A. In vitro concentration-dependent effects of aspirin, with or without concurrent $P2Y_{12}$ inhibition, on platelet aggregation

I. Objective

The objective of this study was to characterise concentration-dependent effects of aspirin on platelet aggregation when added in vitro to human platelet-rich plasma in the presence and absence of potent $P2Y_{12}$ inhibition.

II. Hypothesis

The central hypothesis was that aspirin significantly and concentration-dependently reduces platelet aggregation responses to arachidonic acid and collagen even in the presence of potent $P2Y_{12}$ inhibition.

B. A study of very low dose twice-daily compared to standard low dose once-daily aspirin following acute coronary syndromes

I. Objectives

In order to explore the effects of reducing dose and increasing frequency of administration, a single-centre study of the pharmacodynamic effects of very-low-dose twice-daily aspirin in patients also receiving ticagrelor for ACS, the WILL lOWer dose aspirin be more effective in ACS? (WILLOW ACS) study, was performed.

The primary objective of this study was to assess the effects of two aspirin regimens (very low-dose BD or standard low-dose OD) on the release of arachidonic acid metabolites thromboxane A_2 (measured as the stable metabolite serum TXB_2) and PGI_2 (measured as urinary PGI-M) in ACS patients also receiving ticagrelor.

Secondary objectives were to assess ADP-induced, collagen-induced and arachidonic acid-induced platelet aggregation, as well as bleeding time, in ACS patients receiving two regimens of aspirin against a background of ticagrelor treatment.

The primary safety objective was to estimate the incidence of PLATO-defined major plus minor bleeding at 14 days in patients treated with ticagrelor and either one of the two aspirin dosing regimens. Weaknesses of this measure are the low expected numbers of events in this sample size and timeframe, and the fact that treatment-related bleeds may occur beyond 14 days. In retrospect, bleeding time may have been a more appropriate and well-powered primary safety endpoint. A secondary safety objective was to estimate the incidence of adverse events at 14 days.

II. Hypotheses

The principal hypotheses were that, when receiving aspirin 20 mg BD compared to 75 mg OD, peak (post-dose) TXB_2 would be greater (representing reduced peak COX1 inhibition), PGI-M would be greater (representing reduced COX2 inhibition) and bleeding time would be reduced (representing improved haemostasis).

C. The impact of aspirin dose modification, with or without ticagrelor, on the innate immune response (interim analysis)

I. Study objectives

The primary objective of this study is to assess, in healthy volunteers, the effects of 3 regimens of aspirin, compared with no aspirin, on the release of TNF-α during endotoxaemia. The secondary objectives of this study are to assess, during endotoxaemia, the effects of 3 regimens of aspirin or no aspirin, with and without a loading dose of ticagrelor, on plasma TNF-α (comparisons other than those stated in the primary objective), plasma IL-6, serum CRP, leukocyte count (and subsets), serum TXB_2, serum PGE_2, urine PGI-M, bleeding time and platelet aggregation responses to AA, collagen and ADP.

II. Hypothesis

It is hypothesised that TNF-α will be significantly lower when receiving aspirin 20 mg BD compared to 75 mg OD and 300 mg OD.

Chapter 3: Materials and methods

A. In vitro concentration-dependent effects of aspirin, with or without concurrent $P2Y_{12}$ inhibition, on platelet aggregation

Data from this work can be found in chapter 4.

Ethical approval for this work was granted by the University of Sheffield prior to any participant enrolment. Written informed consent was obtained from participants prior to blood sampling.

Venous blood from 6 healthy volunteers who had received no medicinal products in the preceding 14 days was collected under a gentle vacuum and incubated with aspirin (final concentrations of 0, 1, 10, 100 μmol/L) for 30 minutes in citrated tubes. To prepare these, a stock solution of 2.5 mmol/L aspirin was made by adding 45 mg of pure acetylsalicylic acid powder (Sigma Aldrich, Merck Life Science UK, Gillingham, UK) molar mass 180 g/mol) to 100 mL of 0.9% saline (Baxter Healthcare Ltd, Thetford, UK), stirred and gently warmed until fully dissolved. Appropriate volumes of aspirin stock solution and 0.9% saline were then added to blood tubes containing 500 μL of trisodium citrate (Sigma Aldrich, final concentration once blood added 3.2%) to achieve final aspirin concentrations once blood was added to the 5 mL line (**Table 3.1**).

Table 3.1 Volumes of aspirin and vehicle added to 5 mL citrate blood tubes

Final [Aspirin] in blood (μmol/L)	Volume of 2.5 μmol/L Aspirin stock (μL)	Volume of 0.9 % saline (μL)
0	0	200
1	2	198
10	20	180
30	60	140
100	200	0

Platelet-rich plasma (PRP) was prepared by centrifugation (5804R centrifuge, Eppendorff, Stevenage, UK) at 200 relative centrifugal force (rcf) for 10 minutes at room temperature, aspirating the supernatant (around 2 mL) using a transfer pipette (Cole-Parmer, St Neots, UK) and dispensing into polypropylene tubes (Cole-Parmer). Platelet count of whole blood and PRP was checked using a Sysmex XP-300 automatic haematology analyser (Sysmex Corporation, Milton Keynes, UK) but no adjustment for platelet count of either was made. The remaining blood in the tube (around 3 mL) was centrifuged further (1500 g for 10 minutes at room temperature) and the supernatant (platelet poor plasma, PPP) was again drawn out using a transfer pipette and dispensed into a polypropylene tube (Cole-Parmer).

Light transmittance aggregometry (LTA) was performed using a platelet aggregation profiler (PAP)-8 aggregometer (Bio/Data Corporation, Horsham, PA, USA) using final concentrations of 1 mmol/L AA, 20 μmol/L ADP and 0.5 or 2 μg/mL collagen, in the presence or absence of a final concentration of 1 μmol/L cangrelor (The Medicines Company, Parsippany, NJ, USA, dissolved in 0.9% saline) or vehicle. This concentration of cangrelor is consistent with the highest plasma levels measured during standard patient dosing regimens (Storey et al. 2001). The aggregometer was switched on and allowed to warm to 37^{o}C. 230 μL of PPP and 20 μL of 0.9% saline were mixed in a glass aggregometry tube (Bio/Data). This was used as an optical blank for each test well. For each aspirin concentration and agonist combination, 230 μL of PRP was added to two glass aggregometry tubes with a miniature magnetic stirrer bar (Bio/Data). Tubes were placed in the aggregometer's incubation well at 37^{o}C and immediately 10 μL of cangrelor 25 μmol/L (to give a final concentration of 1 μmol/L at the time of aggregometry) was added to one of the pair and 10 μL 0.9% saline was added to the other. After 1 minute the tubes were transferred to the test wells and recording of light transmittance was started. After a further minute, 10 μL of the appropriate agonist was injected into the tubes. Light transmittance was recorded continuously for 6 minutes. Baseline, maximum aggregation (MA) and final aggregation (FA) were recorded for each tube. It was also confirmed that 1 μmol/L cangrelor provided potent inhibition of ADP-induced platelet aggregation by performing LTA in the presence and absence of cangrelor and aspirin 100 μmol/L using ADP as an agonist (final concentration 20 μmol/L).

B. A study of very low dose twice-daily compared to standard low dose once-daily aspirin following acute coronary syndromes

Data from this work can be found in chapter 5

I. Recruitment of participants

20 patients with a history of recent ACS (between 30 days and 10 months before enrolment) established on aspirin 75 mg OD and ticagrelor 90 mg BD were recruited and their informed consent sought for this study.To proceed to randomisation, participants were required to meet the following inclusion criteria: provision of informed consent prior to any study specific procedures; male or female aged greater than 18 years; previous diagnosis of ACS greater than 30 days and less than 10 months before enrolment; and receiving DAPT with aspirin 75 mg OD and ticagrelor 90 mg BD.

Participants were excluded if they had an indication for DAPT other than IHD; had undergone PCI within 30 days prior to randomisation; had any history of stent implantation to the left main coronary artery; had any history of stent thrombosis during DAPT; had a planned procedure for coronary revascularisation; had any planned surgery or other procedure that might have required suspension or discontinuation of DAPT expected to occur within 3 months of randomisation; if there was prior intention by patient or physician to discontinue aspirin and/or ticagrelor within the study period; if they were receiving doses of aspirin and ticagrelor other than 75 mg OD and 90 mg BD respectively; if they were receiving treatment or planned treatment with antiplatelet medication apart from aspirin or ticagrelor (eg. clopidogrel, prasugrel, dipyridamole, ticlopidine); if they were currently receiving a diuretic agent (including loop, thiazide or potassium sparing diuretic) as these may affect prostanoid assays; if they had any ACS event within 30 days prior to randomisation; if their most recent ACS event was more than 10 months prior to randomisation; if there was current or planned use of an OAC (e.g. warfarin, dabigatran, rivaroxaban, apixaban), parenteral anticoagulant (eg. unfractionated heparin, low molecular weight heparin, bivalirudin), a GP IIb/IIIa inhibitor (eg. abciximab, tirofiban) or a fibrinolytic agent (eg. tissue plasminogen activator); or if they were

requiring or likely to require treatment with a non-steroidal anti-inflammatory drug (NSAID), or COX2 inhibitor, either as regular or intermittent/as required therapy.

To prevent interaction with ticagrelor treatment, those receiving a strong inhibitor of cytochrome P450 (CYP) 3A4 (e.g. ketoconazole, itraconazole, voriconazole, telithromycin, clarithromycin [but not erythromycin or azithromycin], nefazadone, ritonavir, saquinavir, nelfinavir, indinavir, atanazavir, or over 1 litre daily of grapefruit juice); simvastatin or lovastatin at doses higher than 40 mg daily; a CYP3A4 substrate with a narrow therapeutic index (e.g. ciclosporin or quinidine); or a strong inducer of CYP3A4 (e.g. rifampin/rifampicin, rifabutin, phenytoin, carbamazepine, phenobarbital) were excluded.

Participants with any of the following were also excluded: a history of acute or chronic liver disease (e.g. cirrhosis); end-stage renal failure requiring dialysis; alcohol or drug abuse, defined as regular use of an illicit substance for recreational purposes or regular consumption of greater than 50 units (males) or 35 units (females) of alcohol per week in the last year; and any co-morbidity associated with life expectancy less than 1 year or any other condition deemed by the investigator to affect haemostasis, coagulation, bleeding risk or ability to comply with the study protocol.

Females of child-bearing potential were similarly prevented from proceeding to randomisation unless they had a negative pregnancy test at screening and were willing to use effective contraception (i.e. established use of oral, injected or implanted hormonal methods of contraception or placement of an intrauterine device or intrauterine system, or barrier methods of contraception with spermicide or sole male partner with prior vasectomy and confirmed absence of sperm in ejaculate for the duration of treatment with study medication.

II. Study design

After collection of baseline demographic and clinical information, patients were randomised in a 1:1 ratio to one of two sequences of aspirin dosing in an open-label crossover design (**Figure 3.1)**, as follows:

1. Aspirin 20 mg BD for 14 days *then* aspirin 75 mg OD for 14 days; or

2. Aspirin 75 mg OD for 14 days *then* aspirin 20 mg BD for 14 days.

All other usual medications including ticagrelor were continued throughout the study in all participants.

Blood samples for serum TXB_2 and platelet function testing, urine samples for PGI-M and assessment of bleeding time using a standard lancet method were taken at the following time points:

1. At baseline (visit 2 – randomisation) (platelet function and serum TXB_2).
2. After 14 days (visit 4 – end of 1st study medication period), pre-dose (platelet function and serum TXB_2) and 2 hours post-dose (platelet function, serum TXB_2, urinary PGI-M and bleeding time).
3. After 28 days (visit 5 – end of 2nd study medication period) pre dose (platelet function and serum TXB_2) and 2 hours post dose (platelet function, serum TXB_2, urinary PGI-M and bleeding time).

Clinical outcomes were reviewed and adverse events recorded at 14 and 28 days. At the 28-day visit, patients with an indication for ongoing dual antiplatelet therapy were transitioned to standard care with aspirin 75 mg OD and arrangements for ongoing supply of this medication were ensured. A telephone contact was made at 14 days after study medication discontinuation to ensure the successful transition to standard care and to record any adverse events for safety monitoring. Vital sign measurement (heart rate, blood pressure and core temperature) and physical examination were performed at visits 1 to 4. Concurrent medications were recorded at each visit.

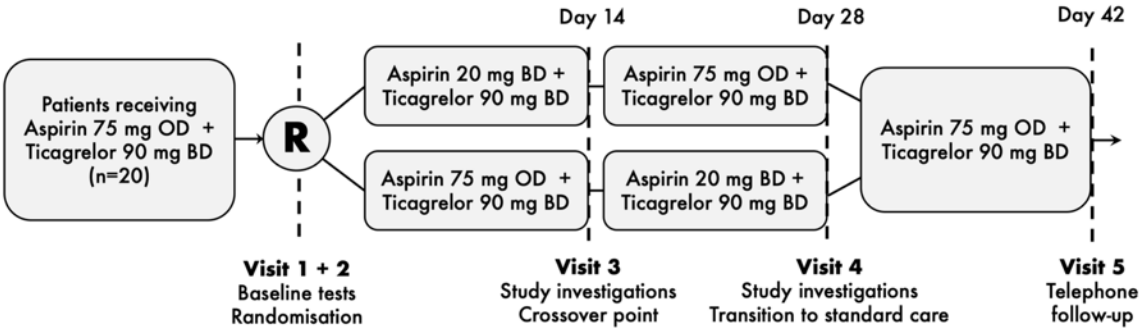

Figure 3.1 Overall design of the WILLOW ACS study. BD, twice daily; OD, once daily; R, randomisation

III. Power calculation

Based on data from our group from patients with a history of MI in the PEGASUS-TIMI 54 platelet substudy, we expected patients receiving ticagrelor 90 mg BD and aspirin 75 mg OD to have post-dose mean serum TXB_2 levels of 2.9 ng/mL and a within-patient standard deviation of approximately 3.0 ng/mL (Storey et al. 2016).

Reviewing data from a previous dosing study of aspirin monotherapy in patients with a history of stroke, mean serum TXB_2 (± SD) was 48.12 ± 40.4 ng/ml when not receiving aspirin, 7.1 ± 10.3 ng/ml after 7 days of aspirin 40 mg OD and 1.8 ± 0.3 ng/ml after 7 days of aspirin 320 mg OD (Tohgi et al. 1992). Data for serum TXB_2 in patients chronically administered doses of around 75 mg once daily vs. lower doses were not readily available in the literature.

Assuming an alpha of 0.05, a mean serum TXB_2 of 2.9 ng/ml, a within-patient SD of 3.0 whilst receiving standard treatment of aspirin 75 mg OD and a similar SD of the difference between the higher and lower dose values, it was determined that 20 patients entering a 2 x 2 crossover design would have 80% power to detect a difference of 1.73 ng/ml in serum TXB_2 when assessed by a one-sided paired t test. This calculation was performed by Kathleen Baster, Statistical Consultant, Statistical Services Unit, University of Sheffield.

No sample size estimation was performed for other primary and secondary outcomes as these were intended to be exploratory and generate pilot data for subsequent studies.

For a standard mid-range concentration (62.5 pg/mL), mean coefficient of variation for serum TXB_2 measurement is 9.5% in our laboratory.

IV. Drug Supply

To ensure accurate titration of aspirin doses, a fully soluble aspirin lysine preparation was used (Aspegic, Sanofi-Aventis), supplied by the Pharmacy Department, Sheffield Teaching Hospitals NHS Foundation Trust. Each ‘100 mg’ sachet contained 180 mg of aspirin lysine, including 100 mg of aspirin. Participants were provided with tuition, written illustrated instructions and dosing equipment (syringes and measuring beakers) at visits 2 and 3.

Participants were asked to dissolve the whole of a 100 mg aspirin sachet in 100 ml of drinking water, measured in a pre-marked beaker, stirring for at least 30 seconds to ensure full dissolution. To prepare 20 mg, they were asked to withdraw 20 ml of the solution using a syringe and take that amount, discarding the remainder. To dispense 75 mg, they were asked to discard 25 ml and take the remainder. A new sachet was used for each dose to minimise drug hydrolysis to salicylate once in solution.

V. Drug Accountability

Participants were provided with a medication diary to record times of aspirin and ticagrelor administration during each period. This was used to assess compliance with study medication. All unused aspirin lysine sachets were also counted by the investigators at visits 3 and 4, before return to pharmacy for disposal.

VI. Assessment of Endpoints

a) Serum thromboxane B_2

Blood was collected into a 5 mL serum separator tube (Becton-Dickinson, Oxford, UK) at visits 2 (baseline), 3 (pre- and post-dose) and 4 (pre- and post-dose). Within 5 minutes this was placed into a 37ºC water bath (Grant Instruments, Cambridge, UK) for 30 minutes, before being centrifuged at 1000 rcf for 15 minutes at room temperature. The supernatant (serum) was transferred into cryovial tubes (Grenier Bio-One, Kermsmunster, Austria) for storage at -80 ºC until analysis could be performed.

Analysis of TXB_2 was performed in batches using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI, USA). Serum samples were thawed to room temperature. Where necessary, dilution was made in ELISA buffer provided with the kit. 15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/mL standards were prepared. 50 µL of standard or sample was added to each test well, followed by 50 µL of TXB_2 acetylcholinesterase tracer and 50 µL of anti-TXB_2 monoclonal antibody. The plates were incubated on an orbital shaker at room temperature for 2 hours, then rinsed 5 times with wash buffer. 200 µL of Ellman's Reagent was added to each well and the plate developed on an

orbital shaker at room temperature in dark conditions. Absorbance at a wavelength of 405 nanometres (nm) was determined using an automated plate reader (Multiskan FC, ThermoFisher Scientfic, Waltham, MA, USA). Final plate readings were taken when the blank-subtracted absorbance of the maximum binding control wells was in the range 0.3-2.0 absorbance units (AU).

Samples were analysed in duplicate and retested at a dilution of 1 in 20 if % sample bound/maximum binding (%B/B_0) was <20% when assayed neat. Assays was repeated if there was a difference in %B/B_0 of >20% between the duplicates.

Mean TXB_2 concentrations were interpolated from the standard concentration curve for each plate using GraphPad PRISM version 6.

b) Urinary prostacyclin metabolite, thromboxane metabolite and 8-iso-$PGF_{2\alpha}$

Urine was collected into a plain universal container (Sterilin, Newport, UK) at visits 3 and 4. This was centrifuged at 1500 rcf for 10 minutes at room temperature to remove any cellular debris. The supernatant was removed by transfer pipette and dispensed into cryovial tubes (Greiner Bio-One, Kermsmunster, Austria) for storage at -80 ^{o}C until analysis could be performed. Samples were transported on dry ice to the laboratory of Professor Bianca Rocca, Catholic University of Rome, under whose supervision the urinary prostanoid measurements were undertaken.

Urine PGI-M (2,3-dinor-6-keto $PGF_{1\alpha}$) was measured by gas chromatography-mass spectrometry, as previously described (Brash et al. 1983; Patrono and FitzGerald 1997). Urinary TX metabolite (11-dehydro-TXB_2, TxM) and 8-iso $PGF_{2\alpha}$, a marker of oxidant stress, were measured by previously-validated immunoassays (Ciabattoni et al. 1989). Urinary prostanoids were corrected for urinary creatinine levels, measured by the Department of Laboratory Medicine, Northern General Hospital, Sheffield, using a clinically accredited automated assay (Roche, Basel, Switzerland).

c) Light transmittance aggregometry

Blood was collected into 5 ml tubes (Becton-Dickinson) pre-filled with 0.5 ml 3.2% sodium citrate solution as an anticoagulant. Whole blood platelet count was measured using an automated haematology analyser (Sysmex XP-300, Sysmex, Milton Keynes, UK). Tubes were centrifuged at 200 rcf for 10 minutes at room temperature and the supernatant (platelet rich plasma, PRP) removed with a transfer pipette and dispensed into a polypropylene tube (Sarstedt, Numbrecht, Germany) with a transfer pipette. Platelet count in the PRP was again measured. The remaining citrated blood was re-centrifuged at 1500 rcf for 10 minutes at room temperature. The supernatant (platelet poor plasma, PPP) was again removed with a transfer pipette and dispensed into polypropylene tubes.

Baseline, maximum and final (6 minute) platelet aggregation responses to AA (final concentrations of 0.1, 0.3, 1 mmol/L), collagen (1, 4, 16 µg/mL) and ADP (20 µmol/L) were determined using a PAP machine (PAP-8 v2.0, Bio/Data Corporation, Horsham, PA, USA). Blank calibration of each test well was performed using a glass cuvette (7.25 x 55 mm, Bio/Data Corporation) containing 240 µL PPP and 10 µL 0.9 % saline. Cuvettes containing 240 µL PRP and a micro-stirrer bar (Bio/Data Corporation) were incubated (unstirred) at 37^{o}C for 1 minute before being transferred to covered stirred wells (1200 revolutions per minute [rpm]) for a further minute, after which 10 µL of the appropriate agonist was added. Tests were performed in duplicate and repeated if there was a difference of ≥10 % in aggregation responses between the duplicate samples.

d) Bleeding time

Bleeding time was assessed at visits 3 and 4, 2 hours after administration of aspirin. The ventral aspect of the forearm to be used for measurement was cleaned with a sterile alcohol wipe. A sphygmomanometer cuff was placed above the ipsilateral elbow and inflated to maintain a pressure of 40 millimetres of mercury (mmHg) in order to occlude venous flow without limiting arterial flow, thus leading to capillary engorgement and enabling standardisation of the measurement. Using a standard sterile sprung lancet (blade depth 1.6 mm; Haemolance Max Flow Plus, Prospect Diagnostics Ltd., Dronfield, UK), a puncture was made and a stopwatch started. Second and third punctures were made medially to the first after 10 and 20 seconds respectively. 30 seconds after each puncture, filter paper (Whatman Grade 1, GE Healthcare, Little Chalfont, UK) was applied to the edge of each blood droplet. This was repeated at further 30 second intervals until bleeding ceased or 30 minutes post-puncture was

reached. The time between puncture and first time that bleeding was noted to have ceased was recorded for each of the 3 punctures and the mean time was determined. In cases where bleeding from a puncture had not ceased after 30 minutes, this was recorded as the bleeding time for that site.

e) Safety endpoints

All adverse events (AEs) occurring between randomisation and visit 5 (6 week telephone call) were recorded. Serious AEs in this study were defined as any which resulted in death, was life-threatening, required hospitalisation or prolongation of existing hospitalisation, resulted in persistent disability or incapacity or consisted of a congenital abnormality or birth defect. Causality was assessed by the investigators. All AEs were followed up until resolved or stable.

Bleeding events were classified as per definitions used in the PLATO study. Major life-threatening bleeding was defined as any fatal bleeding, intracranial haemorrhage, bleeding resulting in cardiac tamponade, bleeding resulting in haemodynamic compromise requiring surgery or inotropic support, clinically apparent bleeding associated with a drop in haemoglobin of >5 g/dL or bleeding requiring transfusion of at least 4 units of blood. Other major bleeding was defined at that resulting in significant disability, associated with a drop in haemoglobin of 3-5 g/dL or requiring 2-3 units of blood transfusion. Minor bleeding was any which required medical intervention but did not meet the criteria for major bleeding.

VII. Statistical analysis

Effects on AA metabolite levels, platelet function testing and bleeding time were determined using the pharmacodynamic analysis set as defined below. The population for pharmacodynamic analysis was defined as all randomised patients who received at least 90 % of intended aspirin doses during the study medication periods.

Pre-specified pharmacodynamic endpoint analysis was performed as follows:

f) Primary outcome measures

1. Post-dose serum TXB_2, compared within-patients between the 2 dosing regimens by a paired t test.
2. Post-dose urinary PGI-M, compared within-patients between the 2 dosing regimens by a paired t test.

g) Secondary outcome measures

1. Pre-dose serum TXB_2, compared within-patients between the 2 dosing regimens by a paired t test.
2. Maximum and final post-dose platelet aggregation induced by 0.1, 0.3 and 1 mmol/L AA; 1, 4 and 16 μg/mL collagen; and 20 μmol/L ADP compared within-patients between the 2 dosing regimens by paired t tests.
3. Maximum and final pre-dose platelet aggregation induced by 0.1, 0.3 and 1 mmol/L AA; 1, 4 and 16 μg/mL collagen; and 20 μmol/L ADP compared within-patients between the 2 dosing regimens by paired t tests.
4. Post-dose bleeding time compared within-patients between the 2 dosing regimens by a paired t test.

All comparisons were made using GraphPad PRISM (Version 6).

VIII. Study support and approval

The study was funded by the University of Sheffield and sponsored by Sheffield Teaching

Hospitals NHS Foundation Trust. Prior to commencement, approval to conduct the study was granted by the Cardiology and Cardiothoracic Directorate, Sheffield Teaching Hospitals NHS Foundation Trust; the NHS Research Ethics Committee (South Yorkshire – Sheffield, reference 16/YH/0119); the Medicines Healthcare Regulatory Authority (MHRA) (reference 21304/0258/001-0001); and the Health Research Authority. The study was registered on the European Clinical Trials Database (EudraCT 2016-000920-25) and clinicaltrials.gov (NCT02741817) prior to enrolment of the first participant.

C. Supplementary analyses from the WILLOW ACS trial

Data from this work can be found in chapter 6

I. Leukocyte count and subsets

Leukocyte count, subsets (neutrophil, lymphocyte and mixed monocyte, eosinophil and basophil counts) and platelet count were measured in citrated whole blood using a Sysmex XP-300 haematology automated analyser within the laboratory of the Cardiovascular Research Unit, University of Sheffield. No adjustment was made for dilution by citrate as this was a constant factor and the results were intended for exploratory analyses only.

II. Measurement of plasma TNF-α and IL-6 levels

Venous blood was collected into citrate tubes (Becton-Dickinson) on and kept on ice until processing. Samples were centrifuged at 1500 rcf for 10 minutes at 4^{o}C then the supernatant (plasma) was removed with a transfer pipette and dispensed into cryovial tubes. Plasma samples were stored at -80^{o}C until analysis.

Plasma levels of TNF-α and IL-6 were determined using commercially available ELISA kits (Biolegend, London, UK). 96-well plates (ThermoFisher) were coated with anti-TNF-α or IL-6 capture antibodies and incubated overnight in a refrigerator. Plates were washed 4 times with

phosphate-buffered saline (PBS) + 0.05% Tween-20 (Sigma Aldrich, used also for subsequent washing steps) then incubated on an orbital shaker with 200 μL of assay diluent (Biolegend) in each well for 1 hour to block non-specific activity. After washing again, 100 μL of neat sample was added to each well and incubated for 2 hours with shaking. Samples were assessed in duplicate. A serial dilution of the appropriate standard was performed and included on each plate in order to plot a standard curve. After further washing, 100 μL of diluted detection antibody was added to each well and incubated with shaking for a further hour. After washing again, 100 μL of avidin-horseradish peroxidase solution was added to each well and incubated for 30 minutes with shaking. After a final prolonged wash step, 100 μL of 3,3',5,5'-tetramethylbenzidine solution was added to the well and incubated in the dark for 15 minutes before adding 100 μL of 2N sulfuric acid as a stop solution. Plates were read using an automated plate reader (ThermoScientific Multiskan FC) at a wavelength of 450 nm as per manufacturer's instructions. Non-specific absorbance (measured at a wavelength of 620 nm) was subtracted. Concentration was interpolated from the standard curve using GraphPad Prism v6. If absorbance of a sample was found to be above the validated range of the assay, it was retested at an appropriate dilution, using the assay diluent provided, as per manufacturer's instructions.

III. Measurement of serum creatinine, uric acid and high-sensitivity CRP

Serum creatinine, uric acid and hsCRP were measured using clinically validated methods within the clinical chemistry laboratory, Sheffield Teaching Hospitals NHS Foundation Trust.

IV. Fibrin clot dynamics

High-throughput turbidimetric analysis was performed as described and validated previously (Sumaya et al. 2018). Plasma samples were mixed with standard lysis and activation mixes to form acellular clots. Serial absorbance was measured using an automated plate reader (ThermoFisher Multiskan FC) until lysis was achieved. Variables recorded were maximum absorbance (a representation of fibrin clot turbidity), lag time (time from addition of clot activation mix to the start of clot formation and lysis time (time taken for turbidity to drop by 50% from maximum as a measure of lysis potential).

D. The impact of aspirin dose modification, with or without ticagrelor, on the innate immune response

Data from this work can be found in chapters 7, 8 and 9

Note. This study began in April 2019 but recruitment was halted in March 2020 due to the coronavirus disease 2019 (COVID-19) pandemic. This write-up makes use of interim results, and explains why the methods are described in the present tense, as it is anticipated that study will restart when safe and feasible to do so.

I. Trial design

A graphical overview of the study, named WILL lOWer dose aspirin Therapy ReducE the response to Endotoxin? (WILLOW TREE) is shown in **Figure 3.2**. The trial is a pharmacodynamic study to determine the effect of aspirin dose modification on immune response, compared to control and compared between the presence and absence of potent $P2Y_{12}$ inhibition with ticagrelor. Healthy volunteers receive either no aspirin (control group) or one of three doses of aspirin with or without a loading dose of ticagrelor on the last day of the first medication period, which lasts 10-14 days. They then receive sterile E. coli endotoxin to induce an immune response. Serial measurements of inflammatory markers, cytokines, leukocyte function, prostanoids and platelet function are be taken over 6 hours. Bleeding time is measured before endotoxin administration and 3 hours after it to assess haemostasis. After a washout period of at least 5 weeks, and not more than 18 weeks, subjects then crossover to receive the same regimen of aspirin (aspirin lysine) (or no aspirin in the control group) without or with a loading dose of ticagrelor on the last day of the second medication period.

A total of 72 eligible subjects are planned to be randomised. There are three regimens of aspirin included in the study, in addition to a control group (no aspirin), each tested with and without a 180 mg loading dose of ticagrelor. Aspirin 75 mg OD represents the current standard regimen in ACS and for primary and secondary prevention of cardiovascular disease. Aspirin 20 mg BD is a novel regimen hypothesised to provide a better profile of inflammatory and antithrombotic effect than 75 mg OD. Lastly, aspirin 300 mg OD represents a regimen commonly given after coronary artery bypass grafting. Randomisation is in a 1:1:1:1:1:1:1:1 fashion and managed by a commercially available service for this purpose, SealedEnvelope, with whom the sponsor has a study-specific contract.

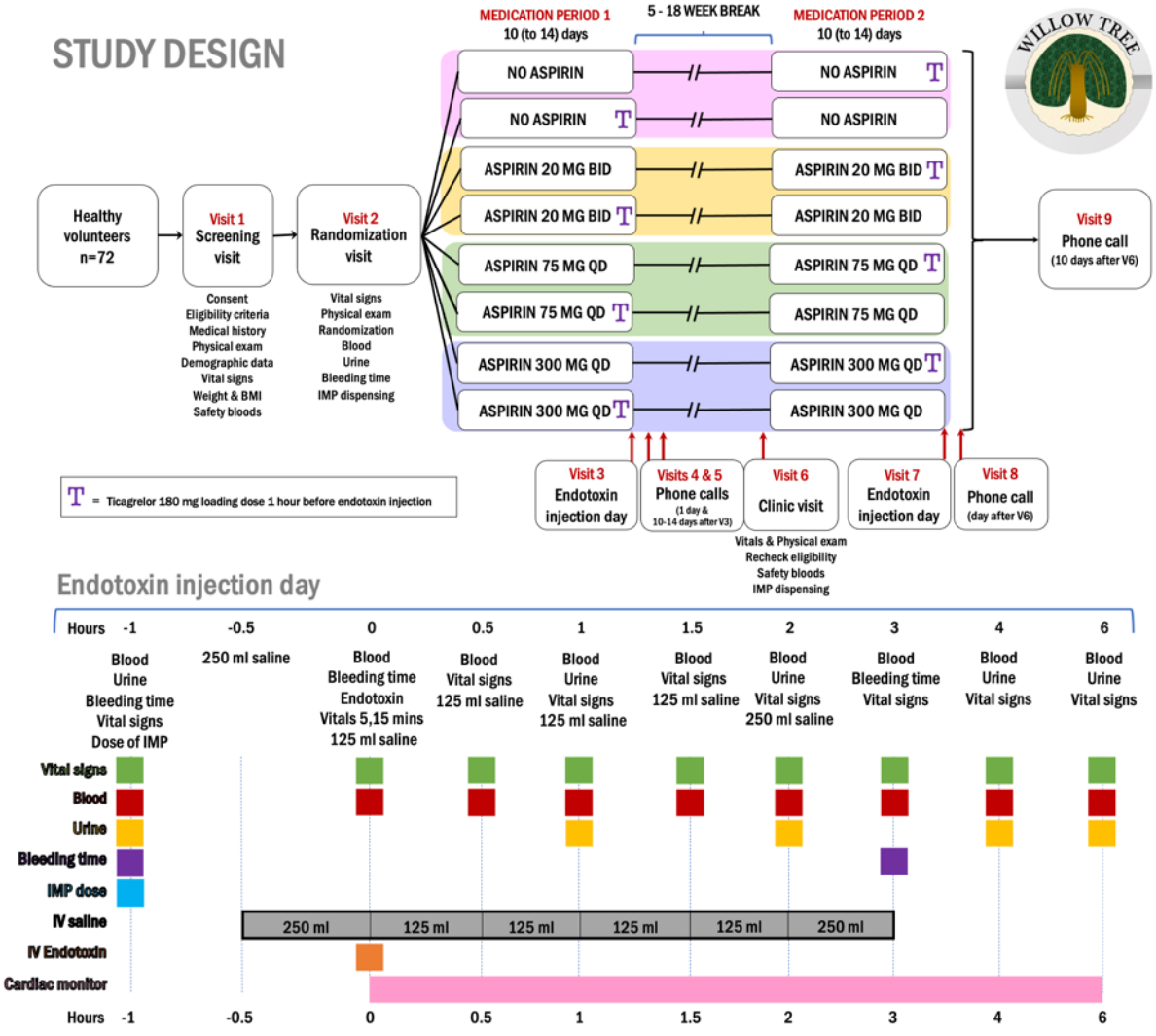

Figure 3.2 Overview of the trial.

BD, twice daily; BMI, body mass index; IMP, investigational medicinal product; OD, once daily.

II. Trial setting

This is a single-centre open-label study, conducted by the Cardiovascular Research Unit, University of Sheffield within the Clinical Research Facility at the Northern General Hospital, Sheffield. Participants are healthy volunteers, recruited primarily from local higher education establishments, but also staff from local healthcare institutions.

III. Participant eligibility criteria

a) Inclusion criteria

To participate in this trial, subjects must be males, or females not of childbearing potential e.g. surgically sterile or post-menopausal. They must be aged between 18 and 65 years with a body mass index (BMI) between 18 and 28 kg/m^2 inclusive and a body weight between 60-100 kg. They must be in good health as determined by a medical history, physical examination, vital signs and clinical laboratory test results, including renal and liver function, and full blood count. Provision of informed consent must be obtained before any trial-related activity.

b) Exclusion criteria

Subjects are excluded if they have any history of cancer, diabetes or, in the opinion of the investigator, clinically-significant cardiovascular, respiratory, metabolic, renal, hepatic, gastrointestinal, haematological, dermatological, neurological, psychiatric or other major disorders; any history of either significant multiple drug allergies or known allergy to the study drugs or any medicine chemically related to the study drugs; a clinically-significant illness within the preceding 2 weeks; any clinically-significant abnormal laboratory test result (full blood count, urea & electrolytes [sodium, potassium, urea and creatinine], liver function tests, clotting screen, urinalysis), at screening (visit 1), in the opinion of the investigator; a supine blood pressure at screening, after resting for 5 minutes, higher than 150/90 mmHg or lower than 105/65 mmHg; a supine heart rate at screening, after resting for 5 minutes, outside the range of 50-100 beats/min; receipt of any prescribed or over-the-counter systemic or topical medication within the preceding 48 hours; planned or expected requirement, during the next 3 months (at randomisation, or 3 weeks at the start of period 2), for any non-study systemic or

topical prescribed drug, or for systemic or topical over-the-counter NSAID, corticosteroid, antihistamine or any other drug that could affect inflammation, thrombosis or haemostasis in the opinion of the investigator; if they have received of an investigational medicinal product, excluding those for the purposes of this study, within the previous four month period (new chemical entity) or three month period (licensed product) or a vaccine within the previous three months; if they have donated blood or plasma in the preceding one month period, excluding for the purposes of this study; a history of alcohol or drug abuse; mental incapacity or language barriers that preclude adequate understanding; or any other factor that, in the opinion of the investigator, would affect the participant's ability to safely and reliably complete the study, or would affect the scientific validity of the results obtained.

IV. Trial procedures

a) Overview of study tests

The aim of the analysis of study samples is to show the dose-dependent effects of aspirin on the immune response to endotoxaemia including on inflammatory markers, cytokines, prostanoids, leucocytes, haemostasis and platelet function, and show whether the addition of ticagrelor modifies these effects. Measurement of inflammatory markers and cytokines that include TNF-α, IL-6 and CRP enable this. To determine the detailed effects of aspirin dose modification on circulating prostanoids in the unstimulated and endotoxin-stimulated states, the eicosanoids and/or their metabolites, including, but not limited to, PGE_2, PGI_2 and TXB_2, are/will be measured. Haemostasis is assessed by measuring bleeding time pre- and 3 hours post-endotoxin using a method shown to be sensitive to additive effects of antiplatelet agents (Teng et al. 2013). In order to confirm the efficacy of the antiplatelet aspects of the drug regimens being studied, the activity of a broad range of pathways of platelet activation and aggregation will be assessed by light transmittance aggregometry pre-medication, post-medication at peak inflammation and during the resolution phase of inflammation, using AA 0.3 and 1 mmol/L, collagen 4 and 16 µg/mL, and ADP 20 µmol/L as agonists.

Study samples are either measured immediately after collection (e.g. LTA) or stored for assays at a later time (other endpoints).

Also stored are acellular samples of serum, plasma, deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and urine within the Cardiovascular Research Unit for future, as yet unplanned, studies. Consent is sought for this at study enrolment.

b) Recruitment

Potential participants self-identify through established channels of advertisement to local Universities and NHS staff and students, using documents approved for this purpose by the relevant research authorities.

c) Screening

Screening occurs at visit 1. This includes taking a medical history, physical examination, collection of demographic data, vital signs (pulse, blood pressure and temperature) measured supine after 5 minutes' rest, weight and BMI, recording of any concomitant medication, safety blood tests (12.5 mL blood sample for full blood count, urea and electrolytes, liver function tests and clotting screen) and urinalysis.

d) Payment

Volunteers who receive at least one endotoxin injection receive £100 per endotoxin injection (maximum £200) to reimburse them for their time, inconvenience and any discomfort caused.

e) Consent

Written, informed consent, is obtained prior to any study procedures being carried out. Participants remain free to withdraw at any time from the trial without giving reasons and without prejudicing his/her further treatment. Samples collected up to the point of withdrawal are only used after withdrawal if the participant consents for this, otherwise they will be destroyed. However, data collected up to that point is used for analysis, and this is explicitly stated in the participant information sheet and consent form.

f) The randomisation scheme

Participants are randomised to one of the following eight treatment sequences, in a 1:1:1:1:1:1:1:1 fashion:

- No IMP for 10-14 days, then ≥ 5-week washout period, then no aspirin for 10-14 days, but a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection (sequence 1).
- No aspirin for 10-14 days, but a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection, then ≥ 5-week washout period, then no IMP for 10-14 days (sequence 2).
- Aspirin (aspirin lysine) 20 mg BD for 10-14 days, then ≥ 5 week washout period, then aspirin (aspirin lysine) 20 mg BD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection (sequence 3).
- Aspirin (aspirin lysine) 20 mg BD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection, then ≥ 5 week washout period, then aspirin (aspirin lysine) 20 mg BD for 10-14 days (sequence 4).
- Aspirin (aspirin lysine) 75 mg OD for 10-14 days, then ≥ 5 week washout period, then aspirin (aspirin lysine) 75 mg OD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection.
- Aspirin (aspirin lysine) 75 mg OD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection, then ≥ 5 week washout period, then aspirin (aspirin lysine) 75 mg OD for 10-14 days (sequence 6).
- Aspirin (aspirin lysine) 300 mg OD for 10-14 days, then ≥ 5 week washout period, then aspirin (aspirin lysine) 300 mg OD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection (sequence 7).
- Aspirin (aspirin lysine) 300 mg OD for 10-14 days plus a loading dose of ticagrelor (180 mg) given one hour before endotoxin injection, then ≥ 5 week washout period, then aspirin (aspirin lysine) 300 mg OD for 10-14 days (sequence 8).

g) Method of implementing the randomisation/allocation sequence

Randomisation is handled by an online interactive web-based randomisation service, sealedenvelope.com. Participants are allocated a three-digit number at enrolment (starting at 001) prefixed with 'E01' (e.g. E01001), then if they proceed to randomisation they are allocated a separate three-digit randomisation number (starting at 001) prefixed with 'R' (e.g. R001). The system generates an immediate email to the investigators stating the treatment allocation and is printed, one copy being placed in the participant's study file and a further copy sent to the Northern General Hospital Pharmacy with, if appropriate, a study-specific prescription for study medication for period 1. A separate prescription is issued for period 2 but using the same randomisation document as evidence of the allocation.

h) Blinding

This study is open label i.e. unblinded to participants and investigators throughout. However, those performing the laboratory assessments are blinded to treatment allocation in order to reduce bias.

i) Trial activities after screening

(1) Visit 2 (Day 0)

Vital signs and findings on physical examination are recorded. Eligibility criteria are reconfirmed then randomisation is performed. 27 ml venous blood is drawn for baseline cytokines, prostanoids, inflammatory markers, platelet function, and plasma, serum, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) for storage. Forearm bleeding time is measured and a urine sample collected for baseline prostanoid measurement. Where allocated, participants are provided with supply of study antiplatelet medication for period 1 as determined by randomisation and dosing training for use of aspirin lysine is provided if required.

(2) Period 1: Day 1 - Day 10 (to 14)

Participants receive one of the following antiplatelet medication regimens for 10 to 14 days:

- No IMP
- No aspirin but a loading dose of ticagrelor (180 mg) taken on the last day of the medication period, 1 hour prior to endotoxin injection
- Aspirin 20 mg BD (only)
- Aspirin 20 mg BD, and a loading dose of ticagrelor (180 mg) taken on the last day of the medication period, 1 hour prior to endotoxin injection
- Aspirin 75 mg OD (only)
- Aspirin 75 mg OD, and a loading dose of ticagrelor (180 mg) taken on the last day of the medication period, 1 hour prior to endotoxin injection
- Aspirin 300 mg OD (only)
- Aspirin 300 mg OD, and a loading dose of ticagrelor (180 mg) taken on the last day of the medication period, 1 hour prior to endotoxin injection

(3) Visit 3 - Period 1: Day 11 (to 15)

(a) On arrival

On arrival, vital signs are recorded and physical examination performed. Any AEs occurring or concomitant medication required in period 1 are noted. Where receiving aspirin in period 1, compliance is checked by counting unused aspirin sachets. A medically qualified member of the research team confirms that the prespecified withdrawal criteria are not met (**Table 3.2**), prior to proceeding further with visit. Withdrawn participants who receive at least one dose of investigational medicinal product (IMP) or endotoxin are followed up by telephone at 10-14 days after withdrawal, and additionally all who discontinue due to adverse events (AEs) will be followed up until resolution or stabilisation. All outcomes of AEs are recorded in the case report form. Withdrawn participants who have proceeded to randomisation are not replaced.

Table 3.2 Trial withdrawal criteria

• Inability to insert intravenous cannula and/or obtain venous blood samples • Withdrawal of consent • Development of an intolerable adverse event due to study participation as determined by the investigator and/or subject • Development of an intercurrent illness, condition or procedural complication that would interfere with the subject's continued participation, unless further study activities can be appropriately postponed • At the time of planned endotoxin injection, receipt of a non-study medication in the preceding 48 hours that, in the opinion of the investigator, is likely to affect inflammation, thrombosis or haemostasis, unless the endotoxin injection visit can be appropriately postponed • Poor compliance (<80% of doses taken) with study aspirin (aspirin lysine) during the preceding medication period, unless the endotoxin visit can be appropriately postponed to achieve 80 % compliance • Violation of the protocol • The investigator feels it is medically in the best interest of the subject to discontinue the subject's participation in the study • Previously unknown data becoming available raising concern about the safety of the study drugs, so that continuation could cause potential risks to the subjects

(b) -1 hour (1 hour before endotoxin injection)

An intravenous (IV) cannula (18 gauge) is inserted into each antecubital fossa using an aseptic non-touch technique. 35.5 mL of venous blood is drawn for lab safety tests (full blood count, urea & electrolytes, liver function tests, clotting screen), cytokines, prostanoids, inflammatory markers and platelet function. Forearm bleeding time is measured and a urine sample obtained for prostanoid assessment. Participants are then asked to take their last dose of aspirin (unless randomised to receive no aspirin), directly witnessed by the investigators to ensure compliance. Where specified by the randomisation allocation, participants are also administered a single 180 mg dose of ticagrelor (orodispersible tablets, AstraZeneca, Cambridge, UK). 250 mL 0.9% saline is then infused over 30 minutes, starting 30 minutes prior to endotoxin injection. 23 mL of venous blood is drawn 60 minutes after last dose of oral antiplatelet medication or equivalent time if not receiving study medication during period 1 (just before endotoxin administration).

(c) 0 hour (time of endotoxin injection)

Before endotoxin injection, continuous cardiac monitoring is started. E.coli endotoxin is then administered by the IV route at a dose of 2 ng/kg given over 1 minute. Vital signs are checked and recorded at 5, 15 and 30 minutes post-endotoxin. A continuous IV infusion of 250 mL 0.9

% saline is given over 1 hour. 14 mL of venous blood sample are drawn at 30 minutes post endotoxin.

(d) 1 hour post endotoxin injection

Vital signs are recorded at 1 and 1.5 hours after endotoxin injection. A further continuous IV infusion of 250 mL 0.9 % saline is given over 1 hour. 14 mL of venous blood is drawn and a urine sample is obtained. A further 14 mL venous blood sample is drawn at 1½ hours post endotoxin.

(e) 2 hours post-endotoxin injection

Vital signs are recorded at 2 hours post endotoxin. A further continuous IV infusion of 250 mL 0.9 % saline over 1 hour is administered. 23 mL of venous blood is drawn and a urine sample is obtained.

(f) 3 hours post-endotoxin injection

Vital signs are recorded at 3 hours post endotoxin. Unless the investigator feels it is clinically indicated, the IV infusion of saline is discontinued. 23 mL of venous blood is drawn and forearm bleeding time is measured.

(g) 4 hours post-endotoxin injection

Vital signs are recorded at 4 hours post endotoxin. 14 mL of venous blood is drawn and a urine sample is obtained.

(h) 6 hours post-endotoxin injection

Vital signs are recorded at 6 hours post endotoxin. 14 mL of venous blood are drawn and a urine sample is obtained. Assuming no clinical concern, continuous cardiac monitoring is discontinued, IV cannulae are removed and participants are discharged home. They are provided with emergency contact details should they need to contact the research team out of hours.

(4) Visit 4 (Period 1, 1 day after visit 3)

Participants are telephoned to follow-up for AEs, with the facility to convert to an in-person visit if any concerns raised.

There then follows a washout period of at least 5 weeks, and not more than 18 weeks, between the first endotoxin challenge day and the beginning of the second medication period. This is to avoid reliably any tolerance of the response to endotoxin, a phenomenon absent by 5 weeks (Rittig et al. 2015).

(5) Visit 5 (10 to 14 days after visit 3)

Participants are followed up by telephone to record AEs.

(6) Visit 6 (Period 2, Day 0)

Vital signs are measured, physical examination performed and concomitant medication recorded. A medically qualified investigator confirms eligibility criteria again and that no withdrawal criteria are met at this point prior to proceeding to medication period 2. 12.5 mL of venous blood is drawn for laboratory safety tests (full blood count, urea & electrolytes, liver function tests and clotting screen). Where allocated, medication is dispensed for period 2.
Participants are advised to begin antiplatelet medication, allocated as follows, for period 2 from the morning after visit 6:

- If received no IMP in period 1, to receive no aspirin, but a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 2.
- If received no aspirin but a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 1, to receive no IMP in period 2.
- If received aspirin (aspirin lysine) 20 mg BD (only) in period 1, to receive aspirin (aspirin lysine) 20 mg BD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 2.
- If received aspirin (aspirin lysine) 20 mg BD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 1, to receive aspirin

(aspirin lysine) 20 mg BD (only) in period 2.

- If received aspirin (aspirin lysine) 75 mg OD (only) in period 1, to receive aspirin (aspirin lysine) 75 mg OD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 2.
- If received aspirin (aspirin lysine) 75 mg OD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 1, to receive aspirin (aspirin lysine) 75 mg OD (only) in period 2.
- If received aspirin (aspirin lysine) 300 mg OD (only) in period 1, to receive aspirin (aspirin lysine) 300 mg OD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 2.
- If received aspirin (aspirin lysine) 300 mg OD plus a loading dose of ticagrelor (180 mg) 1 hour before endotoxin injection in period 1, to receive aspirin (aspirin lysine) 300 mg OD (only) in period 2.

(7) Period 2, Days 1 to 10 (to 14)

Participants take antiplatelet medication during period 2 as determined by the randomisation allocation.

(8) Visits 7,8 & 9

Visits 7, 8 and 9 follow the same form as visits 3, 4 and 5 respectively, other than if a participant did not receive ticagrelor at visit 3, they receive it at visit 7, and vice versa. Assuming there are no reasons for follow up because of AEs, participants are thanked for their involvement in this study, which ends at this point.

j) Study investigations

A summary of sample types, times and volumes obtained from participants during the trial is shown in **Table 3.3**, and a synopsis of methods for endpoint analysis for the whole study is shown in **Table 3.4**.

Table 3.3 Summary of sample types, times and volumes obtained from participants during the trial

EDTA, Ethylenediaminetetraacetic acid; LTA, light transmittance aggregometry; SST, serum separator tube.

Visit	Visit description	Timepoint within visit	Safety (FBC)	Safety (Chemistry)	Safety (Clotting screen)	DNA/RNA for storage	Plasma for cytokines/storage	Serum for prostanoids/CRP /storage	Plasma for LTA	Blood for white cell count and subsets/storage	Total for timepoint	Urine	Bleeding time
		Tube type ⇨	**EDTA**	**SST**	**Citrate**	**EDTA**	**Citrate**	**SST**	**Citrate**	**Citrate**			
			Whole blood to draw (mL)										
1	Enrolment		4	4	4.5						12.5	✓(Dip only)	
2	Randomisation					4	9	5	9	4.5	27	✓	✓
3	Endotoxin day 1	Baseline (pre drug dose)	4	4	4.5		4.5	5	9	4.5	35.5	✓	✓
		Post drug dose/pre endotoxin					4.5	5	9	4.5	23		
		30 mins post endotoxin					4.5	5		4.5	14		
		1 hour post endotoxin					4.5	5		4.5	14	✓	
		1.5 hours post endotoxin					4.5	5		4.5	14		
		2 hours post endotoxin					9	5		9	23	✓	
		3 hours post endotoxin					4.5	5	9	4.5	23		✓
		4 hours post endotoxin					4.5	5		4.5	14	✓	
		6 hours post endotoxin					4.5	5		4.5	14	✓	
4	(Telephone call only)										0		
5	(Telephone call only)												
6	Start of period 2		4	4	4.5						12.5		
7	Endotoxin day 2	Baseline (pre drug dose)	4	4	4.5		4.5	5	9	4.5	35.5	✓	✓
		Post drug dose/pre endotoxin					4.5	5	9	4.5	23		
		30 mins post endotoxin					4.5	5		4.5	14		
		1 hour post endotoxin					4.5	5		4.5	14	✓	
		1.5 hours post endotoxin					4.5	5		4.5	14		
		2 hours post endotoxin					9	5		9	23	✓	
		3 hours post endotoxin					4.5	5	9	4.5	23		✓
		4 hours post endotoxin					4.5	5		4.5	14	✓	
		6 hours post endotoxin					4.5	5		4.5	14	✓	
8	(Telephone call only)										0		
9	(Telephone call only)										0		
		TOTAL	16	16	18	4	99	95	63	94.5	**401**		

Table 3.4 Summary of methods for endpoint analysis. x = measured at timepoint.

				Endotoxin challenge day - Time (hours after endotoxin injection)								
			Randomisation visit (baseline)	-1	0	0.5	1	1.5	2	3	4	6
Endpoint	Sample medium	Method										
Inflammatory markers and cytokines												
TNF-alpha	Plasma	ELISA	x	x	x	x	x	x	x	x	x	x
IL-6	Plasma	ELISA	x	x	x	x	x	x	x	x	x	x
CRP	Serum	Roche Cobas	x	x								x
Prostanoids												
TXB2	Serum	ELISA	x	x	x	x	x	x	x	x	x	x
PGI-M	Urine	Gas chromatography-mass spectrometry	x	x					x		x	x
PGE2	Serum	ELISA	x	x	x	x	x	x	x	x	x	x
Leukocyte phenotype and activation status												
Leukocyte count	Citrated whole blood	Automated Haematology analyser (Sysmex XP-300)	x	x	x	x	x	x	x	x	x	x
Neutrophil count			x	x	x	x	x	x	x	x	x	x
Lymphocyte count			x	x	x	x	x	x	x	x	x	x
Mixed cell count			x	x	x	x	x	x	x	x	x	x
Neutrophil CD11b expression (%)		Flow cytometry	x	x	x	x	x	x	x	x	x	x
Neutrophil CD11b expression (MFI)			x	x	x	x	x	x	x	x	x	x
Neutrophil TLR4 expression (%)			x	x	x	x	x	x	x	x	x	x
Neutrophil TLR4 expression (MFI)			x	x	x	x	x	x	x	x	x	x
Platelet-neutrophil CD42a (MFI)			x	x								x
Platelet-neutrophil co-aggregates (%)			x	x								x

Monocyte CD11b expression (MFI)			x	x	x	x	x	x	x	x	x	x
Monocyte subtype			x	x	x	x	x	x	x	x	x	x
Monocyte TLR4 expression (%)			x	x	x	x	x	x	x	x	x	x
Monocyte TLR4 expression (MFI)			x	x	x	x	x	x	x	x	x	x
Platelet-monocyte CD42a (MFI)			x	x								x
Platelet-monocyte co-aggregates (%)			x	x								x
PGI2 sensitivity			x									
Platelet function												
Max AA-induced PA (1 mmol/L)	Platelet rich plasma	Light transmittance aggregometry	x	x	x					x		
Max ADP-induced PA (20 umol/L)	Citrated platelet rich plasma		x	x	x					x		
Max Collagen-induced PA (4 ug/mL)	Platelet rich plasma		x	x	x					x		
Max Collagen-induced PA (16 ug/mL)	Platelet rich plasma		x	x	x					x		
Final AA-induced PA (1 mmol/L)	Platelet rich plasma		x	x	x					x		
Final ADP-induced PA (20 umol/L)	Platelet rich plasma		x	x	x					x		
Final Collagen-induced PA (4 ug/mL)	Platelet rich plasma		x	x	x					x		
Final Collagen-induced PA (16 ug/mL)	Platelet rich plasma		x	x	x					x		
Platelet P-selectin expression %)	Citrated whole blood		x	x								x
Haemostasis												
Bleeding time	In vivo	Standard lancet method	x							x		
Fibrin clot dynamics												
Lag time	Citrated plasma	Fibrin clot turbidimetry										
Maximum absorbance												
Lysis time												

k) Details of fluid management

The default fluid regimen outlined above totals 1000 mL over the 8 hours of the day (250 mL pre-endotoxin and 750 mL post-endotoxin). Supplemental fluid boluses are also administered if required, typically 250-500 mL of 0.9% saline, given if mean arterial blood pressure is reduced by 20% or more or to maintain a systolic blood pressure over 100 mmHg and/or to relieve symptoms related to hypotension. Fluid administration is recorded on the clinical drug chart and on the case report form (CRF).

l) Long term follow-up

In the event of a participant remaining uncontactable at the end of the follow-up window, the hospital records of the participant are interrogated to clarify vital status and any evidence of untoward events. Participants are declared 'lost to follow up' if they are uncontactable 28 days after the second endotoxin injection day.

m) Provision for postponement of visit in the event of intercurrent illness

There is provision in the protocol in the event a study participant develops an intercurrent illness, a requirement for a course of medication that is likely to affect study endpoint measurement or compliance with aspirin is <80% during the preceding study medication period. If the subsequent endotoxin injection visit cannot be postponed to a time within the permitted window of 10-14 days into the medication period at which the illness or relevant effect of medication has deemed to have resolved or compliance has increased to at least 80% for the medication period, study medication may be stopped and the medication period restarted once the intercurrent illness or requirement for medication is deemed to have resolved, or when compliance is likely to be at least 80%.

n) Study medications

(1) Name and description of investigational medicinal product(s)

Aspirin lysine (e.g. ‘Aspegic’ [Sanofi-Aventis] or ‘Cardirene’ [Sanofi-Aventis] ‘100 mg’ sachets) is the aspirin preparation used in this study. Each aspirin lysine ‘100 mg’ sachet contains 180 mg aspirin lysine, equivalent to 100 mg acetylsalicyclic acid (aspirin). In this thesis, the stated dose refers to the aspirin content. Where directed to take a dose of 20 mg BD, participants are asked to dissolve 100 mg (1 sachet) in 100 mL of drinking water and ingest 20 ml of the solution, measured using a graduated syringe provided to them, discarding the remainder. Where directed to take a dose of 75 mg OD, participants are asked to dissolve 100 mg (1 sachet) in 100 mL of drinking water, remove 25 ml of the solution using a graduated syringe, and ingest the remainder. Where directed to take a dose of 300 mg OD, participants are asked to dissolve 300 mg (3 sachets) in 100 mL of drinking water and ingest the whole amount of the solution.

Participants are asked to take a loading dose of 180 mg ticagrelor 1 hour before endotoxin injection where specified by the randomisation allocation. This is directly observed by the research team in order to verify compliance. The specific ticagrelor preparation used is in the form of 90 mg oro-dispersible tablets (AstraZeneca, 10 tablets per pack), which is in line with local protocols for loading with ticagrelor during an ACS event.

(2) Regulatory status of the drug preparations

Whilst aspirin (aspirin lysine) 100 mg sachets are not licensed in the UK, they are licensed in other EU countries (e.g. Belgium, Italy) for the purposes of analgesia and antipyresis. As there is no marketing authorisation in the UK for aspirin lysine, this is imported from other EU countries facilitated by an existing relationship with Mawdsleys Ltd. For the purposes of regulatory approval and safety monitoring, a notarised translation of the summary of the product characteristics from the original French was obtained. Ticagrelor 90 mg orodispersible tablets are licensed in the UK, in combination with aspirin, for the treatment of ACS.

(3) Concomitant medication

Regular concomitant medication leads to exclusion from randomisation. Recording of any prescribed or over-the-counter medication after randomisation is made at each subsequent visit. If, at visits 3 and 7, any of the withdrawal criteria are met, the participant is withdrawn if the visit cannot be postponed.

The following guidance is observed with regards to specific groups of concomitant medications, should participants require these during the study after randomisation, excluding the period between first endotoxin visit and second medication supply visit. Participants who receive at least one dose of IMP or endotoxin are followed up by telephone 10-14 days after any withdrawal relating to concomitant medication.

(a) Oral antiplatelet drugs

Aspirin use, with the exception of the study medication as prescribed, is prohibited during the study period. At enrolment, patients are asked to not use aspirin as an analgesic and they are made aware of the range of over-the-counter products that contain it. If no contraindication exists, paracetamol is recommended if the need for analgesia arises, but endotoxin injection is not performed if paracetamol has been received in the preceding 24 hours. Patients are asked about extra aspirin use, including over-the-counter supplies, at all visits. If, during the course of study treatment, a participant develops a clinical indication for regular antiplatelet therapy, a clinically appropriate regimen should be prescribed and they are followed up but withdrawn from the study.

Treatment with any other oral antiplatelet therapy apart from the study medication (e.g. clopidogrel, prasugrel, dipyridamole, cilostazol) is prohibited during the course of study medication. However, if during the course of study treatment, a participant develops a contraindication to aspirin or ticagrelor, these are discontinued/withheld and they are withdrawn from the trial.

As all participants receive ticagrelor at some stage during the study, drugs interacting with its

metabolism should be avoided. Strong inhibitors of CYP3A4 substantially increase plasma ticagrelor levels whereas strong inducers of CYP3A4 have the opposite effects. Consequently, strong CYP3A4 inhibitors (eg, ketoconazole, itraconazole, voriconazole, telithromycin, clarithromycin [but not erythromycin or azithromycin], nefazadone, ritonavir, saquinavir, nelfinavir, indinavir, atanazavir, or over 1 litre daily of grapefruit juice) should not be co-administered with ticagrelor as plasma levels of ticagrelor may be substantially increased (or time course of metabolism altered in the case of a single ticagrelor dose). If regular treatment with such therapies is essential, then ticagrelor is withheld and the participant withdrawn.

Concomitant therapy with simvastatin or lovastatin at doses higher than 40 mg daily is not permitted since ticagrelor significantly increases the levels of these statins and theoretically therefore may increase the risk of myopathy. There are no restrictions to other statin therapies (i.e. doses of simvastatin or lovastatin ≤40 mg daily or any dose of any other statin is permitted) but if a participant fails to meet the eligibility criteria for the study at the next check (visit 3, 6 or 7), they are withdrawn if the visit cannot be postponed.

Co-administration of ticagrelor with CYP3A4 substrates with a narrow therapeutic index (e.g. cyclosporine and quinidine) should be avoided. Co-administration of ticagrelor with strong inducers of CYP3A4 should also be avoided (e.g. rifampin/rifampicin, rifabutin, phenytoin, carbamazepine, phenobarbital). If regular treatment with such therapies becomes essential during the study medication period then they are withdrawn from the study.

(b) Non-steroidal anti-inflammatory drugs

NSAIDs may affect the antiplatelet and immunomodulatory effects of aspirin whilst increasing the risk of gastric irritation/ulceration and renal impairment. Levels of arachidonic acid metabolites may also be affected. Requirement for regular treatment with an NSAID at enrolment meets the exclusion criteria of the study. Treatment with NSAIDs during the study period is discouraged. COX2 inhibitors are prohibited in combination with study medication. Paracetamol is safe in combination with both aspirin and ticagrelor and therefore will be the recommended analgesic/antipyretic agent if required. In the case of a participant requiring treatment with an NSAID/paracetamol/COX2 inhibitor, if they fail to meet the eligibility criteria for the study at the next check (visit 3, 6 or 7), they are withdrawn if the visit cannot be postponed.

(c) Other anti-inflammatory/immunomodulatory drugs

In the case of a participant requiring regular treatment with oral, topical or inhaled corticosteroids; disease-modifying anti-rheumatic drugs; immunosuppressants; chemotherapy drugs; oral or topical antihistamines, they are withdrawn if the visit cannot be postponed to avoid meeting the withdrawal criteria.

(d) Diuretics, ACE inhibitors and angiotensin receptor blockers

Diuretics, including loop (e.g. furosemide, bumetanide), thiazide (e.g. bendroflumethiazide, indapamide) and potassium-sparing agents (e.g. spironolactone, eplerenone, amiloride); angiotensin-converting enzyme (ACE) inhibitors; and angiotensin receptor blockers exert effects on renal prostaglandin synthesis and therefore may interfere with urinary prostaglandins. If a requirement for regular diuretic treatment develops during the study, a participant who fails to meet the eligibility criteria for the study at the next check (visit 3, 6 or 7), is withdrawn if the visit cannot be postponed.

(e) Parenteral anticoagulants, glycoprotein IIb/IIIa antagonists and oral anticoagulants

In the event of an indication for parenteral anticoagulation, oral anticoagulation or GP IIb/IIIa inhibitor developing, the participant is withdrawn from the study.

(4) Trial restrictions

Whilst undergoing sampling during visits 3 and 7, subjects are asked to have a light breakfast before 8 am then remain nil by mouth until 2 hours post-endotoxin.

(5) Non-Investigational Medicinal Products

Although not a medicinal product, also administered in this study is sterile bacterial endotoxin, 2 ng/kg intravenously. As a challenge agent, this is classified as a non-IMP (NIMP), as per European Commission Guidance Document SANCO/C/8/SF/cg/a.5.001(2011)332855. This is reconstituted with water for injection as per the manufacturer's instructions. Endotoxin specifically designed for human challenge studies is obtained from Dr Anthony Suffredini, National Institutes of Health, Bethesda, USA. Endotoxin is securely held separately in cold storage within the Cardiovascular Research Unit, Northern General Hospital. The temperature of the storage environment is monitored by recording a daily maximum and minimum temperature log (with the exception of weekends). The allowable temperature range is 2 to 8^{o}C. An accountability log to track each vial is in the trial master file. Endotoxin is prescribed by a medically qualified investigator on a Sheffield Teaching Hospitals Drug Administration Record. Administration is recorded on the same chart.

(6) Recording and reporting of adverse events

Serious AEs (SAEs)/suspected unexpected serious adverse reactions (SUSARs) are recorded and reported from randomisation to 10 days after visit 3 (first endotoxin injection day), and from visit 6 (start of period 2) to 10 days after visit 7 (second endotoxin injection day). Thus, AEs occurring in the break between the first and second medication periods, excepting those originating in the 10 days after endotoxin injection, are not recorded or reported.

These are recorded on the Sheffield Teaching Hospitals NHS Foundation Trust SAE reporting Form and emailed to the Sponsor's dedicated email address for this purpose within 24 hours of the research staff becoming aware of the event. For any SAE or SUSAR, the full details in medical terms and case description, event duration (start and end dates, if applicable), action taken, outcome, seriousness criteria, causality (i.e. relatedness to trial drug / investigation), in the opinion of the investigator and whether the event would be considered anticipated are recorded. Any SAEs assigned by the investigator as both suspected to be related to IMP-treatment and unexpected are be classified as SUSARs and are subject to expedited reporting

to the MHRA. Any SAEs related to the NIMP (endotoxin) are reported by the investigators to the sponsor, but further forwarding to other agencies is not required, as per European Directive 2011/C 172/01. NIMP-related SAEs are evaluated by the sponsor and investigator team with regard to ongoing safety implications. If there are ongoing safety implications for the trial then these are addressed via urgent safety measure, substantial amendment or termination of the trial.

If, on adjudication by the investigators, there is a possibility that an SAE is related to interaction between the NIMP and at least one of the IMPs, the process is followed for reporting this as an IMP-related event, including any required onward notification by the sponsor concerning the relevant IMP. If an SAE is judged as possibly related to both NIMP and an IMP then it is assessed with regard to expectedness with regard to the IMP and reported onwards as a SUSAR if it is judged to be unexpected in relation to the IMP.

V. Statistical plan

With the exception of the interim analysis, the statistical plan refers to the final analysis of the study data to be performed on completion (once all 72 randomised participants have finished active involvement in the study). The trial results presented later in this thesis result from the protocol-defined interim analysis and supplementary exploratory analyses of data collected at this point, therefore for reasons of maximising the ability to draw interpretations, do not necessarily follow the same form.

a) Sample size calculation for primary endpoint

In a previous study of endotoxaemia in 30 participants receiving ticagrelor, clopidogrel or placebo, after log transformation, mean 2 hour post-endotoxin plasma TNF-α was 1.88 pg/mL with a standard deviation of 0.410 pg/mL (Thomas et al. 2015).

There is no available raw data and no reliable estimate of the means or standard deviations of the log transformed TNF-α values for any of the three aspirin doses using the same methods. However, a study using a different endotoxin regimen showed that aspirin 80 mg OD (which is not expected to be significantly different in effect to 75 mg OD) increased peak plasma TNF-α by around 40% compared to those receiving no drug (Kiers et al. 2017).

There is a clear hypothesis that increasing aspirin dose increases TNF-α, therefore a one-sided test will suffice.

It is estimated that clinically relevant differences in plasma TNF-α at 2 hours post-endotoxin between the dosing regimens might be a 20% increase with 20 mg BD vs. no aspirin, 40% increase with 75 mg OD vs. no aspirin and 60% with 300 mg OD vs. no aspirin, based on the raw data. These assumptions provide the following sample size estimate in **Table 3.5**.

Table 3.5 Sample size calculation

Test significance level, α	0.050
Number of groups, G	4
Variance of means, $V=S(m_i-m)^2 / G$	0.031
Common standard deviation, s	0.410
Effect size, $D^2 = V/s^2$	0.1828
Power (%)	80
n per group	16

Therefore, when the sample size in each of the 4 groups is 16, a one-way analysis of variance will have 80% power to detect at the 0.050 level a difference in means characterised by a Variance of means of 0.031, assuming that the common standard deviation is 0.410.

To allow for up to 10% drop-out, the sample size per group will be 18. Drop-outs are not replaced but it is expected that there will be at least 16 per group after withdrawals.

This sample size calculation was performed with the assistance of Mrs Kathleen Baster CStat, Senior Consultant, Statistical Services Unit, University of Sheffield, to whom the candidate is grateful.

b) Statistical analysis plan

(1) Summary of baseline data and flow of patients

The following baseline data are collected and will be reported:

From visit 1

- Demographic data (age, sex, ethnicity)
- Vital signs: pulse, blood pressure and temperature
- Weight and BMI
- Full blood count, urea & electrolytes, liver function tests

At visit 2

- TNF-α, IL-6, serum TXB_2, urine PGI-M, platelet aggregation responses to AA, ADP and collagen
- Bleeding time

Categorical data will be reported as proportions and percentages. Differences between the groups will be assessed using Fisher's exact contingency test. Continuous data will be reported as mean and standard deviation if normally distributed otherwise median and interquartile range. Differences between the parallel groups will be assessed with ANOVA, with Bonferroni pairwise comparisons made if any ANOVA reaches $p<0.05$. A CONSORT flow diagram will be prepared for inclusion in the report of study findings.

(2) Primary outcome analysis

The primary endpoint will be plasma TNF-α at 2 hours following endotoxin administration assessed between aspirin groups (control vs. 20 mg BD vs. 75 mg OD vs. 300 mg OD, without ticagrelor) by one-way ANOVA with treatment as a factor.

(3) Secondary outcome analyses

Secondary analyses will be carried out for each endpoint between aspirin dose regimens and

DAPT regimens by two-way mixed ANOVA with treatment as a between-subjects factor and timepoint as a within-subjects factor, followed by pairwise comparisons if found to exhibit significant relationships. Each aspirin regimen (control, 20 mg BD, 75 mg OD, 300 mg OD, each without ticagrelor) and each corresponding DAPT regimen (control, 20 mg BD, 75 mg OD, 300 mg OD, each with ticagrelor 90 mg BD) will be compared by paired t-tests of area under the curve. To model the effects of all variables, each endpoint will be compared between each aspirin regimen (control, 20 mg BD, 75 mg OD, 300 mg OD, each without ticagrelor) and each corresponding DAPT regimen (control, 20 mg BD, 75 mg OD, 300 mg OD, each with ticagrelor 90 mg BD) with a mixed three-way ANOVA with aspirin-dose and presence or absence of ticagrelor as between-subjects factors and timepoint as a within-subject factor.

(4) Interim analysis and criteria for the premature termination of the trial

After discussion between the investigators and the Sponsor, face-to-face participant study visits were halted on 17th March 2020 due to the unprecedented circumstances arising during the coronavirus disease 2019 (COVID-19) pandemic. It was considered by the investigators that the results of the study may have important implications for the management of cardiovascular disease patients who contracted COVID-19 in terms of optimal antiplatelet medication, in view of the morbidity and mortality in COVID-19 associated with dysregulated inflammatory response. It was noted that the recruitment and study activities to date had reached the level that had originally been planned for the study, as per the application funded by the British Heart Foundation. Given the uncertainty regarding restarting the trial, the need to preserve healthcare resources and to limit face-to-face encounters, the investigators and Sponsor decided that an interim analysis should be performed.

(a) Sample size for interim analysis

Data from all participants who have completed visit 3 (first endotoxin day) were included in the interim analysis. This was expected to include 46 participants who had completed visit 3, 37 of whom have completed the whole study giving a total of 83 endotoxin day visits, anticipated to be split into approximately equal numbers receiving each of the eight study

treatment regimens.

(b) Statistical procedure for interim analysis

The primary endpoint of the interim analysis was plasma TNF-α measured at 2 hours after endotoxin administration assessed between aspirin treatment groups (control vs. 20 mg BD vs. 75 mg OD vs. 300 mg OD, without ticagrelor) using one-way ANOVA. If this demonstrated a significant difference between the groups ($p<0.05$), the following pre-specified pairwise comparisons would be performed, in hierarchical fashion: 300 mg OD vs. no aspirin, then, if significant ($p<0.05$) 75 mg OD vs. no aspirin, then, if significant ($p<0.05$) 300 mg OD vs 20 mg BD, then, if significant ($p<0.05$) 75 mg OD vs 20 mg BD, then, if significant ($p<0.05$) 20 mg BD vs no aspirin. Other analyses were deemed exploratory and at the discretion of the investigators.

(c) Criteria for premature termination

The trial was to be prematurely terminated if the primary interim analysis demonstrated a statistically significant ($p<0.05$) effect of aspirin dosing on plasma TNF-α two hours after endotoxin injection. Additionally, continuing the study will be considered futile, and therefore discontinued, if the lower limit of the 95% confidence interval of the mean change in TNF-α at 2 hours after endotoxin from the 300 mg aspirin OD group to no aspirin group is less than (more negative than) -2000 pg/mL. If neither of these criteria were met, the protocol stated that the investigators should meet to review the results of the interim analysis and, in discussion with the Sponsor, decide on whether to continue the study, taking into account feasibility, including consideration of local and national restrictions.

(d) Secondary analyses to be carried out in the event of premature termination

If the trial was prematurely terminated, data concerning other endpoints were to be analysed using mixed effect linear models with patient as a random effect, aspirin dose, presence or

absence of ticagrelor treatment and treatment period (first or second) as fixed effects, and baseline value as covariate. This method was felt best to take into account the cases of participant data missing from one period.

(5) Participant population

The pharmacodynamic analysis set will include all participants who achieve at least 80% compliance with study medication during each of the two periods and who complete both endotoxin injection days. The safety analysis set (for the purposes of adverse event reporting etc.) will include any participant randomised into the trial that received at least one dose of trial drug or one dose of IV endotoxin.

VI. Ethical and regulatory considerations

The trial protocol obtained Sponsor approval from the Clinical Research and Innovation Office at Sheffield Teaching Hospitals NHS Foundation Trust prior to submitting for external regulatory review. The trial was reviewed by the NHS Research Ethics Service East of England - Cambridge (East) Committee on 11 December 2018 and approval was issued on 27 December 2018 (Ref 18/EE/0401). A Clinical Trial Authorisation for the study was granted by the MHRA on 18 January 2019 (Ref 21304/0268/001-0001) and the study received approval from the NHS Health Research Authority on 25 January 2019 (ref 254420). The trial was registered with the European Union Drug Regulating Authorities Clinical Trials Database (EudraCT, ref 2018-004285-34) and clinicaltrials.gov (ref NCT03869268) prior to enrolment of the first participant. All investigators hold current Good Clinical Practice certification.

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VII. Public and Patient Involvement

Members of the Sheffield Cardiovascular Patient Panel were involved in reviewing the design of the study and key participant documents during development of this protocol and associated documentation.

VIII. Handling of samples and methods of pharmacodynamic endpoint assessment

Detailed methods are only included in this thesis where endpoints have formed part of the interim analysis.

a) Serum thromboxane B_2

Venous blood was drawn into serum separator tubes (Becton-Dickinson) and immediately incubated in a water bath for 30 minutes at 37ºC. Tubes were then centrifuged at room temperature, serum drawn off and stored at -80ºC prior to anaylsis. Serum TXB_2 was measured using a commercially available ELISA kit (Cayman Chemical) as described previously in this thesis. At completion of the study, it is planned to measure serum TXB_2 in samples obtained at baseline then -1, 0, 0.5, 1, 1.5, 2, 3, 4 and 6 hours after endotoxin. However, for the interim analysis, only samples drawn at 1 hour after endotoxin injection were included. In animal models, serum TXB_2 is elevated during endotoxaemia, levels peaking between 40 and 70 minutes after injection, hence why the 1 hour timepoint was the one chosen (Wise et al. 1981).

b) Platelet aggregation responses

Maximum and final platelet aggregation responses were measured at baseline, -1, 0 and 3 hours after endotoxin injection by LTA, as described earlier in this thesis. AA (final concentration 1 mmol/L), ADP (20 μmol/L) and collagen (4 and 16 μg/mL) were used as agonists.

c) Measurement of neutrophil and monocyte CD11b and TLR4 expression

Four antibody cocktails were prepared (**Table 3.6**) prior to receiving study samples. Venous blood was collected into citrate tubes (Becton-Dickinson) and processed within 15 minutes of venepuncture to avoid artefactual platelet-monocyte aggregate formation. 25 μL of each antibody cocktail was mixed with 100 μL of blood. The tubes were gently mixed by tapping to ensure good antibody binding. Tubes were incubated in the dark for 20 minutes at room temperature, after which 1 mL of FACSlyse® (Becton-Dickinson, diluted 1:10 with distilled water) was added to each. Tubes were vortexed briefly then incubated for a further 10 minutes at room temperature before being centrifuged at 300 rcf for 5 minutes. The supernatant was discarded by inverting the tube and blotting. The pellet was resuspended in 200 μL of FACSfix® (Becton-Dickinson) and then processed on a 6-colour flow cytometer (Accuri C6, Becton-Dickinson). 100,000 events were collected using a high forward scatter threshold (approximately 200,000). Results were analysed for median fluorescence intensity (MFI) using the cytometer's native software package.

Table 3.6 Antibody cocktails prepared for each tube

A: Monocyte phenotyping	**B: Platelet-leukocyte coaggregates**	**C: Isotype control (A)**	**D: Isotype control (B)**
10 μL **FITC anti-human CD11b** (Biolegend 301330)	25 μL **FITC anti-human CD42a** (BD 558818)	10 μl **FITC mouse IgG** (Biolegend 400110)	25 μ **FITC mouse IgG** (Biolegend 400110)
5 μl **PE anti-human CD14** (Biolegend 367104)	5 μl **PE anti-human CD14** (Biolegend 367104)	5 μl **PE anti-human CD14** (Biolegend 367104)	5 μl **PE anti-human CD14** (Biolegend 367104)
2.5 μl **PE-Cy7 anti human CD16** (Biolegend 302016	2.5 μl **PE-Cy7 anti human CD16** (Biolegend 302016	2.5 μl **PE-Cy7 anti human CD16** (Biolegend 302016	2.5 μl **PE-Cy7 anti human CD16** (Biolegend 302016
25 μl **APC anti-human TLR4** (Biolegend 312816)		25 μl **APC anti-mouse IgG1** (Biolegend 406610)	
50 μl **Mouse serum** (Sigma Aldrich 18765)		50 μl **Mouse serum** (Sigma Aldrich 18765)	
157.5 μl PBS	217.5 μl PBS	157.5 μl PBS	217.5 μl PBS

d) Platelet P-selectin expression

Venous blood was collected into citrate tubes (Becton-Dickinson). 10 μL of anti-CD42a antibody (FITC, Becton-Dickinson 558818) and 10 μL of anti-P-selectin (also known as CD62P) (R-PE, Becton-Dickinson 555524) were added to a polypropylene tube ('ADP tube'). 10 μL of anti-CD42a and mouse IgG R-PE control (Becton Dickinson 555749) were added to another ('control tube'). All antibodies had been diluted 1:10 in PBS prior to the experiment. 23 μL of PBS was added to the ADP and control tubes. 2 μL of ADP dissolved in 0.9% saline (to achieve a final concentration of 30 μmol/L) was added to the ADP tube and 2 μL of 0.9% saline to the control. 5 μL of blood was then added to each tube and gently mixed, before being incubated in the dark for 20 minutes. 1 mL of FACSfix® (Becton-Dickinson) was then added to each tube and gently mixed. Contents of the tubes were analysed using an Accuri C6 flow cytometer (Becton-Dickinson). 5,000 P-selectin positive events were collected. % of positive events and median fluorescence were determined for each tube.

e) Bleeding time

Forearm bleeding time was determined using a simple lancet method as detailed earlier in this thesis.

f) Plasma TNF-α and IL-6

Venous blood was collected into citrate tubes (Becton-Dickinson) that were kept on ice until processing. Samples were centrifuged at 1500 rcf for 10 minutes at 4°C then the supernatant (plasma) was removed using a transfer pipette and dispensed into cryovial tubes. Plasma samples were stored at -80°C until analysis.

Plasma levels of TNF-α and IL-6 were determined using commercially available ELISA kits (Biolegend) as detailed earlier in this thesis.

E. Attribution of work presented in this thesis

Of the work described in this chapter, in section A (in vitro work), the candidate designed the experiment under the supervision of Professor Robert Storey and Dr Heather Judge, took consent from participants, obtained blood samples, performed the laboratory work and analysed the results.

In section B (the WILLOW ACS study), the candidate designed the trial under the supervision of Professor Storey, authored the protocol and related documents, developed these in collaboration with the Sheffield Teaching Hospitals Clinical Research Office, wrote and submitted forms for ethical and MHRA approval, attended the ethics committee meeting, recruited and consented patients, performed randomisation, obtained study samples, performed bleeding time measurement and reviewed adverse events. Dr Rachel Orme performed study visits on the few occasions that the candidate was not available to. Kathleen Baster of the Statistical Services Unit, University of Sheffield performed the sample size calculation. With regards to laboratory analyses, the candidate performed ELISAs for TXB_2 with the assistance of Hannah Stokes. 'Real time' laboratory procedures during study visits (LTA, plasma, serum and urine preparation), performed whilst the candidate was supervising the participant, were carried by Dr Heather Judge, Jessica Hanson and Hannah Stokes. Measurement of urinary prostanoids was performed by a team under the supervision of Professor Bianca Rocca at the Catholic University of Rome. The candidate analysed the data.

In section C (supplementary analyses from WILLOW ACS), the candidate conceived and performed the statistical analyses. The candidate performed the ELISAs for IL-6 and TNF-α. Fibrin clot turbidimetry was performed by the candidate under the supervision of Dr Wael Sumaya. Measurement of serum hsCRP, creatinine and uric acid was performed by the Sheffield Teaching Hospitals Clinical Chemistry Laboratory.

In section D (the WILLOW TREE study), the candidate designed the trial under the supervision of Professor Robert Storey, authored the protocol and related documents, developed these in collaboration with the Sheffield Teaching Hospitals Clinical Research Office, wrote and submitted forms for ethical and MHRA approval, attended the ethics committee meeting, recruited and consented participants, performed randomisation, administered study IMP and

NIMP, obtained study samples, performed bleeding time measurements and reviewed adverse events. Dr Tom Nelson and Dr Hazel Haley performed study visits on the few occasions that the candidate was not available to. Kathleen Baster of the Statistical Services Unit, University of Sheffield performed the sample size calculation. The candidate performed ELISAs for TNF-α. 'Real time' laboratory procedures during study visits (LTA, flow cytometry, plasma, serum and urine preparation), performed whilst the candidate was supervising the participant, were carried by Dr Heather Judge, Cameron May and Sasha Lucie-Smith. Blood safety parameters were measured by the Sheffield Teaching Hospitals Department of Laboratory Medicine. The candidate performed statistical analyses.

Chapter 4: In vitro concentration-dependent effects of aspirin, with or without concurrent $P2Y_{12}$ inhibition, on platelet aggregation

A. Baseline characteristics

Samples from 6 healthy volunteers were included in this study. Mean (± SD) platelet counts in citrated whole blood and PRP were 226 ± 23 $x10^9$/L and 363 ± 107 $x10^9$/L respectively.

B. Light transmittance aggregometry

A summary of the maximum and final aggregation responses to each agonist is shown in **Figure 4.1**. In the absence of potent $P2Y_{12}$ inhibition with cangrelor, aspirin concentration-dependently inhibited maximum and final collagen- and arachidonic acid-induced platelet aggregation. In the presence of cangrelor, aspirin remained additive in effect on maximum aggregation responses to arachidonic acid (1 mmol/L) and collagen (2 μg/mL), and on final aggregation responses to collagen (2 μg/mL). In all cases of significant effect, the lowest concentration of aspirin that exerted a maximal inhibitory response was 10 μmol/L, with higher concentrations failing to achieve any significant further inhibition. Addition of cangrelor did not appear to shift the concentration-response curve for aspirin either to the left or right.

Aspirin had no significant effect on maximal or final ADP-induced platelet aggregation responses, whether in the presence or absence of cangrelor.

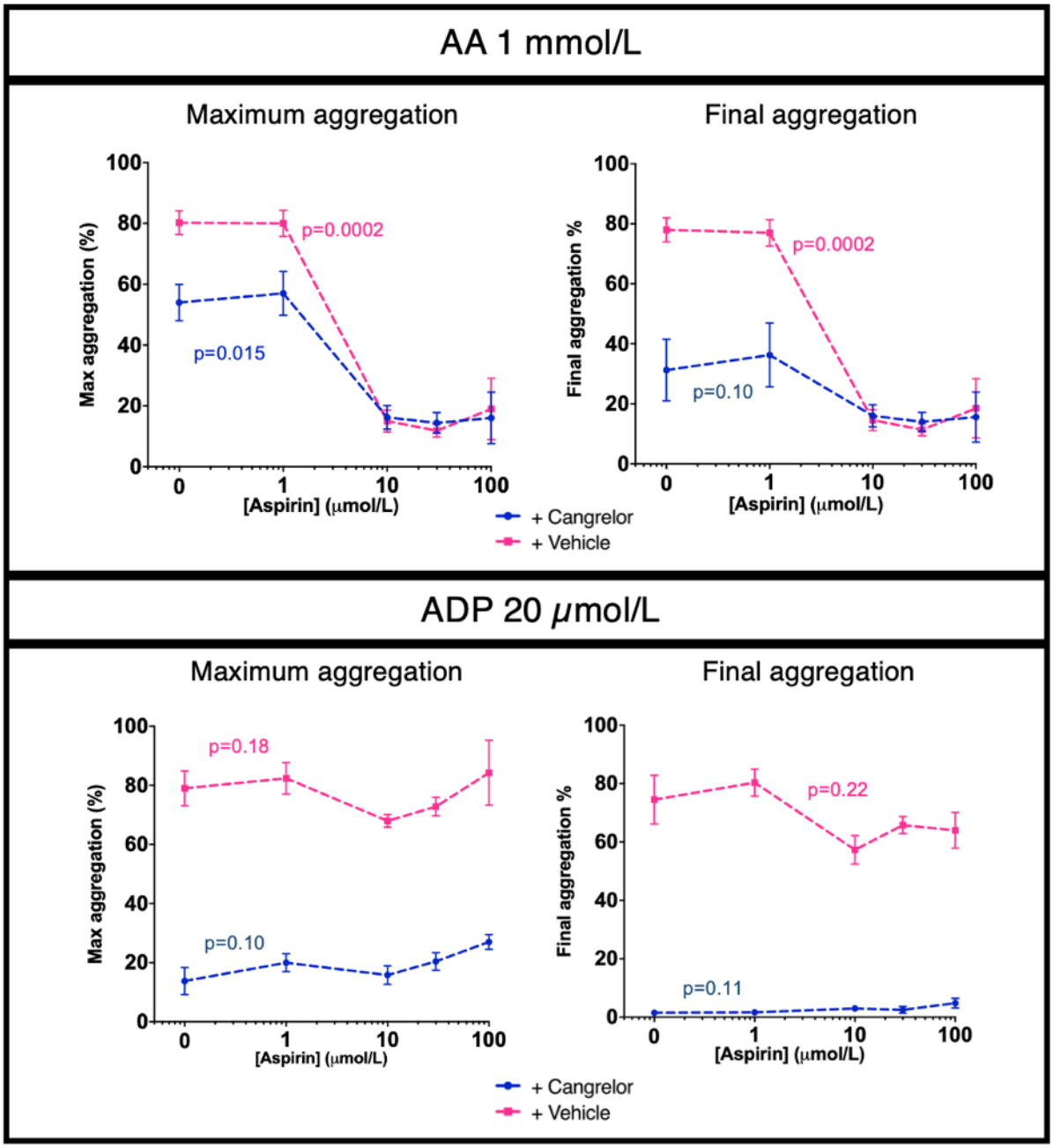


Figure 4.1 Concentration-response curves demonstrating aspirin's effect on final and maximum platelet aggregation responses using 1 mmol/L-arachidonic acid (AA) or 20 µmol/L-adenosine diphosphate (ADP) as agonists, in the presence of cangrelor (1 µmol/L) or vehicle (0.9% saline).

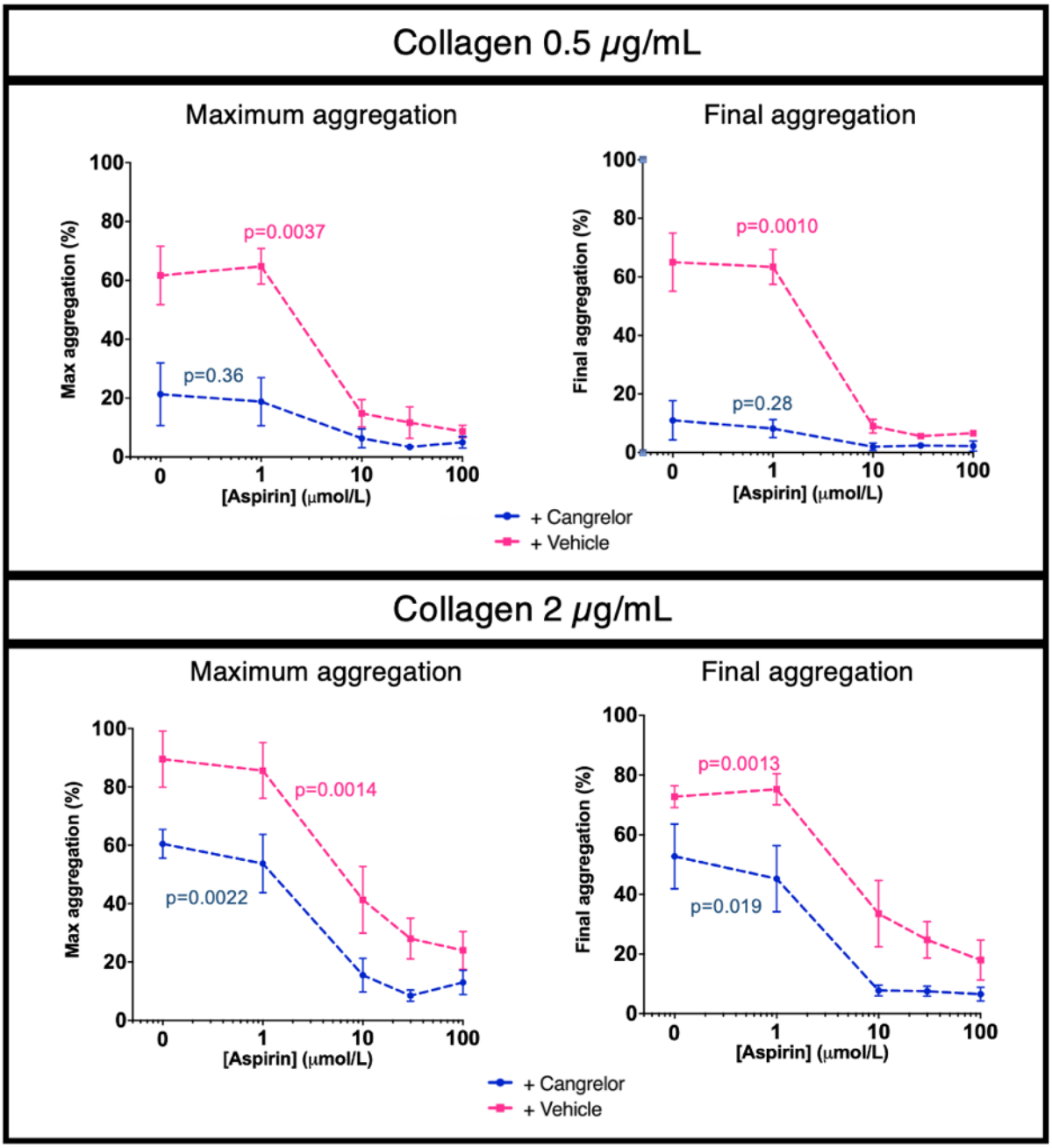


Figure 4.2 Concentration-response curves demonstrating aspirin's effect on final and maximum platelet aggregation responses using collagen (0.5 μg/mL or 2 μg/mL) as an agonist, in the presence of cangrelor (1 μmol/L) or vehicle (0.9% saline).

C. Discussion

It has been proposed that aspirin confers little additional antiplatelet effect in the presence of potent $P2Y_{12}$ inhibition (Armstrong et al. 2011). This assertion has largely been based on the results of aggregometry techniques that may not adequately simulate physiological levels of shear stress, such as microplate assays.

This study used the 'gold standard' method of determining platelet aggregation responses: LTA. The results of this study reassert the fact that, in more representative physiological shear stress conditions, aspirin offers significant additive effects of platelet inhibition on top of those conferred by potent $P2Y_{12}$ inhibition. This was particularly notable with respect to collagen-induced aggregation responses. As collagen is a key initiator of atherothrombosis and present at high levels during a plaque event, this may be a particularly important synergistic effect of aspirin and $P2Y_{12}$ inhibition.

Whilst of course clinical outcomes were not studied in these experiments, the results suggest that use of both aspirin and a potent $P2Y_{12}$ inhibitor, in combination, is likely to provide a stronger antithrombotic effect than either agent alone. This supports the hypothesis that for those patients at highest risk of ischaemic events, dual antiplatelet therapy is likely to offer a better degree of protection against atherothrombosis and its sequelae than SAPT; however, this must be balanced with any anti-haemostatic effects.

Aspirin exerted significant and reliable antiplatelet effect, including in the presence of cangrelor, at concentrations as low as 10 μmol/L. Reliable measurements of the aspirin concentration that platelets are exposed to in vivo is difficult to determine due to the fact that portal concentrations are likely to be higher than those in the systemic circulation (Pedersen and FitzGerald 1984). However, it has been estimated that maintenance therapy with 75 mg OD leads to a peak concentration of around 30 μmol/L (Warner et al. 2011). Furthermore, when studying aspirin's effect, aggregometry neglects to take into account inhibition of the release of endothelial prostacyclin, which has a very short half-life (Patrono et al. 2017).

Chapter 5: A study of very low dose twice-daily compared to standard low dose once-daily aspirin following acute coronary syndromes (the WILLOW ACS study)

A. Participant characteristics

Twenty-one participants were enrolled between July and December 2016. 1 participant withdrew consent before randomisation and therefore was replaced, as per the study protocol. 20 participants (16 males and 4 females) therefore proceeded to randomisation. The demographics and baseline clinical parameters are summarised in **Table 5.1**. Mean age (± SD) was 64.3 ± 11.9 years and BMI was 28.3 ± 3.9 kg/m^2. Blood pressure (BP) and pulse appeared well controlled by secondary preventative medications (mean systolic BP was 134.8 ± 20.1 mmHg and diastolic BP 71.7 ± 11.7 mmHg; pulse rate was 59.2 ± 5.6 beats per minute [bpm]).

Participants had previously experienced an ACS event, which was either STEMI (35%) or NSTEMI (65%), a mean of 123.8 ± 82.8 days prior to randomisation. Their ACS was managed either by PCI (80%) or medical therapy alone (20%). No patients had undergone CABG. 2 participants (10%) were current smokers, 11 (55%) had smoked in the past and 7 (35%) had never smoked. 3 (15%) had type 2 DM, 11 (55%) had hypertension and 9 (45%) hypercholesterolaemia. Key concomitant medications are summarised in table 4. 13 (65%) were receiving a regular proton pump inhibitor. No participant reported any use of additional aspirin or NSAIDs during the study medication periods.

B. Study conduct and compliance

There were no significant differences in the times of sampling blood or urine and measurement of bleeding time between the two regimens (**Table 5.2**). Both doses were taken for an average of at least 14 days (14.95 +/- 2.0 days [20 mg BD] vs. 14.80 +/- 1.47 days [75 mg OD], p=0.55). Time from the participant's last dose of ticagrelor to the pre-aspirin dose venepuncture was also similar (214.0 +/- 208.5 mins vs. 184.9 +/-160.2 mins, p=0.36). Compliance with both aspirin and ticagrelor doses was >99% for both regimens. There were no differences in vital signs between the regimens (**Table 5.1**).

Table 5.1. Baseline demographics of the 20 randomised patients. Where appropriate mean values ± SD have been quoted. ACE, angiotensin converting enzyme; ACS, acute coronary syndrome; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; CI, confidence interval; F, female M, male; NSTEMI, non-ST elevation myocardial infarction; PCI, percutaneous coronary intervention; PPI, proton pump inhibitor; STEMI, ST-elevation myocardial infarction; UA, unstable angina.

Category		**n**	**%**
Sex		16M:4F	80%M:20%F
Age (years)		64.3 ± 11.9	
Ethnic group	Caucasian	18	90%
	Black	2	10%
	Asian	0	0%
Height (cm)		171.4 ± 10.4	
Weight (kg)		83.6 ± 15.5	
BMI (kg/m^2)		28.3 ± 3.9	
Systolic BP (mmHg)		134.8 ± 20.1	
Diastolic BP (mmHg)		71.7 ± 11.7	
Pulse rate (bpm)		59.2 ± 5.6	
Diagnosis	STEMI	7	35%
	NSTEMI	13	65%
	Unstable angina	0	0%
Management	PCI	16	80%
	CABG	0	0%
	Medical	4	20%
Smoking	Current	2	10%
	Past	11	55%
	Never	7	35%
Diabetes mellitus		3	15%
Hypertension		11	55%
Hypercholesterolaemia		9	45%
Days from ACS event to randomisation		123.8 ± 82.8	
Medication			
Beta-blocker		17	85%
ACE inhibitor		14	70%
ARB		5	25%
Statin		19	95%
Regular nitrate		1	5%
Nicorandil		1	5%
PPI		13	65%

Table 5.2. Comparison of study medication period duration, sampling times and compliance between the 20 mg BD and 75 mg OD aspirin regimens. Values are shown as mean ± SD and p values were generated by paired t-tests. BD, twice daily; BP, blood pressure; bpm, beats per minute; hrs, hours; mmHg, millimetres of mercury; mins, minutes; OD, once daily.

Category	**Aspirin 20 mg BD**	**Aspirin 75 mg OD**	**p value**
Time from aspirin dose to post-aspirin venepuncture (mins)	124.7 ± 18.2	123.1 ± 10.2	0.68
Time from last ticagrelor dose to pre-aspirin venepuncture (mins)	214.0 ± 208.5	184.9 ± 160.2	0.36
Time from aspirin dose to bleeding time measurement (mins)	122.2 ± 21.0	122.8 ± 19.6	0.88
Time from aspirin dose to collection of urine sample (mins)	133.9 ± 24.7	136.2 ± 24.6	0.76
Time of aspirin dose on day of sampling from previous dose (hrs)	13.7 ± 2.4	26.2 ± 1.8	NA
Compliance with aspirin therapy (% of doses taken)	99.9 ± 0.7	100.0 ± 0.0	0.33
Compliance with ticagrelor therapy (% of doses taken)	100.0 ± 0.0	99.7 ± 1.3	0.33
Time on study medication (days)	14.95 ± 2.0	14.80 ± 1.47	0.55
Systolic BP (mmHg)	127.3 ± 12.9	128.8 ± 15.5	0.67
Diastolic BP (mmHg)	72.2 ± 10.2	71.1 ± 10.2	0.53
Heart rate (bpm)	60.7 ± 6.0	62.3 ± 10.3	0.49

C. Pharmacodynamic endpoints

I. Effects on thromboxane A_2 release

The principal stable metabolite of TXA_2, TXB_2, was measured in serum obtained before and after aspirin dosing on the last day of each study medication period. Results are summarised in **Table 5.3** and **Figure 5.1**.

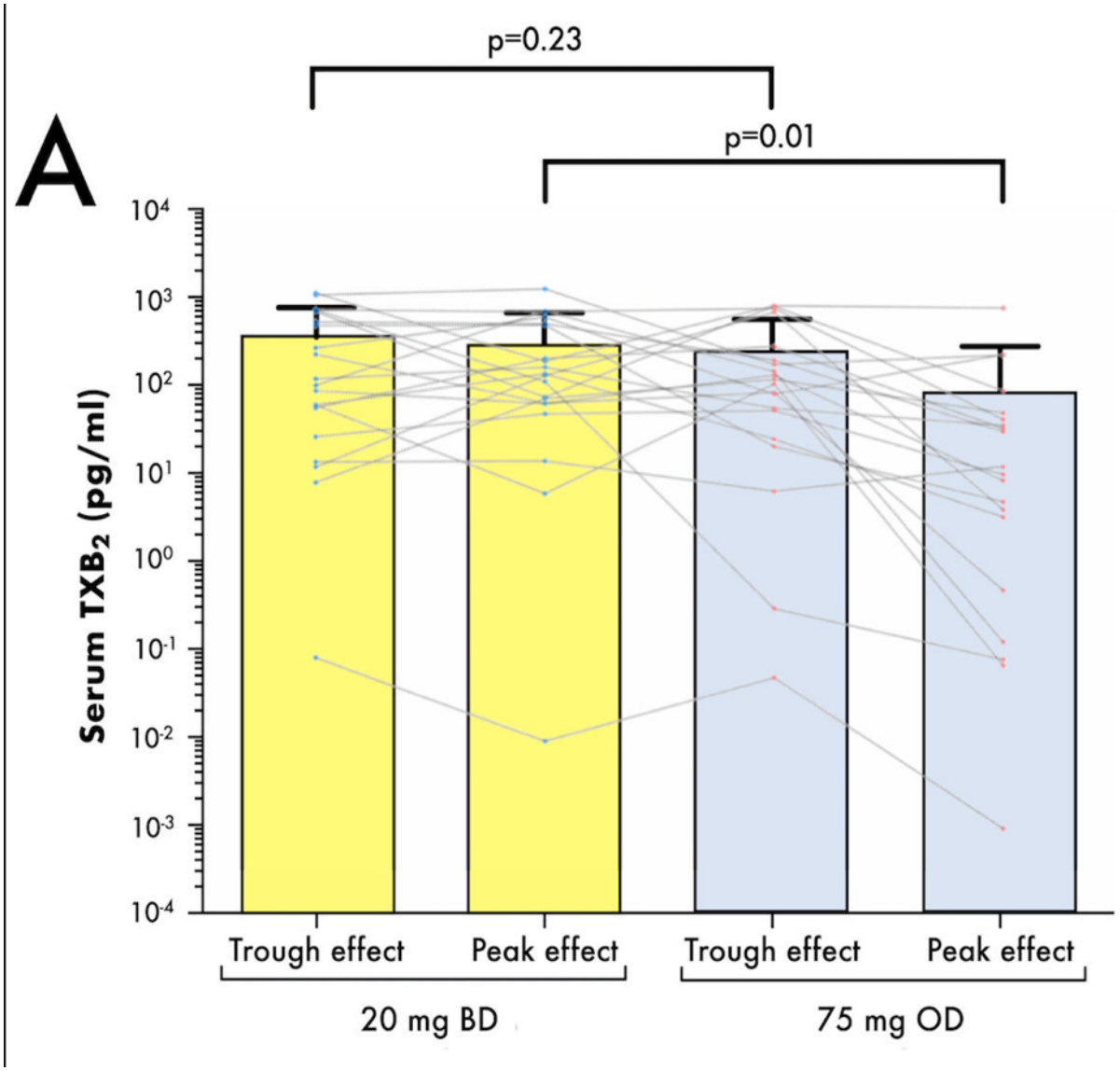
Serum TXB_2 was significantly greater post-dose when participants were receiving aspirin 20 mg BD compared to 75 mg OD (3.03 ± 3.64 ng/ml vs. 0.83 ± 1.93 ng/ml, p=0.018); however, there was no significant difference in pre-dose levels between the 2 regimens (3.51 ± 4.07 ng/ml vs. 2.48 ± 3.14 ng/ml, p=0.23).

Table 5.3 Pre- and post-dose serum TXB_2 in ACS patients receiving ticagrelor and aspirin 20 mg BD or 75 mg OD. Mean ± SD is shown.

Aspirin regimen		**sTXB$_2$ (ng/ml)**
20mg BD	Pre-dose	3.51 ± 4.07
	Post-dose	3.03 ± 3.64
75mg OD	Pre-dose	2.48 ± 3.14
	Post-dose	0.83 ± 1.93

p= 0.23

p= 0.01



BD, twice daily; mg, milligrams; ng/ml, nanograms per millilitre; OD, once daily; pg/ml. picograms per millilitre; $sTXB_2$, serum thromboxane B_2

Figure 5.1 Pre- and post-dose serum thromboxane B_2 in ACS patients receiving ticagrelor and aspirin 20 mg twice daily (BD) or 75 mg once daily (OD). Mean ± SD is shown. P values were generated using paired t-tests.

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II. Effects on urinary prostanoids

Urine was collected 2 hours after the last dose of each treatment period. Urinary TxM, which represents COX1 activity over a broader time range than serum TXB_2, showed no significant difference comparing the regimens (aspirin 20 mg BD: 430.0 ± 269.7 vs. 75 mg OD: 371.5 ± 176.6 pg/mg creatinine, p=0.17 for paired comparison). Urine PGI-M and 8-iso-$PGF_{2\alpha}$ levels did not significantly differ between the two groups (**Table 5.4, Figure 5.2**).

Table 5.4 Urinary levels of TX metabolite, PGI_2 metabolite 8-iso-$PGF_{2\alpha}$ measured at the end of each treatment period. Values quoted are mean ± SD. p values were generated using paired t-tests.

Parameter	20 mg BD	75 mg OD	p
Urinary TxM (pg/mg creatinine)	430.0 ± 269.7	371.5 ± 176.6	0.17
Urinary PGI-M (pg/mg creatinine)	109.9 ± 143.3	86.68 ± 54.58	0.41
Urinary 8-iso-$PGF_{2\alpha}$ (pg/mg creatinine)	2713 ± 1534	2834 ± 1945	0.77

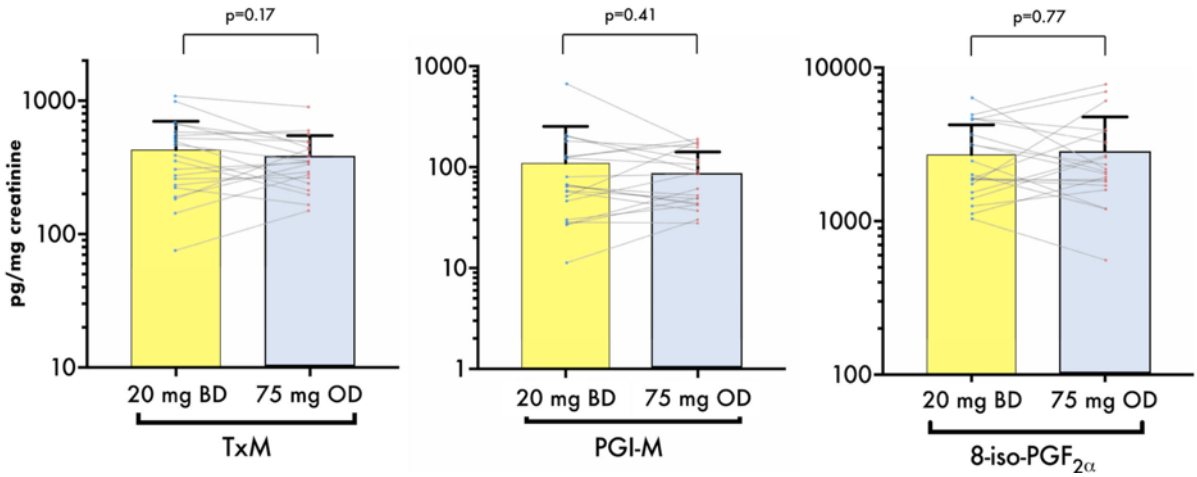

Figure 5.2 Urinary levels of TX metabolite, PGI_2 metabolite 8-iso-$PGF_{2\alpha}$ measured at the end of each treatment period. Bars represent mean + SD. Dots and lines represent paired values for the individual participants. P values shown were generated by paired t tests between the groups. Scale on the y axis is logarithmic. 8-iso-$PGF_{2\alpha}$, 8-iso prostaglandin $F_{2\alpha}$; BD, twice-daily; PGI-M, prostacyclin metabolite; OD, once-daily; TxM, thromboxane metabolite.

III. Effects on platelet aggregation responses

MA and FA responses to AA (0.1, 0.3, 1 mmol/L), collagen (1, 4, 16 μg/mL) and ADP (20 μmol/L) were assessed using LTA. Results are summarised in **Tables 5.5 and 5.6**, and in **Figures 5.3 and 5.4**.

There were no significant differences in AA or ADP-induced MA responses between the regimens. Post-dose (peak effect) MA responses to collagen 1 and 4 μg/mL (but not 16 μg/mL) were greater (when receiving aspirin 20 mg BD compared to 75 mg OD (**Table 5.5, Figure 5.3**). Pre-dose MA to collagen 1 μg/mL, but not 4 or 16 μg/ml, was greater when receiving the novel regimen (**Table 5.5, Figure 5.3**).

FA responses followed a broadly similar pattern (**Table 5.6, Figure 5.4**).

Table 5.5 Maximum platelet aggregation responses to AA, ADP and collagen assessed by LTA pre- and post-aspirin dose at the end of each treatment period. AA, arachidonic acid; ADP, adenosine diphosphate; BD, twice-daily; mg, milligrams; µg/ml, micrograms per milliliter; µM, micromolar; OD, once daily.

		Aspirin 20 mg BD				**Aspirin 75 mg OD**							
		Maximum aggregation (%)	Maximum aggregation (%)	Mean difference (Pre to Post)	p-value	Maximum aggregation (%)	Maximum aggregation (%)	Mean difference (Pre to Post, %)	p-value	Mean difference (20mg Pre to 75mg Pre, %)	p-value	Mean difference (20mg Post to 75mg Post, %)	p-value
Agonist	**Concentration**	**Pre-dose**	**Post-dose**			**Pre-dose**	**Post-dose**						
AA (mM)	0.1	2.53 (1.68 to 3.37)	2.30 (1.60 to 3.00)	-0.225 (-0.885 to 0.435)	0.484	2.35 (1.61 to 3.09)	2.55 (1.82 to 3.29)	0.237 (-0.350 to 0.824)	0.408	-0.175 (-0.892 to 0.542)	0.616	0.211 (-0.4287 to 0.8497)	0.498
	0.3	2.15 (1.54 to 2.76)	2.08 (1.32 to 2.84)	-0.075 (-0.608 to 0.458)	0.772	1.65 (0.96 to 2.34)	1.71 (1.28 to 2.15)	0.053 (-0.713 to 0.819)	0.887	-0.500 (-1.112 to 0.112)	0.104	-0.421 (-1.127 to 0.2851)	0.226
	1	8.68 (2.03 to 15.32)	8.48 (1.78 to 15.17)	-0.200 (-1.114 to 1.514)	0.754	6.60 (4.12 to 9.08)	5.11 (3.37 to 6.85)	-1.263 (-2.296 to -0.230)	0.019	-2.075 (-7.241 to 3.091)	0.411	-3.474 (-9.501 to 2.554)	0.242
ADP (µM)	20	44.63 (39.14 to 50.11)	40.85 (33.62 to 48.08)	-3.775 (-11.86 to 4.308)	0.341	41.50 (35.88 to 47.12)	40.55 (34.70 to 46.40)	-0.421 (-4.456 to 3.614)	0.829	-3.125 (-8.195 to 1.945)	0.213	-0.632 (-7.824 to 6.561)	0.856
Collagen (µg/ml)	1	26.33 (16.83 to 35.82)	16.45 (9.16 to 23.75)	-9.875 (-15.85 to -3.896)	0.003	15.45 (11.04 to 19.86)	9.03 (6.29 to 11.76)	-6.605 (-10.21 to -3.002)	0.001	-10.88 (-20.68 to -1.068)	0.032	-8.158 (-15.30 to -1.021)	0.027
	4	57.03 (49.69 to 64.36)	44.55 (33.63 to 55.47)	-12.48 (-23.35 to 1.599)	0.027	51.03 (42.54 to 59.51)	29.58 (22.40 to 36.76)	-21.42 (-26.12 to 16.73)	<0.0001	-6.00 (-14.64 to 2.639)	0.162	-15.55 (-26.34 to -4.763)	0.007
	16	72.08 (66.54 to 77.61)	65.63 (56.74 to 74.51)	-6.450 (-15.61 to 2.71)	0.157	73.48 (69.22 to 77.73)	58.37 (51.78 to 64.96)	-14.95 (-20.56 to -9.33)	<0.0001	1.40 (-4.141 to 6.941)	0.603	-7.71 (-17.40 to 1.976)	0.112

Table 5.6 Final platelet aggregation responses to AA, ADP and collagen assessed by LTA pre- and post-aspirin dose at the end of each treatment period. AA, arachidonic acid; ADP, adenosine diphosphate; BD, twice-daily; mg, milligrams; µg/ml, micrograms per millilitre; µM, micromolar; OD, once daily.

		Aspirin 20 mg BD				**Aspirin 75 mg OD**							
		Final aggregation (%)	Final aggregation (%)	Mean difference (Pre to Post)	p-value	Final aggregation (%)	Final aggregation (%)	Mean difference (Pre to Post, %)	p-value	Mean difference (20mg Pre to 75mg Pre, %)	p-value	Mean difference (20mg Post to 75mg Post, %)	p-value
Agonist	**Concentration**	**Pre-dose**	**Post-dose**			**Pre-dose**	**Post-dose**						
AA (mM)	0.1	1.23 (0.194 to 2.26)	1.8 (-0.11 to 3.71)	0.575 (-0.734 to 1.884)	0.369	1.10 (0.327 to 1.873)	1.63 (0.658 to 2.61)	0.553 (-0.165 to 1.271)	0.123	-0.125 (-0.97 to 0.72)	0.760	-0.263 (-2.14 to 1.62)	0.772
	0.3	0.785 (0.049 to 1.501)	0.825 (0.104 to 1.55)	0.05 (-0.365 to 0.465)	0.804	0.075 (-0.662 to 0.812)	0.237 (-0.034 to 0.508)	0.158 (-0.696 to 1.01)	0.702	-0.70 (-1.46 to 0.061)	0.069	-0.632 (-1.438 to 0.175)	0.117
	1	7.75 (1.29 to 14.21)	7.55 (1.00 to 14.10)	-0.20 (-1.635 to 1.235)	0.774	5.38 (2.75 to 8.00)	4.13 (2.11 to 6.15)	-1.00 (-2.18 to 0.18)	0.091	-2.375 (-7.56 to 2.81)	0.349	-3.474 (-9.2 to 2.25)	0.219
ADP (µM)	20	23.68 (16.13 to 31.22)	25.90 (18.22 to 33.58)	2.23 (-6.39 to 10.84)	0.595	20.40 (14,16 to 26.64)	19.16 (11.95 to 26.36)	-0.579 (-5,25 to 4.09)	0.797	-3.275 (-8.28 to 1.73)	0.186	-7.342 (-14.34 to -0.342)	0.041
Collagen (µg/ml)	1	17.95 (9.51 to 26.40)	11.05 (4.81 to 17.29)	-6.90 (-11.86 to -1.94)	0.009	9.70 (6.96 to 12.44)	6.00 (3.89 to 8.14)	-3.632 (-6.04 to -1.23)	0.005	-8.25 (-16.79 to 0.288)	0.057	-5.632 (-11.4 to 0.138)	0.055
	4	51.80 (43.71 to 59.89)	40.00 (29.15 to 50.85)	-11.80 (-22.13 to -1.468)	0.027	45.50 (37.07 to 53.93)	24.74 (18.37 to 31.10)	-20.66 (-25.08 to -16.24)	<0.0001	-6.3 (-14.64 to 2.04)	0.130	-16.00 (-25.97 to 6.03)	0.003
	16	67.85 (61.56 to 74.14)	63.68 (54.92 to 72.43)	-4.18 (-13.27 to 4.92)	0.349	71.13 (66.78 to 75.47)	56.26 (49.40 to 63.13)	-14.68 (-20.34 to -9.028)	<0.0001	3.275 (-3.06 to 9.610	0.292	-7.90 (-17.57 to 1.78)	0.104

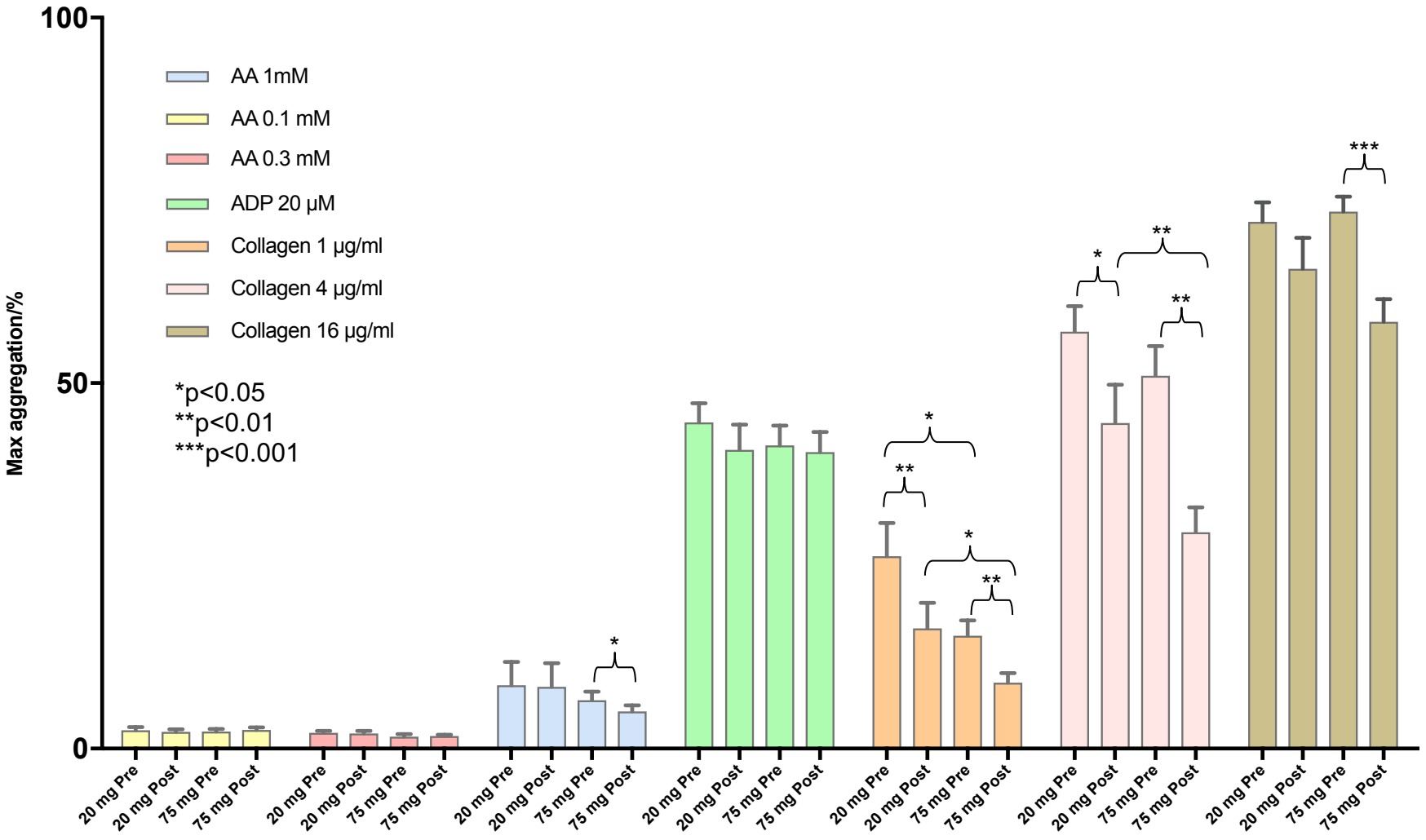

Figure 5.3 Maximum platelet aggregation responses to AA, ADP and collagen assessed by light transmittance aggregometry pre- and post-aspirin dose at the end of each treatment period.

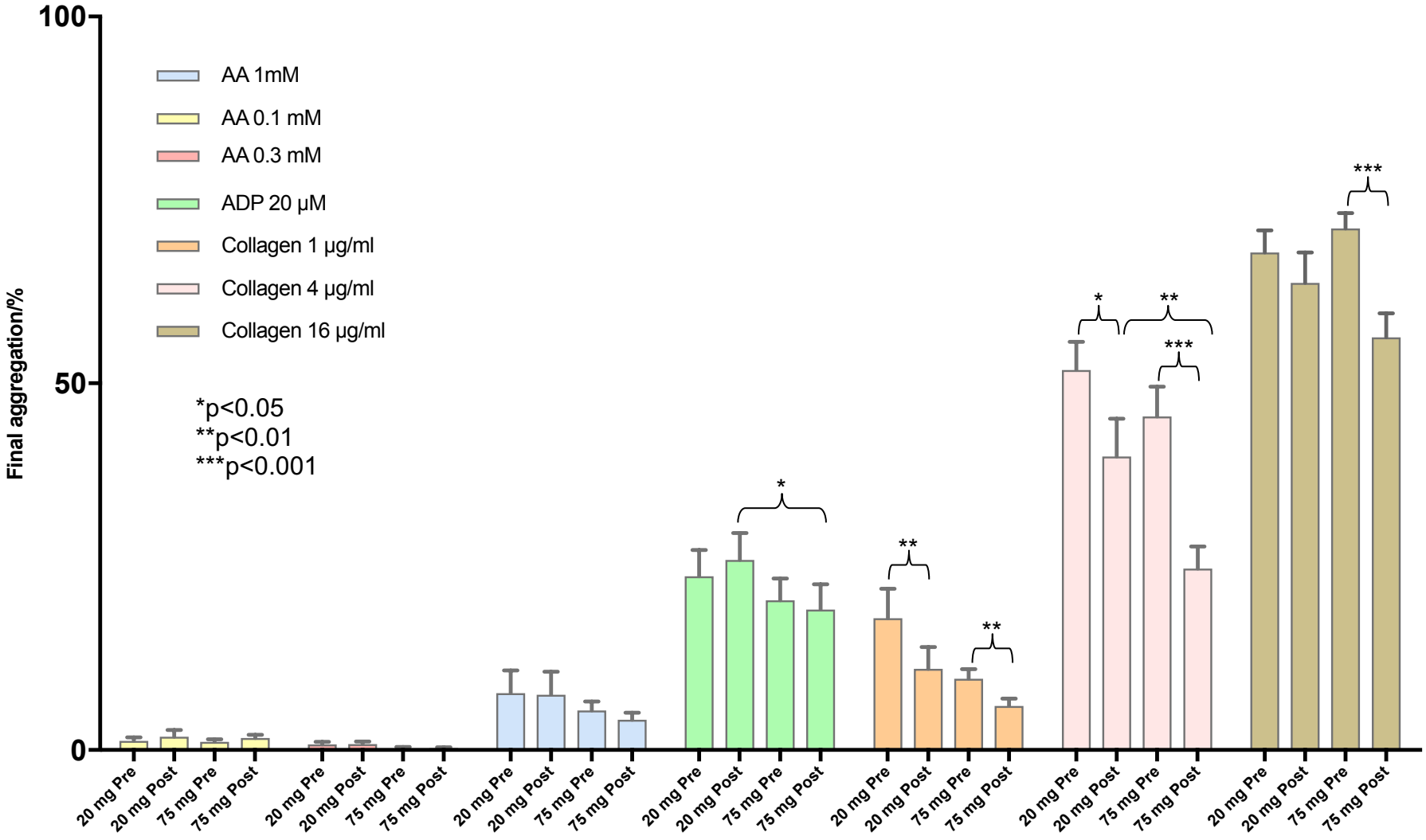

Figure 5.4 Maximum platelet aggregation responses to AA, ADP and collagen assessed by light transmittance aggregometry pre- and post-aspirin dose at the end of each treatment period.

Post-dose bleeding time was measured using a standard lancet method on the last day of each study medication treatment period (**Figure 5.5**).

Mean bleeding time was significantly shorter when participants were receiving aspirin 20 mg BD compared to 75 mg OD (679.5 ± 305.5 seconds vs. 833.9 ± 385.7 seconds, p=0.04) with a mean reduction of 154.5 seconds (95% CI 6.5 to 302.4).

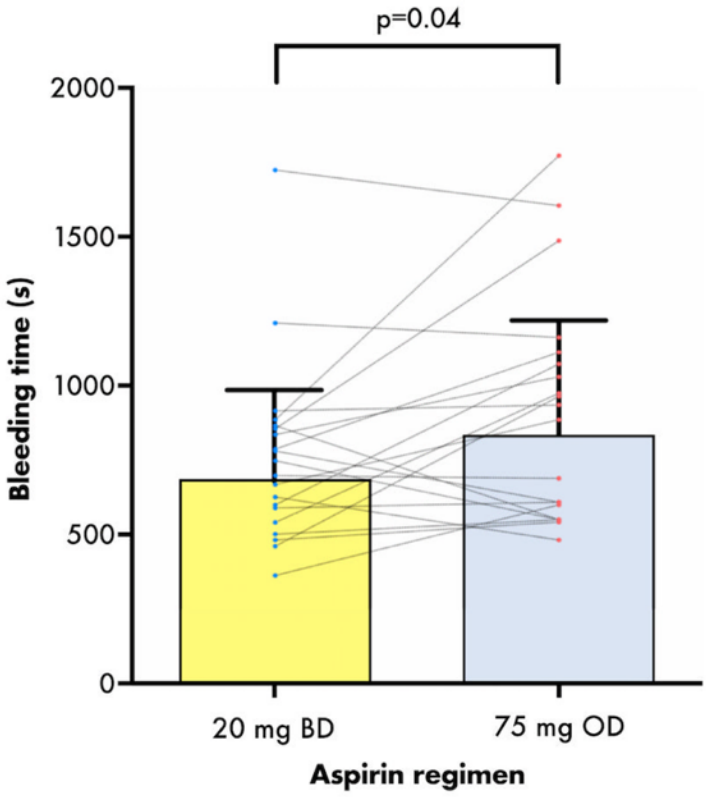

Figure 5.5 Post-dose bleeding time measured in ACS patients receiving ticagrelor and aspirin 20 mg BD or 75 mg OD. Mean ± SD is shown.

D. Safety data

During the study, there were no PLATO-defined life-threatening major, other major or minor bleeding events. In addition, no serious adverse events occurred during the study.

All adverse events were recorded during each treatment period and are summarised in **Table 5.7**. No participant stopped study medication prematurely.

Table 5.7 Adverse events occurring during each treatment period.

	Aspirin regimen		Severity	Serious?
	20 mg BD (n=20)	75 mg OD (n=20)		
ADVERSE EVENTS				
Possibly related to study medication				
Epistaxis (not requiring medical attention)	0	1	Mild	No
Spontaneous cutaneous bruising	0	1	Mild	No
Unlikely to be related to study medication				
Non-cardiac chest pain*	1	0	Mild	No
Pedal oedema	0	1	Mild	No
Lower respiratory tract infection	0	1	Moderate	No

*In addition, 1 participant developed non-cardiac chest pain (secondary to trauma) after transition back to standard-of-care aspirin 75 mg OD, which was noted at the telephone follow-up visit.

E. Discussion

DAPT with aspirin and ticagrelor represents standard maintenance antithrombotic therapy that is recommended as first-line treatment following ACS (Roffi et al. 2015; Windecker et al. 2014; Steg et al. 2012). Typically given for at least 1 year, continuation for longer remains an effective strategy for preventing MACE in high-risk patients but comes at the price of increased bleeding (Bonaca et al. 2015). Even though the overall balance of mortality risks appears to favour use of longer-term ticagrelor-based DAPT in high-risk patients (Bhatt et al. 2016; Bonaca, Bhatt, Storey, et al. 2016; Bansilal et al. 2018), clinicians and patients alike may be reluctant to extend DAPT therapy due to the bleeding risk. It has been proposed that aspirin can safely be stopped in ticagrelor-treated patients with a history of PCI; however, even if ticagrelor monotherapy is effective in preventing stent thrombosis, there are likely to remain a significant group of patients at high risk of ongoing native plaque rupture events in whom DAPT is needed to optimize protection against future MACE.

Strategies maintaining the combined antithrombotic effect of DAPT, whilst reducing bleeding tendency, therefore have the potential to improve overall clinical outcomes. This study showed that a novel regimen of very-low-dose BD aspirin given to ticagrelor-treated ACS patients broadly maintained the inhibitory effects of aspirin on TXA_2 synthesis and arachidonic acid-induced platelet activation, but reduced peak inhibition and was associated with a significant reduction in bleeding time (**Figure 5.6**). It remains to be determined whether this would translate into a reduction in clinical bleeding events, but similar doses have previously been shown to have clinical antithrombotic efficacy. The European Stroke Prevention Study 2 investigated a similar very-low-dose, BD aspirin regimen (25 mg BD) alone or in combination with another antiplatelet drug (dipyridamole) in 6,602 stroke patients, showing a significant benefit of BD aspirin, alone or in combination, vs. placebo in preventing recurrent cerebrovascular events (Diener et al. 1996). Giving aspirin BD may also improve symmetry of DAPT effect when given with ticagrelor, which is also given BD, and may simplify drug intake, including the possibility of developing a combination tablet. This might help to address the under-recognised issue of treatment compliance that can limit the efficacy of treatment strategies in coronary artery disease patients (Du et al. 2017), and can be improved by reducing the number of tablets they receive (Castellano et al. 2014).

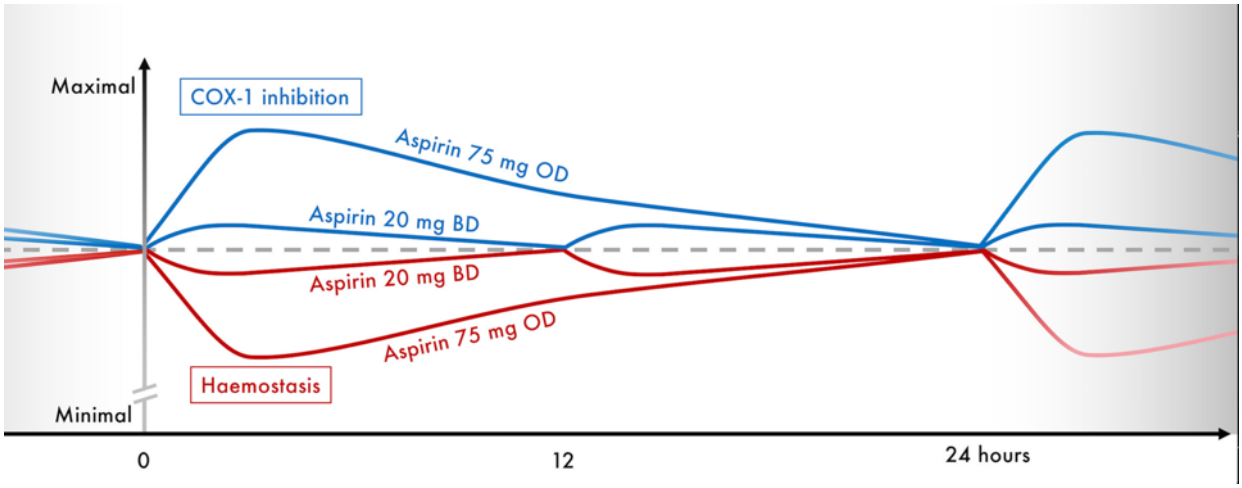

Figure 5.6 Illustrative figure showing differential profiles of COX1 inhibition over 24 hours provided by maintenance aspirin doses of 75 mg OD and 20 mg BD in ticagrelor-treated patients.

The use of very-low-dose BD aspirin in this setting is only considered feasible when given in combination with ticagrelor which, in contrast to older drugs, provides potent and reliable $P2Y_{12}$ inhibition (Joshi et al. 2014). $P2Y_{12}$ inhibitors reduce AA-induced platelet aggregation independently of aspirin (Armstrong et al. 2011), meaning there may be a rationale for reducing the intensity of aspirin therapy. However, the results of this study also illustrate the fact that aspirin continues to provide additional antiplatelet effects even in the presence of potent $P2Y_{12}$ inhibition, given that we saw differences in TX-related biomarkers and platelet function between the two dosing regimens at 2-hours post-dose. This is consistent with the in vitro studies reported in chapter 4, and with previous studies of the relationship between aspirin and $P2Y_{12}$ receptor inhibition or deficiency (Scavone et al. 2016). Hence, there is justification for an approach that continues to include aspirin but seeks to reduce its intensity to improve haemostasis whilst maintaining adequate levels of platelet inhibition.

In pre-dose samples, there were similar levels of overall platelet inhibition with the two regimens, but it is possible there may be differences at the platelet level in the pattern of inhibition between the two regimens at this timepoint given that some newly-formed platelets will be more rapidly exposed to aspirin with the BD regimen. Even a small number of uninhibited platelets can form the basis for thrombosis (Hoefer et al. 2015). However, the clinical efficacy and safety of a BD aspirin regimen remains to be explored.

The study was limited by a small sample size and therefore was unable to determine whether the very-low-dose aspirin regimen significantly improves in vivo PGI_2 biosynthesis: this would require assessment in a larger study. The goal of the current study was to provide reassurance from a pharmacodynamic study that such a larger study is appropriate. Similarly, it only compared one novel regimen with standard therapy, rather than including multiple permutations; however, the results suggest that aspirin 20 mg BD and ticagrelor 90 mg BD achieve the goal of reducing peak-trough variation in effect and improving haemostasis.

In conclusion, this study suggests that aspirin dose modification represents a novel and feasible strategy to be investigated for optimising the balance of antithrombotic benefits and bleeding-related risks in ticagrelor-treated ACS patients, and demands further study to determine whether this translates into improvements in net clinical outcomes.

Chapter 6: Supplementary analyses from the WILLOW ACS study

This chapter details the results of post-hoc analyses carried out on existing data from the WILLOW ACS trial or those generated from additional sample analyses. These were intended to be only exploratory in nature but proved invaluable in generating hypotheses for further prospective study. Interpretation of unadjusted p values reflects this.

A. Haematology parameters

Data from one participant was excluded from this analysis because of a history of chronic lymphocytic leukaemia with a raised baseline leukocyte count.

I. Haemoglobin

There was no evidence of a significant difference in haemoglobin between dosing regimens or sampling timepoints (p value for all pairwise comparisons >0.05) (**Figure 6.1**).

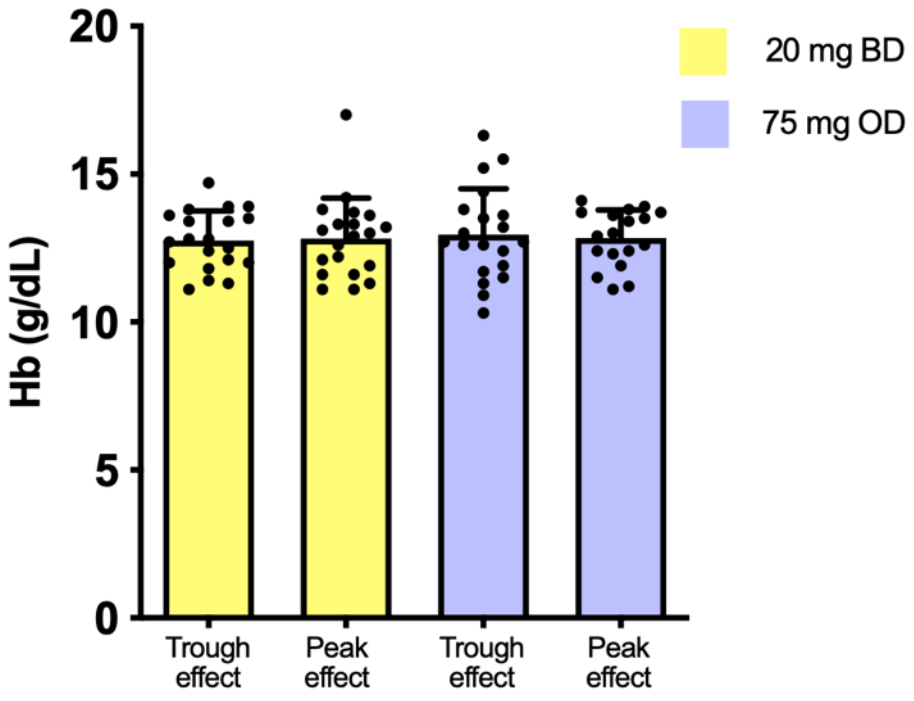

Figure 6.1 Haemoglobin levels at each sampling timepoint during treatment with each dosing regimen.

II. Leukocyte counts

a) Total leukocyte count

Mean leukocyte count was significantly greater at peak aspirin effect than trough effect when receiving 75 mg OD (p=0.018) but not 20 mg BD (p=0.60) (**Figure 6.2**). Furthermore, at peak effect, leukocyte count was lower when receiving 20 mg BD than 75 mg OD (p=0.002, **Figure 6.3**).

b) Leukocyte subset counts

To explore the differences in leukocyte count further, subset data were analysed. There were no significant differences between the regimens in peak effect (post-dose) neutrophil, lymphocyte or mixed (including monocytes, eosinophils and basophils) cell counts when measured as cells per litre (**Figure 6.4**) or % of leucocytes present (**Figure 6.5**). However, there was some evidence of a trend when receiving aspirin 75 mg OD, compared with 20 mg BD, towards higher proportions of neutrophils, but this narrowly missed the level of statistical significance (p=0.06).

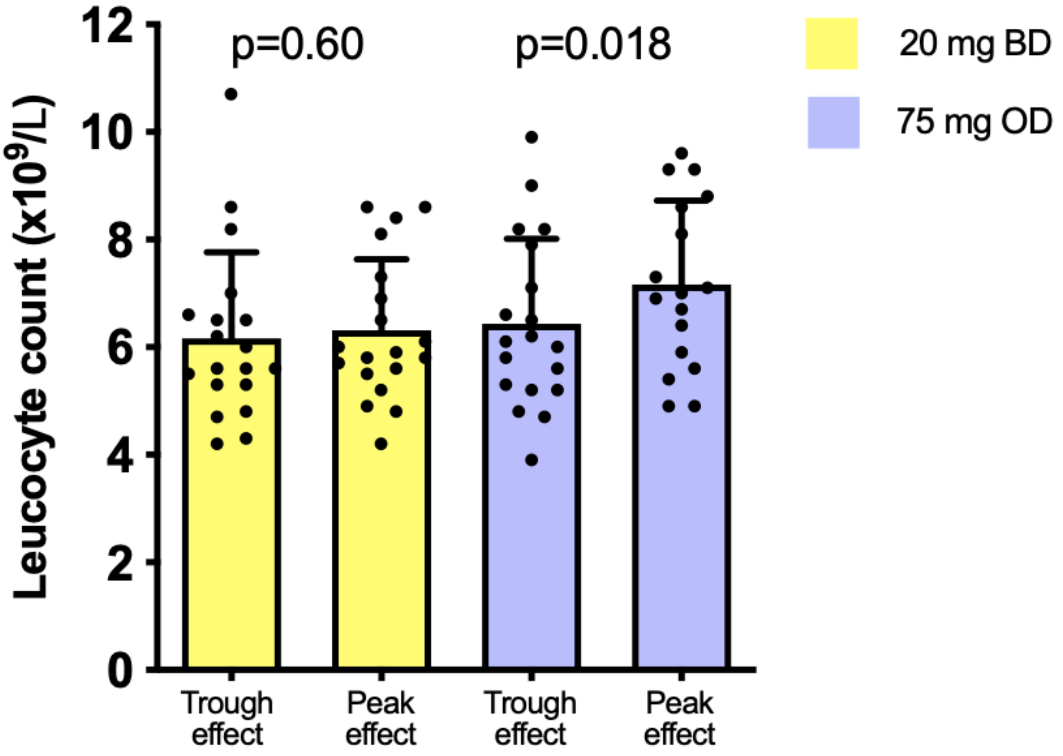

Figure 6.2 Total circulating leukocyte count at each sampling timepoint during treatment with each dosing regimen. P-values were generated by paired t-tests. Bars represent mean + SD.

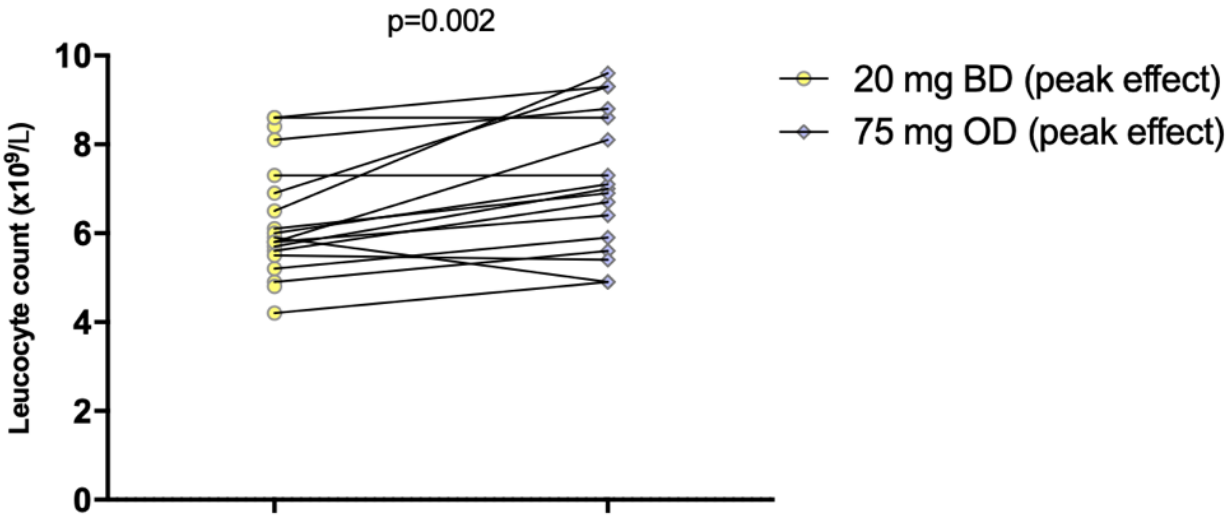

Figure 6.3 Paired data showing total circulating leukocyte count during peak drug effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P-value was generated using a paired t-test.

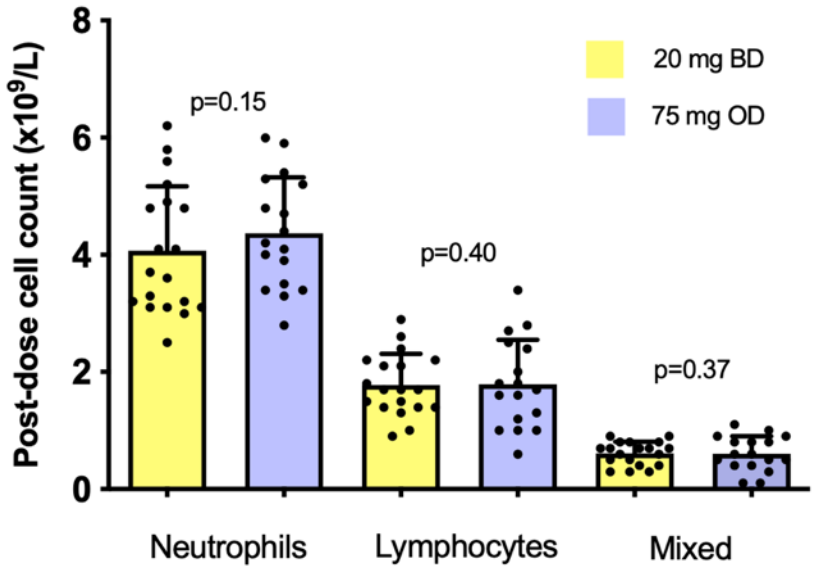

Figure 6.4 Circulating neutrophil, lymphocyte and mixed (monocyte, eosinophil and basophil) counts during peak drug effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P-values were generated using paired t-tests. Bars represent mean + SD.

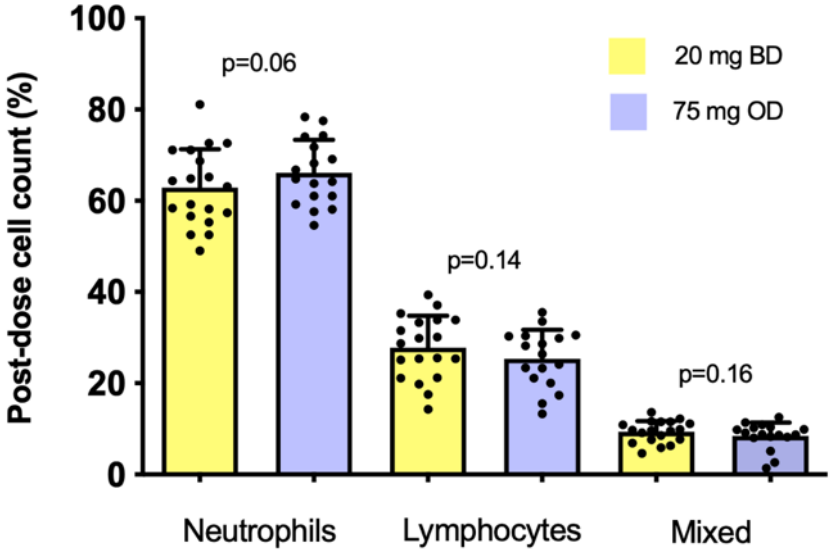

Figure 6.5 Circulating neutrophil, lymphocyte and mixed (monocyte, eosinophil and basophil) counts (expressed as proportion of total leukocyte count) during peak drug effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P-values were generated using paired t-tests. Bars represent mean + SD.

III. Markers of platelet turnover

a) Platelet count

At peak aspirin effect, there were no significant differences in platelet count between the timepoints for each regimen though there was a trend towards reduction of platelet count between trough and peak effect when receiving aspirin 20 mg BD (p=0.068) but not 75 mg OD (p=0.34, **Figure 6.6**). Moreover, platelet count checked at peak effect was significantly lower when receiving aspirin 20 mg BD compared with 75 mg OD (p=0.048, **Figure 6.7**).

b) Mean platelet volume

There were no differences between mean platelet volume (MPV) measurements between timepoints when receiving aspirin 75 mg OD. Whilst there was no significant difference between trough and peak effect MPV when receiving 20 mg BD, there was a trend towards higher levels at the latter timepoint (p=0.074, **Figure 6.8**).

c) Platelet distribution width

There were no differences in platelet distribution width between the timepoints for either regimen. However, platelet distribution width was significantly greater when receiving aspirin 20 mg BD compared to 75 mg OD, assessed at peak aspirin effect (p=0.039, **Figure 6.10**).

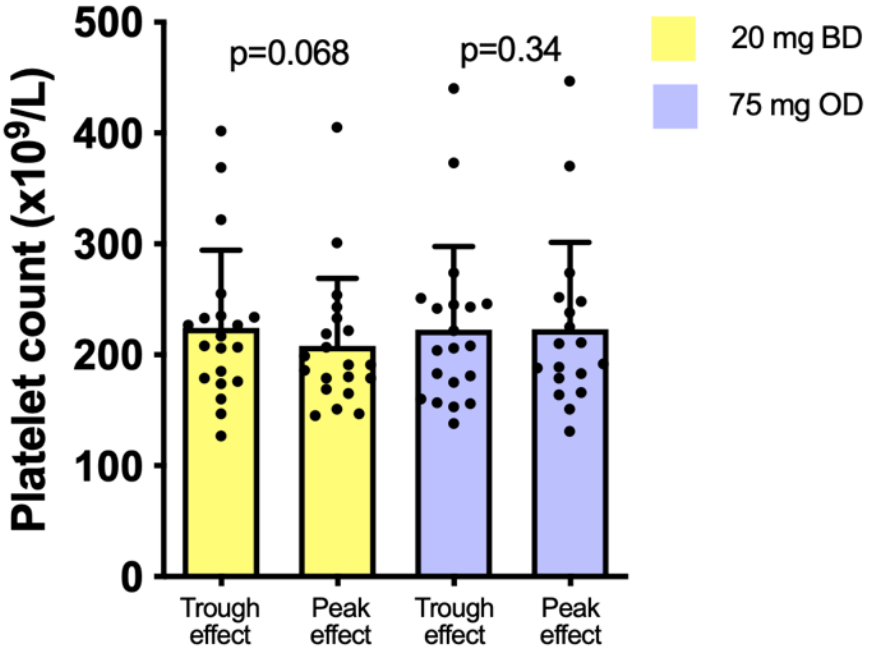

Figure 6.6 Circulating platelet count at trough and peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P values represent the results of Wilcoxon matched pair tests. Bars represent mean + SD.

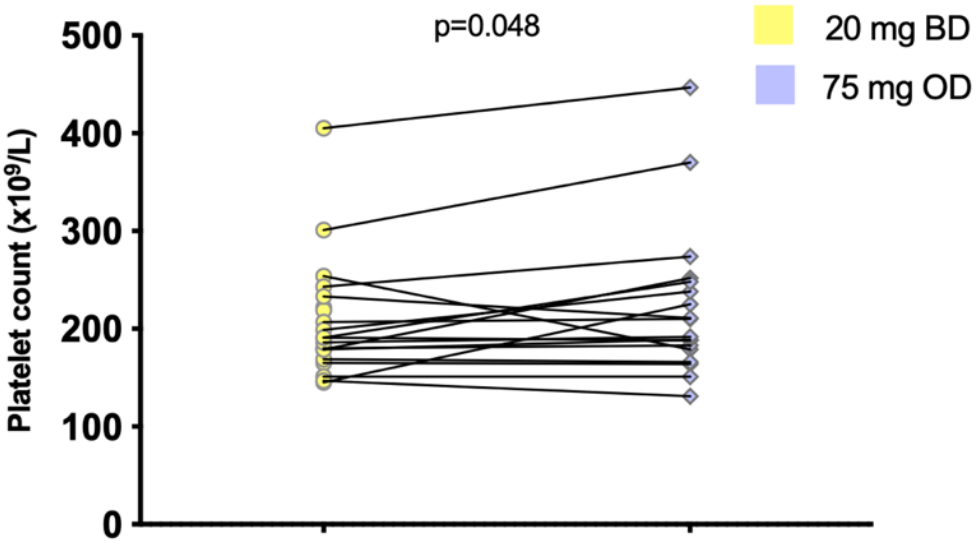

Figure 6.7 Circulating platelet count at peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P value represent the results of a Wilcoxon matched pair test.

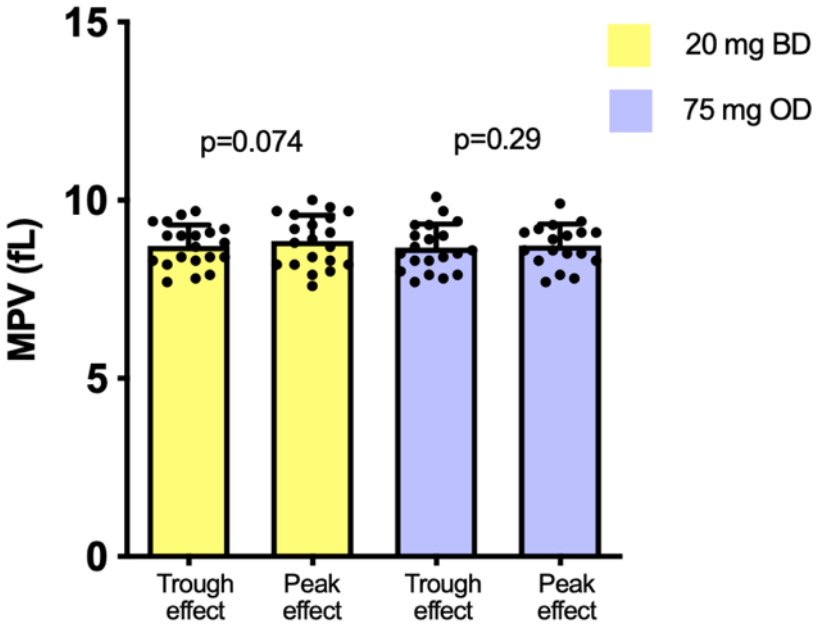

Figure 6.8 Mean platelet volume (MPV) at trough and peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P values represent the results of paired t-tests. Bars represent mean + SD.

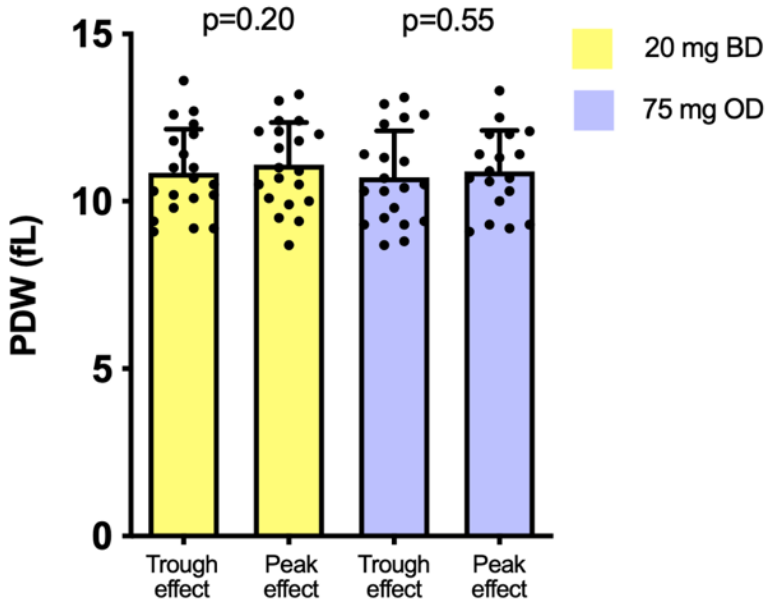

Figure 6.9 Platelet distribution width (PDW) at trough and peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P values represent the results of paired t-tests. Bars represent mean + SD.

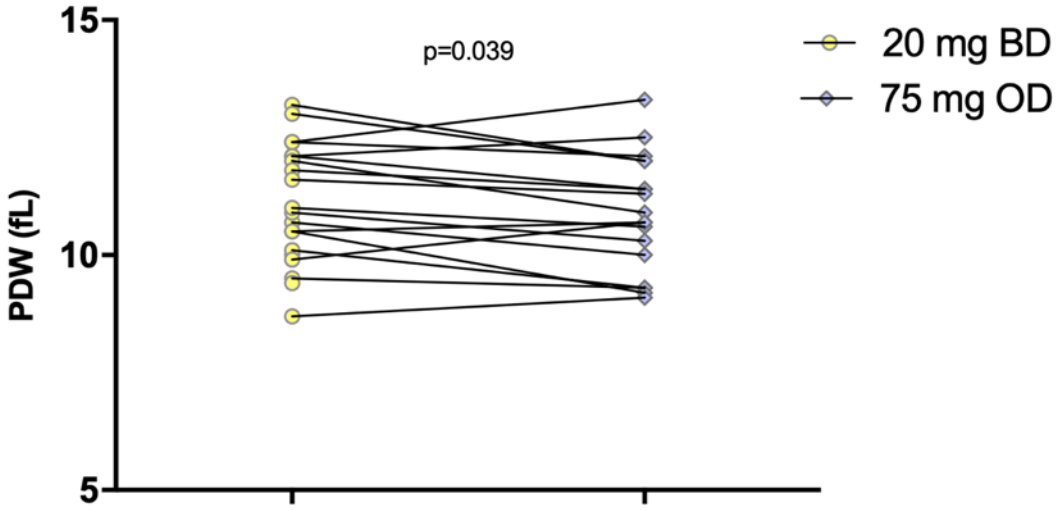

Figure 6.10 Platelet distribution width (PDW) at peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P value represent the results of a paired t-test.

B. Pro-inflammatory cytokines

Data from one participant was excluded from these analyses because of a clinically significant lower respiratory tract infection (bacterial, requiring oral antibiotics) that developed during study treatment.

I. Plasma interleukin-6

Mean plasma levels of IL-6 increased between trough and peak effect when receiving either regimen (**Figure 6.11**). This was statistically significant in the case of aspirin 75 mg OD (p=0.016) but not 20 mg BD (p=0.090). There was no significant difference between trough effect levels between the regimens (p=0.24), but there was a trend towards lower levels of IL-6 at peak effect when receiving aspirin 20 mg BD compared with 75 mg OD that narrowly missed the threshold for statistical significance (p=0.052, **Figure 6.12**). There was a statistically significant interaction between timepoint and regimen when assessed using two-way repeated measures ANOVA (p=0.03).

II. Plasma tumour necrosis factor α

Mean plasma TNF-α was not significantly different between timepoints or regimens though, contrary to that observed for IL-6, there was a trend towards reduced levels at peak aspirin effect compared to trough effect when receiving aspirin 75 mg OD. There were no significant differences between the regimens (**Figure 6.13**).

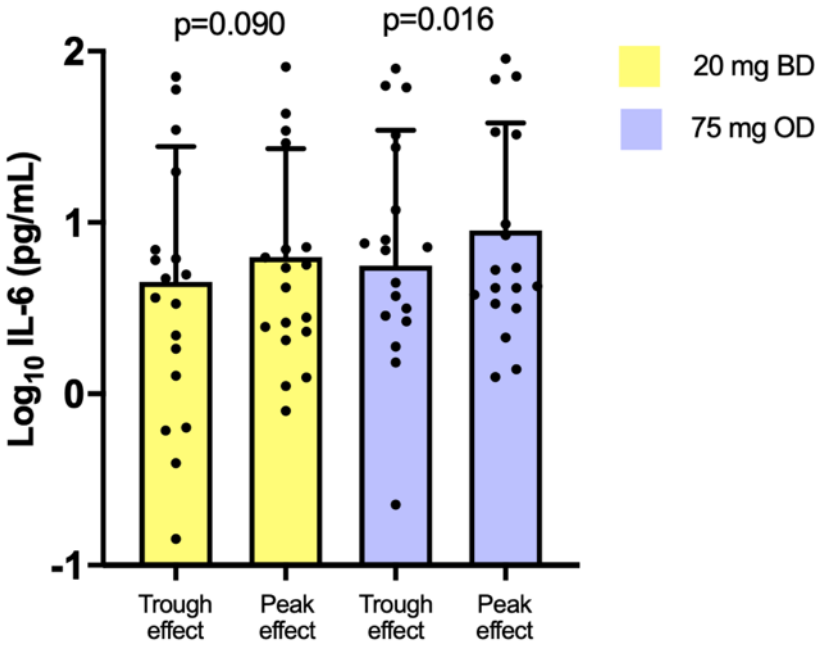

Figure 6.11 Plasma interleukin (IL)-6 levels at trough and peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P values represent the results of paired t-tests. Bars represent mean + SD.

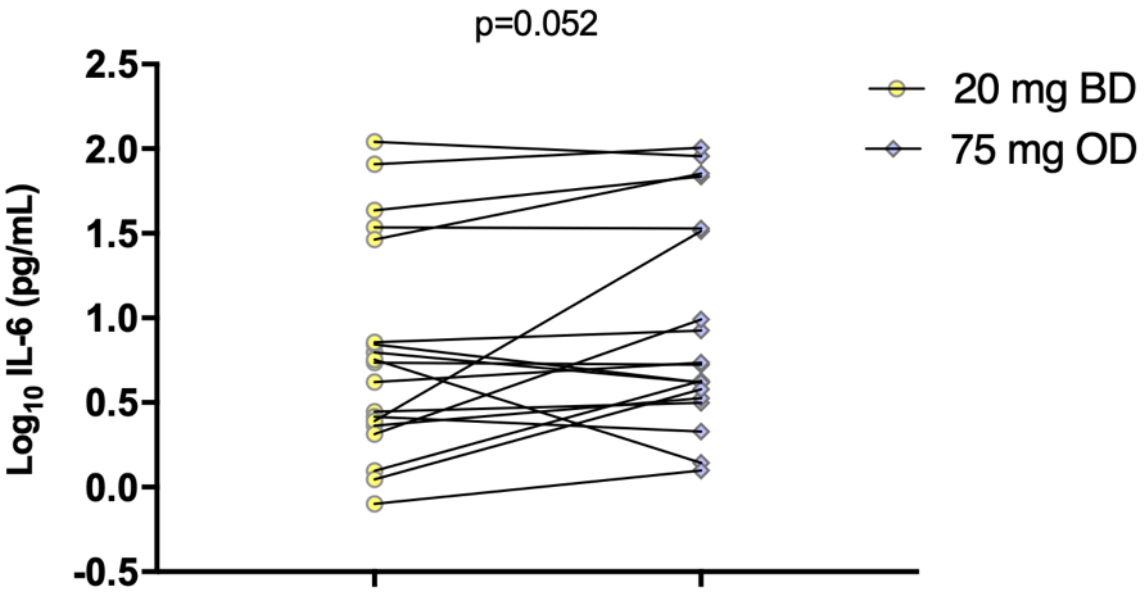

Figure 6.12 Plasma interleukin (IL)-6 level at peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P value represent the results of a paired t-test.

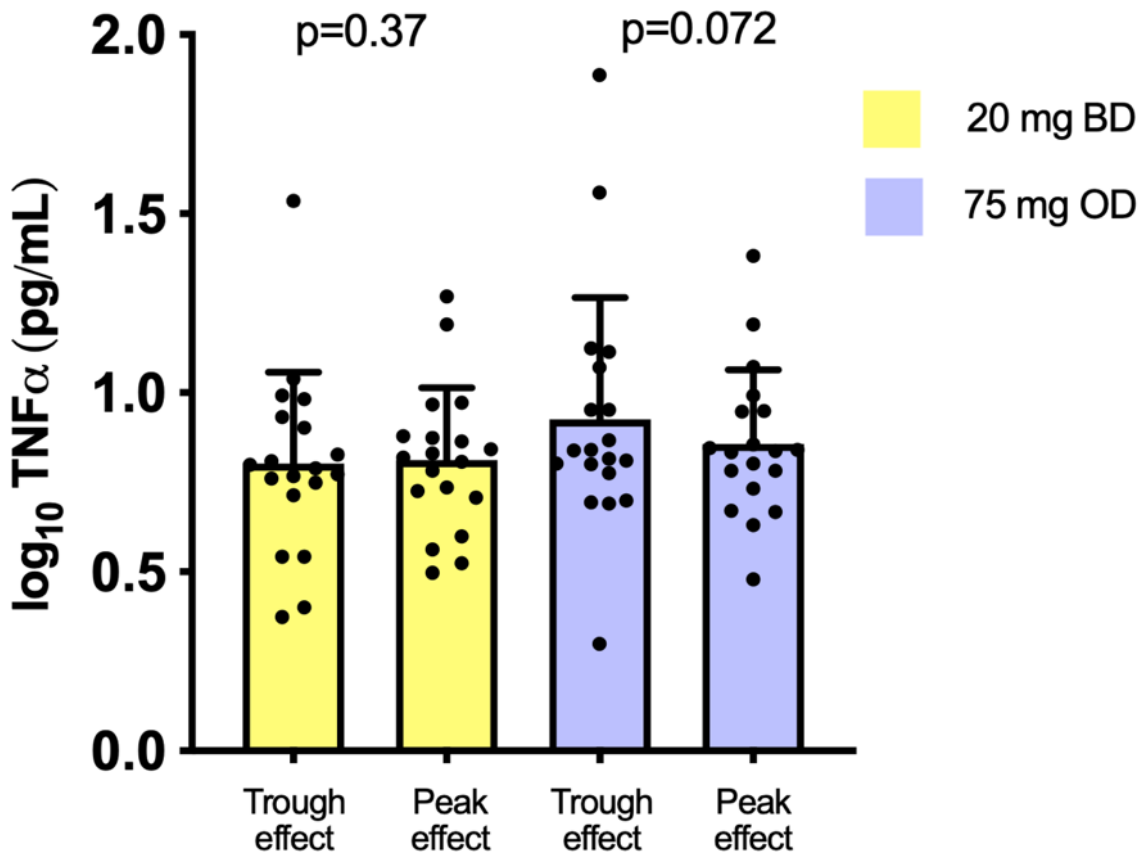

Figure 6.13 Plasma tumour necrosis factor (TNF) α levels at trough and peak aspirin effect when ticagrelor-treated ACS patients were receiving aspirin 20 mg BD or 75 mg OD. P values represent the results of paired t-tests. Bars represent mean + SD.

III. High-sensitivity C-reactive protein

Serum hsCRP was measured in serum obtained from participants 2 hours after the last dose of each treatment period. When receiving aspirin 20 mg BD, mean hsCRP was lower than when receiving 75 mg OD, though this did not reach the statistical level of significance (p=0.083, **Figure 6.14**). However, when stratified by whether hsCRP was <1 mg/L (normal) or ≥1 mg/L (raised) whilst receiving aspirin 75 mg OD, hsCRP appeared to significantly lower during treatment with aspirin 20 mg BD in those with raised levels (p=0.023), but not those with normal levels (p=0.13, **Figure 6.15** and **6.16**). A further observation was that those participants with DM appeared to have a greater tendency to have higher hsCRP than those without DM, and similarly hsCRP seemed to be reduced when taking aspirin 20 mg BD compared to 75 mg OD, though statistical analysis was not performed due to the small numbers in the diabetes group.

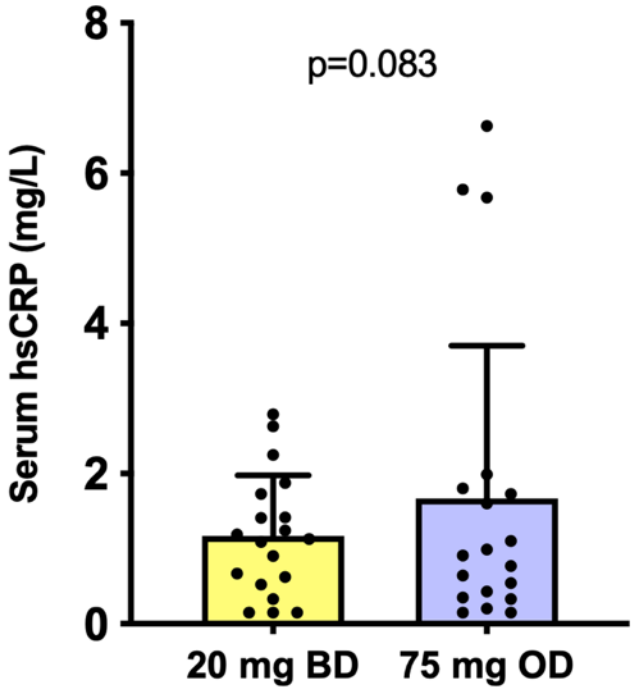

Figure 6.14 Serum high sensitivity C-reactive protein (hsCRP) measured in ticagrelor-treated ACS patients during maintenance treatment with aspirin 20 mg BD or 75 mg OD. P value generated using Wilcoxon matched pair test. Bars represent mean + SD.

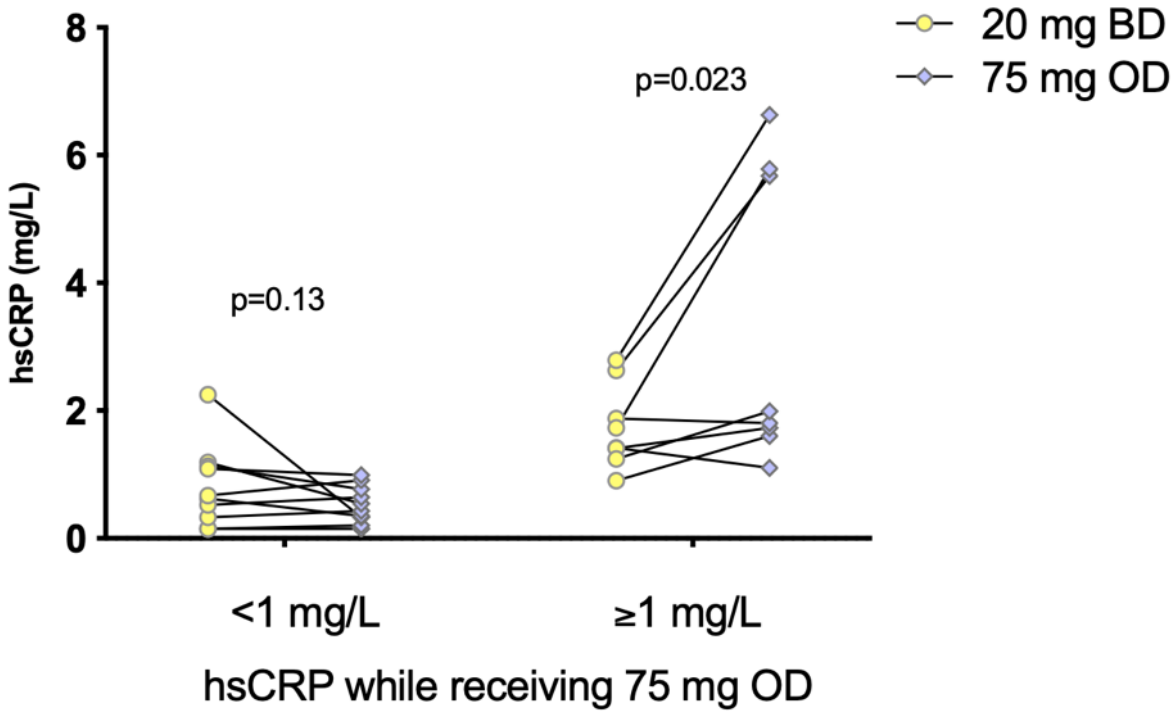

Figure 6.15 Serum high sensitivity C-reactive protein (hsCRP) measured in ticagrelor-treated ACS patients during maintenance treatment with aspirin 20 mg BD or 75 mg OD, stratified by hsCRP level when receiving 75 mg OD. P values generated using Wilcoxon matched pair tests.

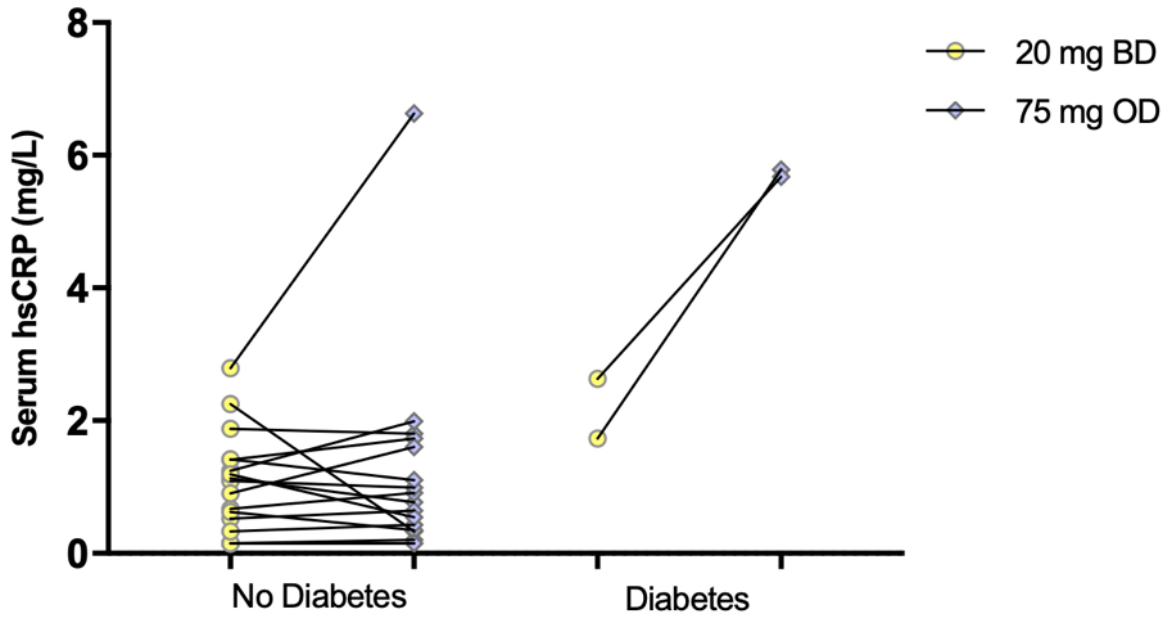

Figure 6.16 Serum high sensitivity C-reactive protein (hsCRP) measured in ticagrelor-treated ACS patients during maintenance treatment with aspirin 20 mg BD or 75 mg OD, stratified by diabetes status.

C. Fibrin clot dynamics

Aspirin is known to affect fibrin clot dynamics (Ajjan et al. 2009; Antovic et al. 2005). Hypofibrinolysis is an independent predictor of MACE after an ACS event (Sumaya et al. 2018). To study the effects of the two aspirin regimens on clot formation and lysis, high-throughput turbidimetric analysis was performed. There were no significant differences in the studied parameters between the aspirin dosing regimens (**Table 6.1, Figure 6.17**).

Table 6.1 Dynamics of fibrin clot formation and lysis in ticagrelor-treated ACS patients receiving maintenance therapy with aspirin 20 mg BD or 75 mg OD, assessed by turbidimetry. Values represent mean ± SD. P values were generated using paired t-tests. AU, absorbance units.

	20 mg BD	**75 mg OD**	**p value**
PRE-DOSE (TROUGH EFFECT)			
Maximum turbidity (AU)	0.410 ± 0.089	0.397 ± 0.087	0.51
Lag time (s)	475.5 ± 86.8	494.7 ± 107.6	0.70
Lysis time (s)	759.9 ± 334.3	745.3 ± 470.0	0.97
POST-DOSE (PEAK EFFECT)			
Maximum turbidity (AU)	0.394 ± 0.089	0.411 ± 0.111	0.52
Lag time (s)	496.3 ± 107.5	500.8 ± 78.94	0.87
Lysis time (s)	680.7 ± 313.1	618.6 ± 151.4	0.63

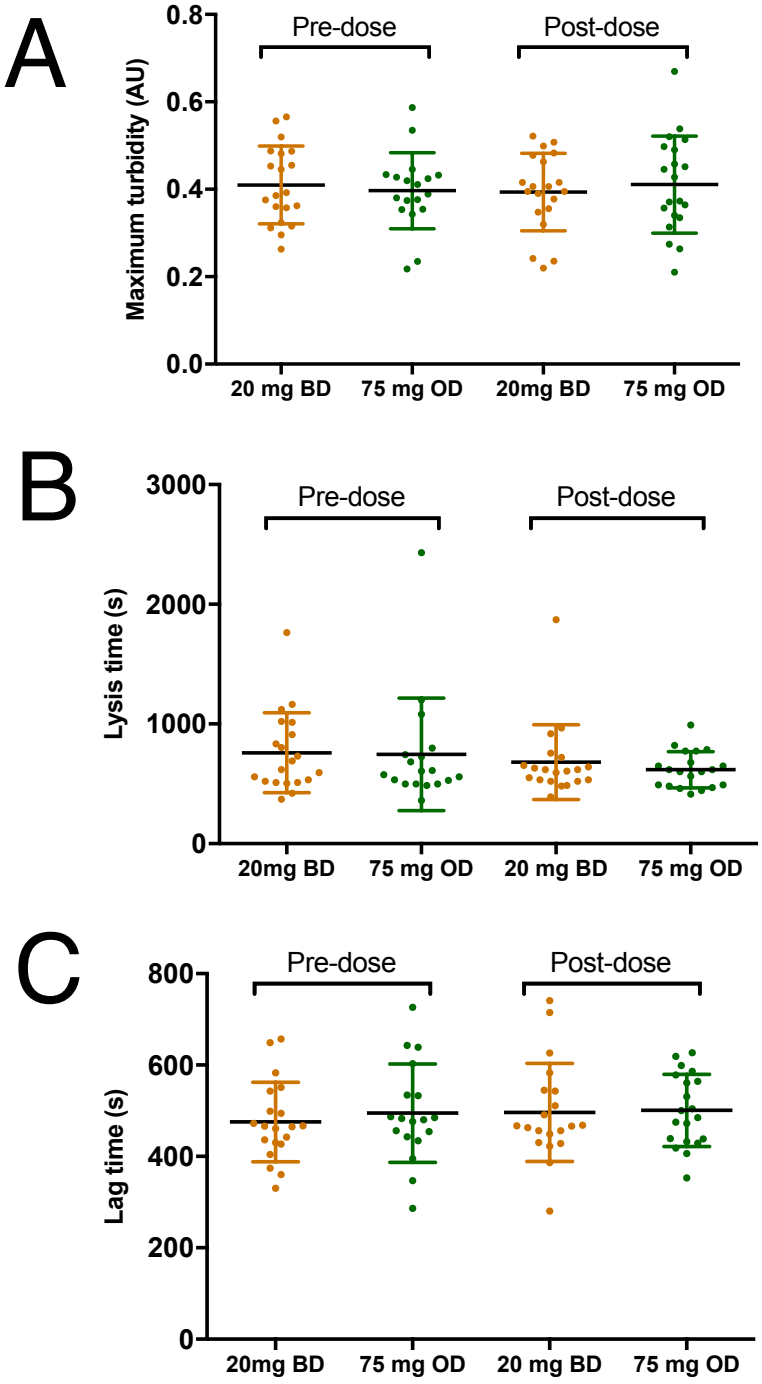

Figure 6.17 Maximum turbidity (A), lysis time (B) and lag time (C) of fibrin clot in ticagrelor-treated ACS patients receiving aspirin 20 mg BD or 75 mg OD, assessed by turbidimetry. Bars represent mean ± SD. No comparisons between 20 mg BD (pre-dose) and 75 mg (pre-dose), or between 20 mg BD (post-dose) and 75 mg OD (post-dose) showed a significant difference. AU, absorbance units; s, seconds.

D. Markers of renal function

Data from one participant with chronic kidney disease stage 4 (associated with marked elevation of serum creatinine) was excluded from this analysis.

I. Serum creatinine

Aspirin, like other NSAIDs, can cause elevations in serum creatinine, representing an underlying reduction in the glomerular filtration rate, including at a dose of 75 mg OD (Segal et al. 2003). Serum creatinine was significantly lower when receiving aspirin 20 mg BD compared to 75 mg OD (p=0.048, **Figures 6.18, 6.19**).

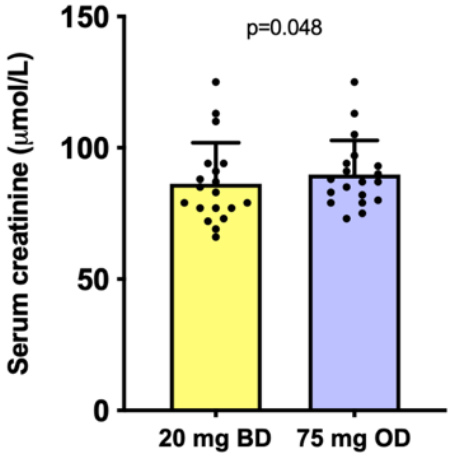

Figure 6.18 Serum creatinine measured in ticagrelor-treated ACS patients during maintenance therapy with aspirin 20 mg BD or 75 mg OD. P value generated using a paired t-test. Bars represent mean + SD.

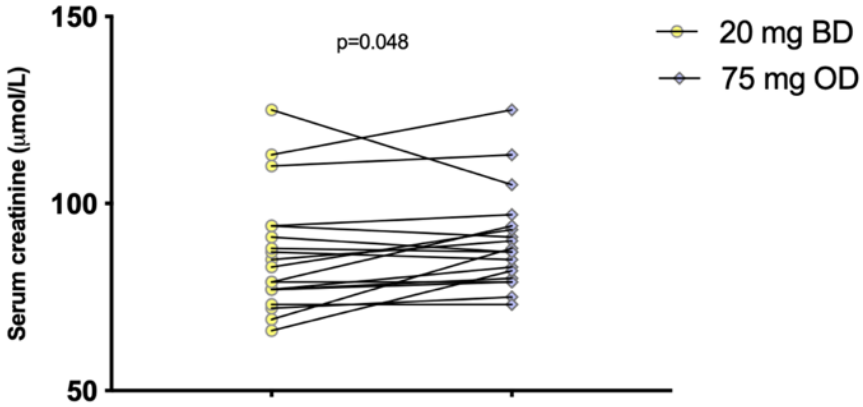

Figure 6.19 Paired measurements of serum creatinine in ticagrelor-treated ACS patients during maintenance therapy with aspirin 20 mg BD or 75 mg OD. P value generated using a paired t-test.

II. Serum uric acid

Both aspirin and ticagrelor can increase circulating uric acid levels (Wallentin et al. 2009; Zhang et al. 2014). There was no evidence of a significant difference in serum uric acid levels between the two dosing regimens (p=0.80, **Figure 6.20**).

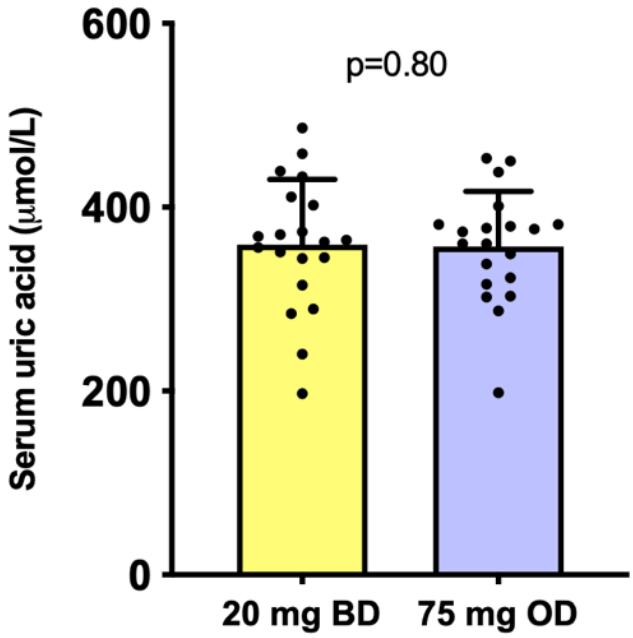

Figure 6.20 Serum uric acid measured in ticagrelor-treated ACS patients during maintenance therapy with aspirin 20 mg BD or 75 mg OD. P value generated using a paired t-test. Bars represent mean + SD.

E. Interaction of body weight and BMI with thromboxane suppression

Study participants had a median enrolment weight of 86 kg (range 55 - 106) and BMI of 28.6 kg/m^2 (22.6 - 37.1). There were no significant correlations between body weight or BMI and TxM or PGI-M when receiving either aspirin regimen (**Figure 6.21**). This suggests the efficacy of the novel regimen was maintained across the range of weight and BMI.

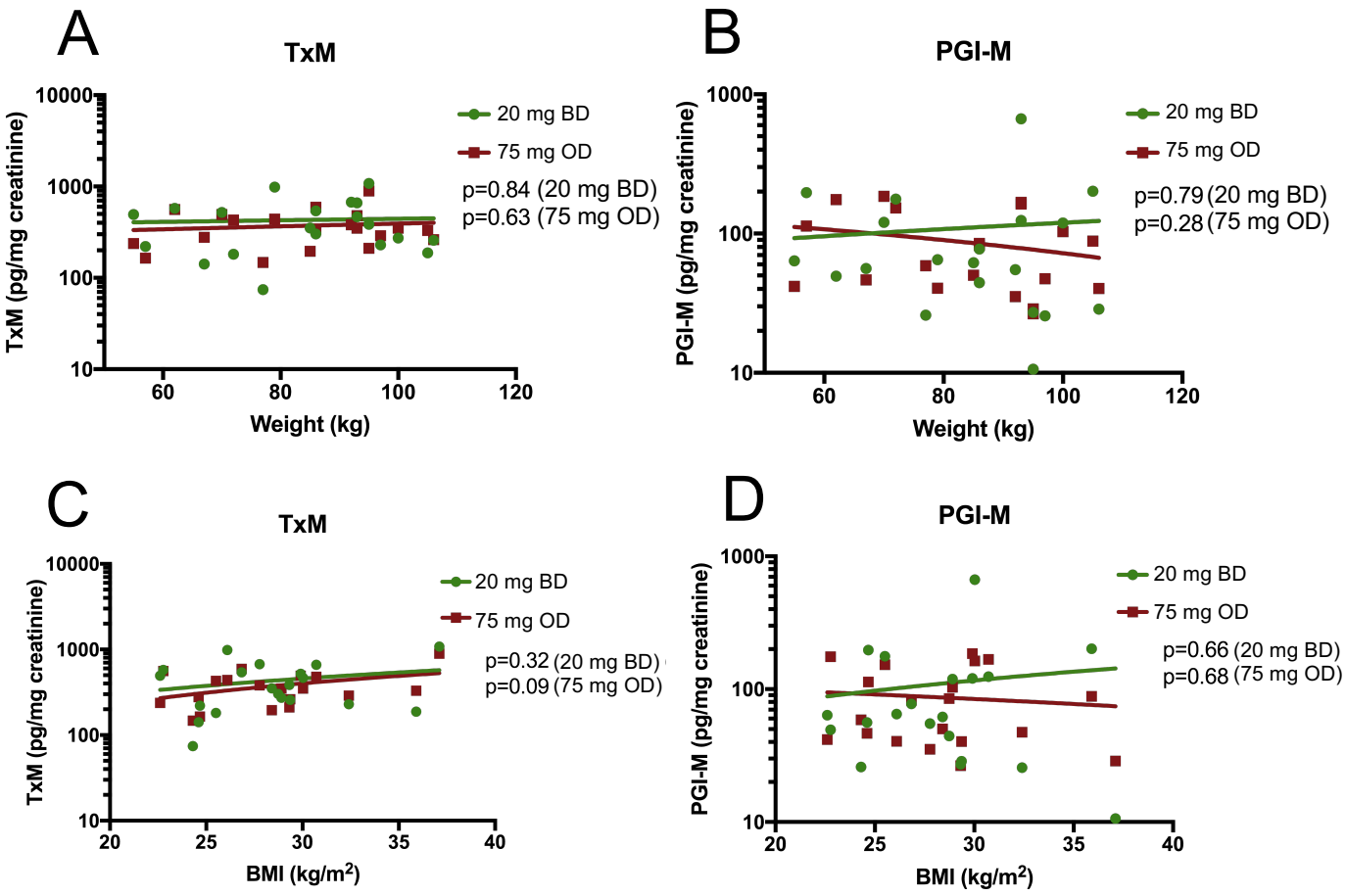

Figure 6.21 Correlation between body weight and urine TX metabolite (TxM) (panel A) or PGI_2 metabolite (PGI-M) (panel B); and body mass index (BMI) and TxM (panel C) or PGI-M (panel D) in ticagrelor-treated patients receiving aspirin 20 mg BD or 75 mg OD, assessed by linear regression.

F. Discussion

The post-hoc, exploratory analyses detailed in this chapter provided insights into the wider potential effects of aspirin dose modification in patients receiving DAPT for ACS and provoked hypotheses for further study.

The data relating to cell counts and cytokines show evidence of the lower dose aspirin regimen being associated with a lower inflammatory state with potential relevance to atherogenesis and atherothrombosis. The novel regimen was associated with significantly lower leukocyte count and platelet distribution width. There was also a trend towards lower levels of IL-6 and hsCRP, and greater MPV. Interestingly, the differences in hsCRP appeared to be only present in those with a high on-treatment level, which also included all the patients with diabetes included in the trial, though this was still a small number.

These data were obtained before the publication from Kiers et al (2017), which showed that maintenance treatment with aspirin 75 mg OD potentiated the response to endotoxaemia in healthy volunteers. In contrast to that study, we saw no significant differences in levels of TNF-α but levels of cytokines were generally very low. In the study by Kiers et al, concurrent ticagrelor 90 mg BD, which all of the patients in our study were receiving, abated the proinflammatory effect of aspirin on TNF-α, but not IL-6.

There was also evidence of a small but significant decrease in serum creatinine when receiving the novel regimen compared to standard-of-care. Aspirin, as an NSAID, can increase serum creatinine by reducing glomerular filtration rate, which may be associated with development and progression of chronic kidney disease, itself a major risk factor for IHD (Segal et al. 2003). The novel regimen, therefore, may hypothetically improve the renal risk profile of DAPT, thus potentially translating into reduced risk of MACE.

Given that the hyperuricaemic effect of aspirin has been reported to be inversely related to dose within the current therapeutic range (Zhang et al. 2014), it was reassuring that there was no evidence of a difference in uric acid levels between the regimens.

Similarly, as aspirin is known to have effects on fibrin clot parameters predictive of MACE in patients with ACS, it was reassuring that there was no evidence of a reduction in this potentially

important effect when receiving the novel regimen compared to standard-of-care.

There has been concern that patients with higher BMI may require greater doses of aspirin for sufficient effect when used as antiplatelet monotherapy (Rothwell et al. 2018; Rocca et al. 2018). However, we saw no significant correlation between either weight or BMI and COX1 inhibition in the present study. Furthermore, there were no significant differences in clinical outcomes between those with BMI ≥30 and <30 kg/m^2 or those above and below 81 kg in patients treated with low-dose aspirin in the PLATO or PEGASUS TIMI 54 studies, respectively, suggesting this may not be relevant when low-dose aspirin is used in combination with ticagrelor (Wallentin et al. 2009, Bonaca et al. 2015).

Whilst differences observed between trough and peak effect timepoints may be due to direct drug effects, particularly as plasma aspirin concentrations fall rapidly after absorption, it is also possible that this is due to or superimposed upon circadian variation in these factors (Keller et al. 2009). Further studies would require valid controls for this.

However, most importantly these findings led to the hypothesis that, as well as potentially beneficial effects on haemostasis, the novel regimen of DAPT might lead to reduced potentiation of the inflammatory response when compared to standard regimens and it was decided to study these in a robust and prospective manner during the next phase of the project.

Chapter 7: The WILLOW TREE study: baseline characteristics and physiological responses to endotoxaemia

A. Recruitment

Recruitment for the study opened in April 2019. At the point of halting of the trial on 16th March 2020 due to resource and infection control implications of the COVID-19 pandemic, 69 participants had been enrolled and 54 randomised. A total of 83 endotoxin challenges had been successfully completed, approximately evenly divided between the regimens (**Figure 7.1**).

B. Baseline characteristics

Baseline characteristics of the 46 participants who underwent at least one endotoxin challenge are shown in **Table 7.1**. These were assessed at enrolment, with the exception of bleeding time, which was measured at the randomisation visit (prior to receiving any study medication). For those who underwent at least one endotoxin challenge, there were no significant differences in baseline characteristics between those randomised to receive regimens containing no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (**Table 7.2**).

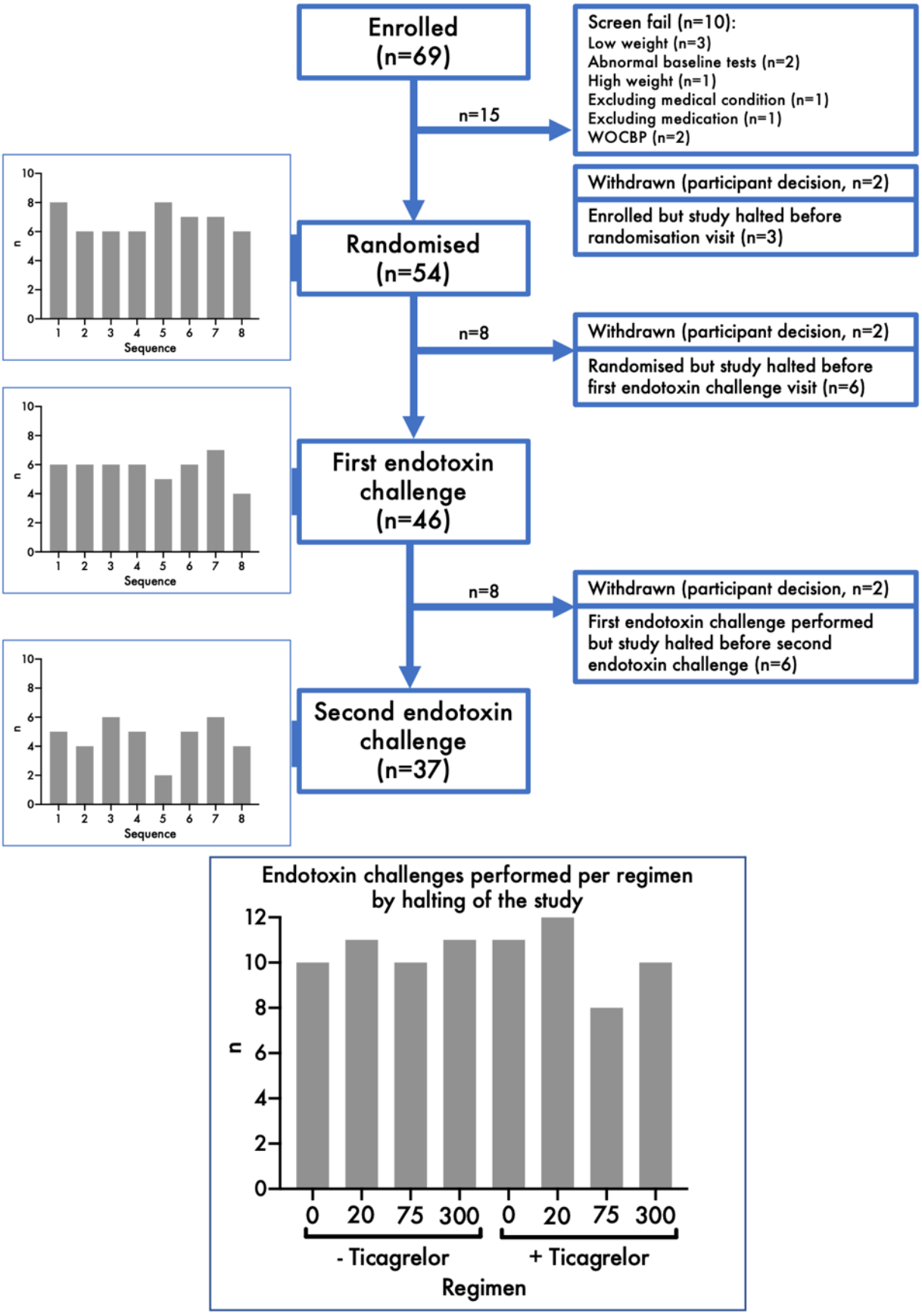

Figure 7.1 Recruitment flow chart for the WILLOW TREE study up to the point of the trial halting due to COVID-19.

Table 7.1 Baseline characteristics of participants who underwent at least one endotoxin challenge. ALP, alkaline phosphatase; ALT, alanine transferase; APTT, activated partial thromboplastin time; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure.

Parameter	**n**
Sex	
Male	43
Female	3
Ethnicity	
Caucasian	40
Asian	5
Afro-Caribbean	1
Smoking status	
Never	41
Ex-smoker	5

Parameter	**Mean +/- SD**
Age (years)	27.2 +/- 10.1
Height (cm)	175.8 +/- 8
Weight (kg)	73.1 +/- 8.2
BMI (kg/m^2)	23.7 +/- 2.4
SBP (mmHg)	127.4 +/- 8.5
DBP (mmHg)	72 +/- 5.8
Heart rate (bpm)	66.4 +/- 10.2
Temperature (^oC)	36.2 +/- 0.5
Haemoglobin (g/L)	145.5 +/- 10
Leukocyte count ($x10^9/L$)	5.6 +/- 1.4
Platelet count ($x10^9/L$)	233.2 +/- 50.8
Sodium (mmol/L)	141.2 +/- 1.4
Potassium (mmol/L)	4.4 +/- 0.3
Urea (mmol/L)	5.1 +/- 1.4
Creatinine (μmol/L)	80.9 +/- 12.8
Bilirubin (μmol/L)	10.4 +/- 4.7
ALP (IU/L)	66.6 +/- 14.3
ALT (IU/L)	21.1 +/- 11
Albumin (g/L)	48 +/- 1.8
Prothrombin time (s)	11.2 +/- 0.7
APTT (s)	25.3 +/- 1.8
Fibrinogen (g/L)	2.6 +/- 0.5
Bleeding time (s)	252.4 +/- 71.6

Table 7.2 Baseline characteristics of participants who underwent at least one endotoxin challenge, stratified by each pair of randomisation sequences. P values were generated using Kruskal-Wallis tests. ALP, alkaline phosphatase; ALT, alanine transferase; APTT, activated partial thromboplastin time; BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure;

	Mean +/- SD				
	Sequence 1 or 2	**Sequence 3 or 4**	**Sequence 5 or 6**	**Sequence 7 or 8**	**p**
	(n=12)	**(n=12)**	**(n=11)**	**(n=11)**	
Age (years)	26.3 +/- 7.9	23.8 +/- 5.1	29.4 +/- 13.7	29.7 +/- 12.1	0.47
Height (cm)	178 +/- 10.7	175.3 +/- 7	174.6 +/- 1.4	175 +/- 6.5	0.74
Weight (kg)	70.9 +/- 10.8	74.2 +/- 6.9	75.9 +/- 7.4	71.3 +/- 7	0.43
BMI (kg/m^2)	22.4 +/- 2.8	24.2 +/- 2.4	24.9 +/- 2	23.4 +/- 1.4	0.07
SBP (mmHg)	129 +/- 6.7	129.5 +/- 9.2	127.5 +/- 9.6	123.5 +/- 8.2	0.33
DBP (mmHg)	71.3 +/- 8.2	74.8 +/- 4	71.4 +/- 4.2	70.5 +/- 5.6	0.29
Heart rate (bpm)	66.7 +/- 8.7	72.1 +/- 14.3	64.3 +/- 6.8	62.2 +/- 7	0.1
Temperature (ºC)	36.3 +/- 0.4	36.3 +/- 0.4	36.3 +/- 0.5	35.9 +/- 0.8	0.13
Haemoglobin (g/L)	144.7 +/- 7.7	149.1 +/- 9.4	143.9 +/- 11.6	143.9 +/- 11.7	0.55
Leukocyte count ($x10^9/L$)	5.5 +/- 1.4	6.1 +/- 1.6	5.4 +/- 1.4	5.4 +/- 1.3	0.56
Platelet count ($x10^9/L$)	211.2 +/- 41	232.8 +/- 58.5	252.6 +/- 47.7	238.4 +/- 52	0.27
Sodium (mmol/L)	141.3 +/- 1.1	141.5 +/- 1.6	141 +/- 1.7	141 +/- 1.3	0.78
Potassium (mmol/L)	4.3 +/- 0.2	4.3 +/- 0.2	4.3 +/- 0.4	4.5 +/- 0.3	0.21
Urea (mmol/L)	5.1 +/- 1.6	5.2 +/- 1.4	5.0 +/- 1.1	5.1 +/- 1.6	0.99
Creatinine (µmol/L)	83 +/- 12.9	81.4 +/- 11.9	80.5 +/- 17.7	78.4 +/- 8.6	0.86
Bilirubin (µmol/L)	11 +/- 4.8	8.4 +/- 2.9	10.7 +/- 4.5	11.5 +/- 6.2	0.42
ALP (IU/L)	66.7 +/- 12.7	66.9 +/- 11.9	71.9 +/- 20.3	60.7 +/- 10.5	0.35
ALT (IU/L)	20.7 +/- 6.8	25.2 +/- 19.1	19.8 +/- 6.7	18.4 +/- 4.4	0.49
Albumin (g/L)	48.1 +/- 1.5	48.8 +/- 1.1	47.7 +/- 1.2	47.5 +/- 2.9	0.36
Prothrombin time (s)	11.0 +/- 0.7	11.1 +/- 0.5	11.1 +/- 0.8	11.5 +/- 0.8	0.5
APTT (s)	24.4 +/- 2.3	26 +/- 1.9	25.4 +/- 1.6	25.4 +/- 1.3	0.22
Fibrinogen (g/L)	2.6 +/- 0.6	2.6 +/- 0.3	2.5 +/- 0.4	2.6 +/- 0.6	0.94
Bleeding time (s)	265.3 +/- 80.6	229.1 +/- 72.5	244.2 +/- 65.3	270 +/- 68.1	0.51

C. Physiological response to 2 ng/kg intravenous endotoxin

Vital signs (blood pressure, heart rate, temperature), haematology parameters and markers of leukocyte activation were recorded before and during endotoxin administration. Data presented in this section are pooled from all endotoxin challenges performed, regardless of study medication allocation. Comparisons between treatment regimens are explored later in this thesis. Doses were administered at a quotient of 2 ng/kg ranging from 123 to 194 ng (**Figure 7.2**), and also appeared to be well adjusted for body surface area (**Figure 7.3**).

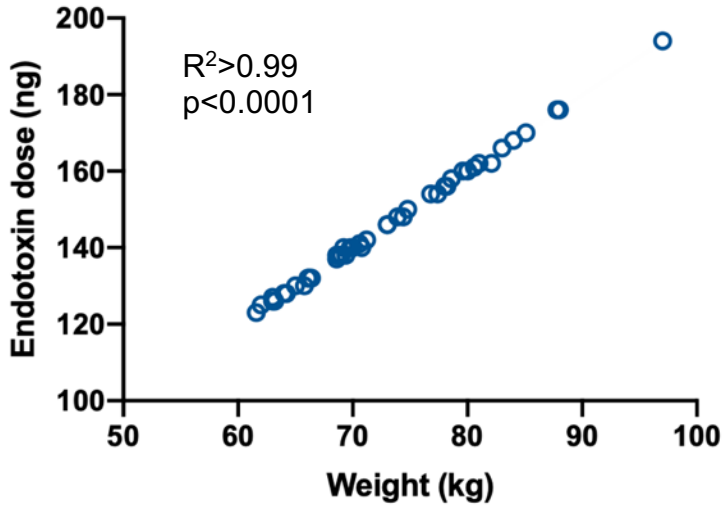

Figure 7.2 Range of endotoxin weight-adjusted doses administered to participants. Correlation assessed by simple linear regression.

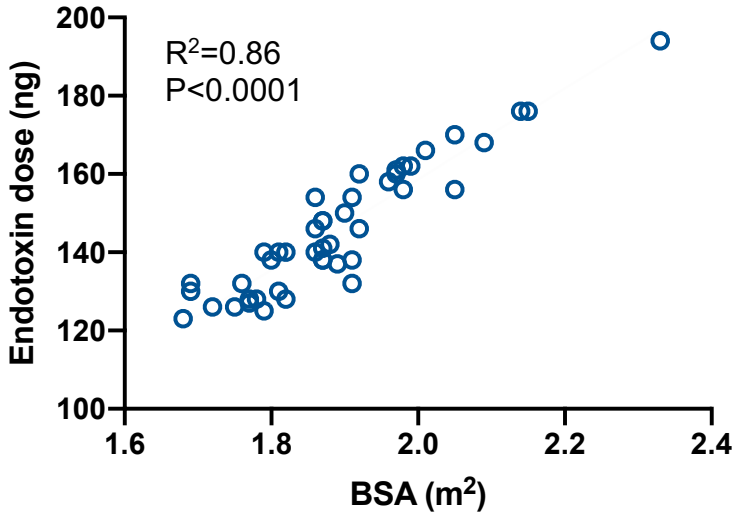

Figure 7.3 Correlation between endotoxin dose administered and body surface area (BSA) calculated using the Dubois formula. Correlation assessed by simple linear regression.

I. Effects on vital signs

Administration of intravenous endotoxin was associated with statistically significant changes in heart rate, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP, calculated as DBP + [(SBP-DBP)/3]) and core body temperature (all $p<0.0001$ using one-way ANOVA) (**Figures 7.4 to 7.8**).

After injection, heart rate increased, peaking at around 4 hours, after which this began to normalise. Similarly, there was a rise in SBP, DBP and MAP after endotoxin administration, peaking at around 60 to 90 mins, before a fall to below baseline by six hours. Core body temperature rose by 90 minutes after endotoxin, peaking at 3-4 hours before beginning to normalise by 6 hours. Assessed using one-way ANOVA, there were also notable decreases in heart rate and SBP, and an increase in temperature between -60 and +5 minutes. This was unlikely to be related to endotoxin administration, but may have been contributed to by factors such as a prolonged period of bed rest between the measurements, IV fluid administration and perhaps circadian variation in these parameters.

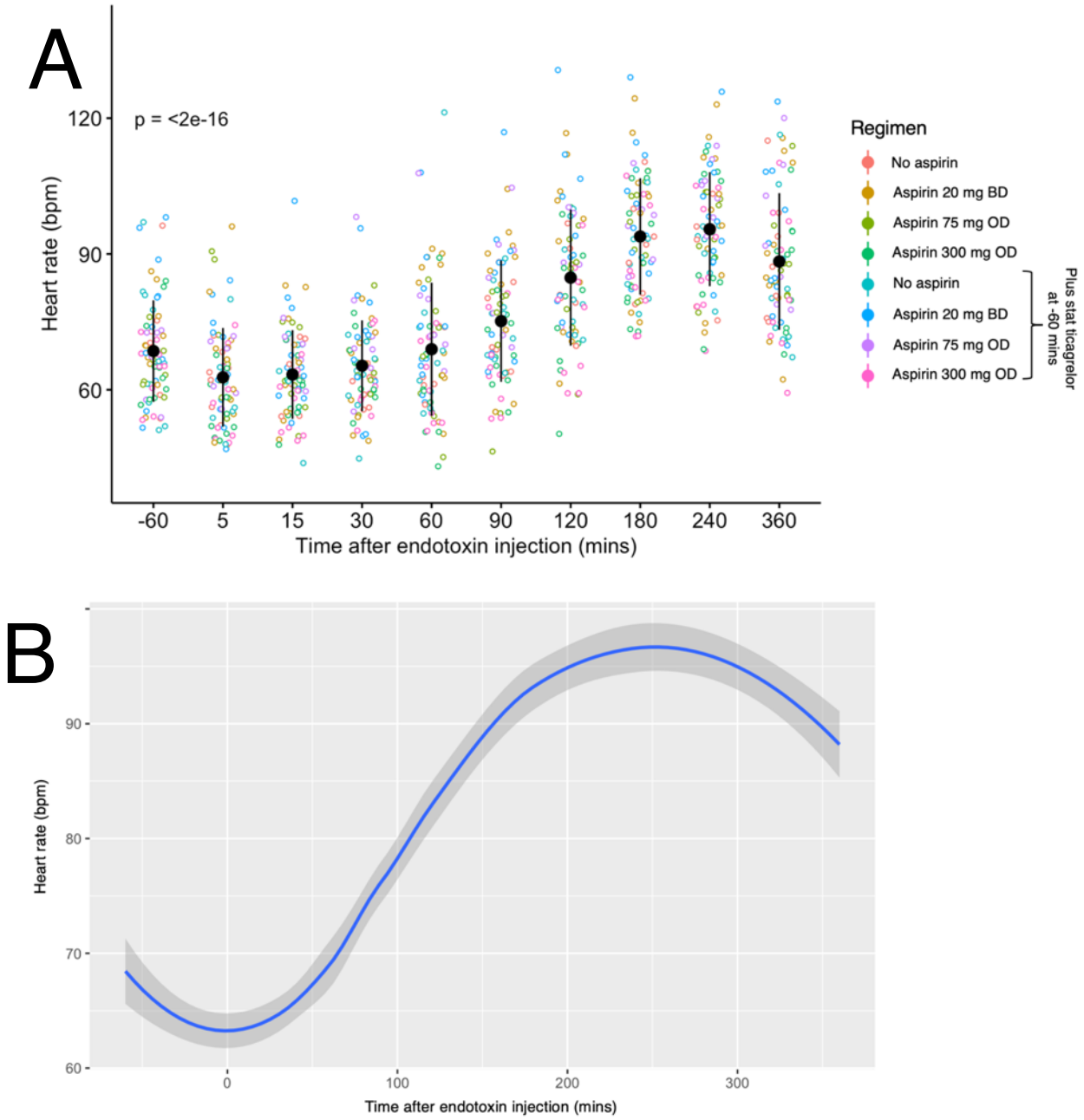

Figure 7.4 Heart rate (beats per minute, bpm) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

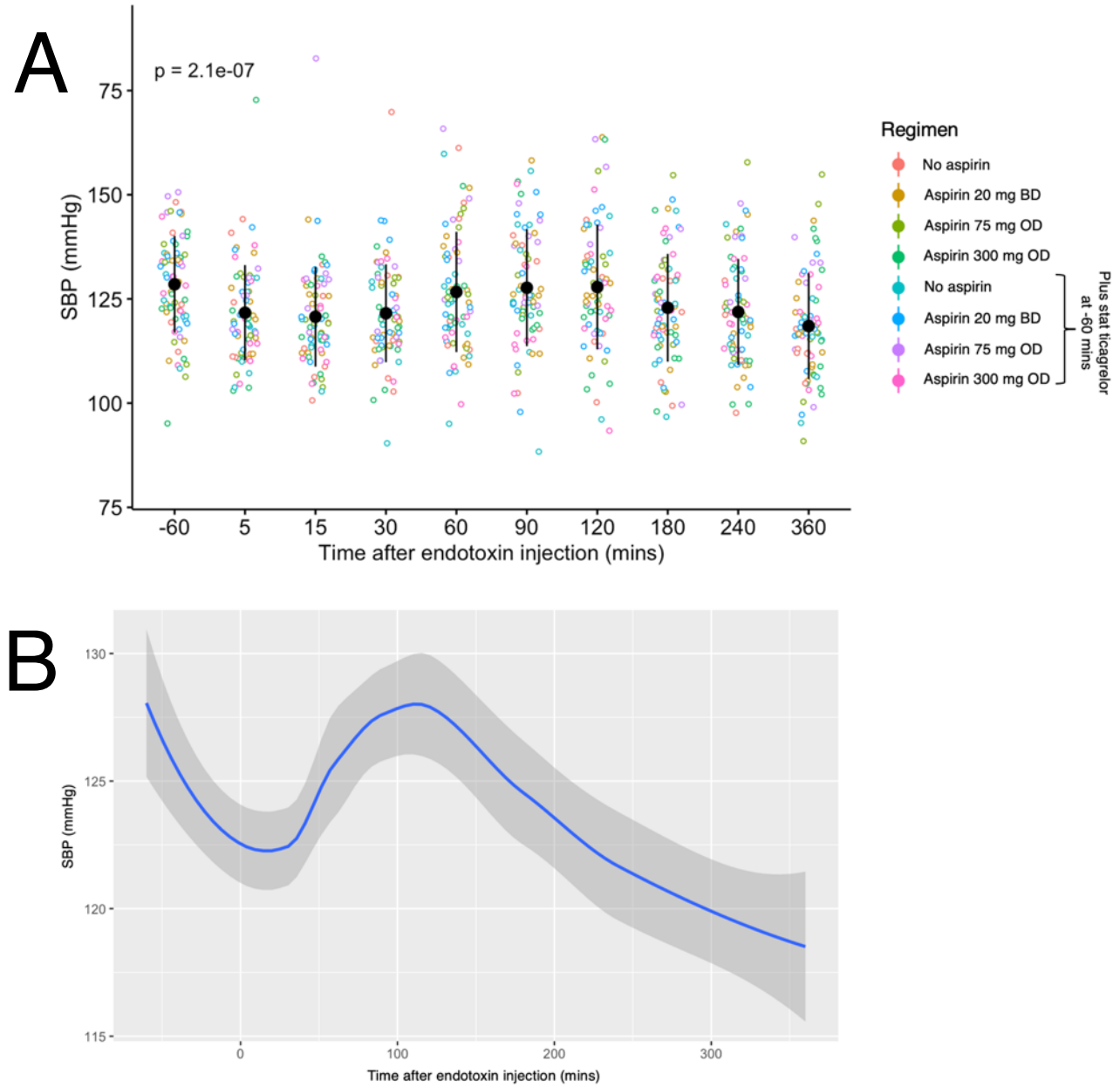

Figure 7.5 Systolic blood pressure (SBP) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

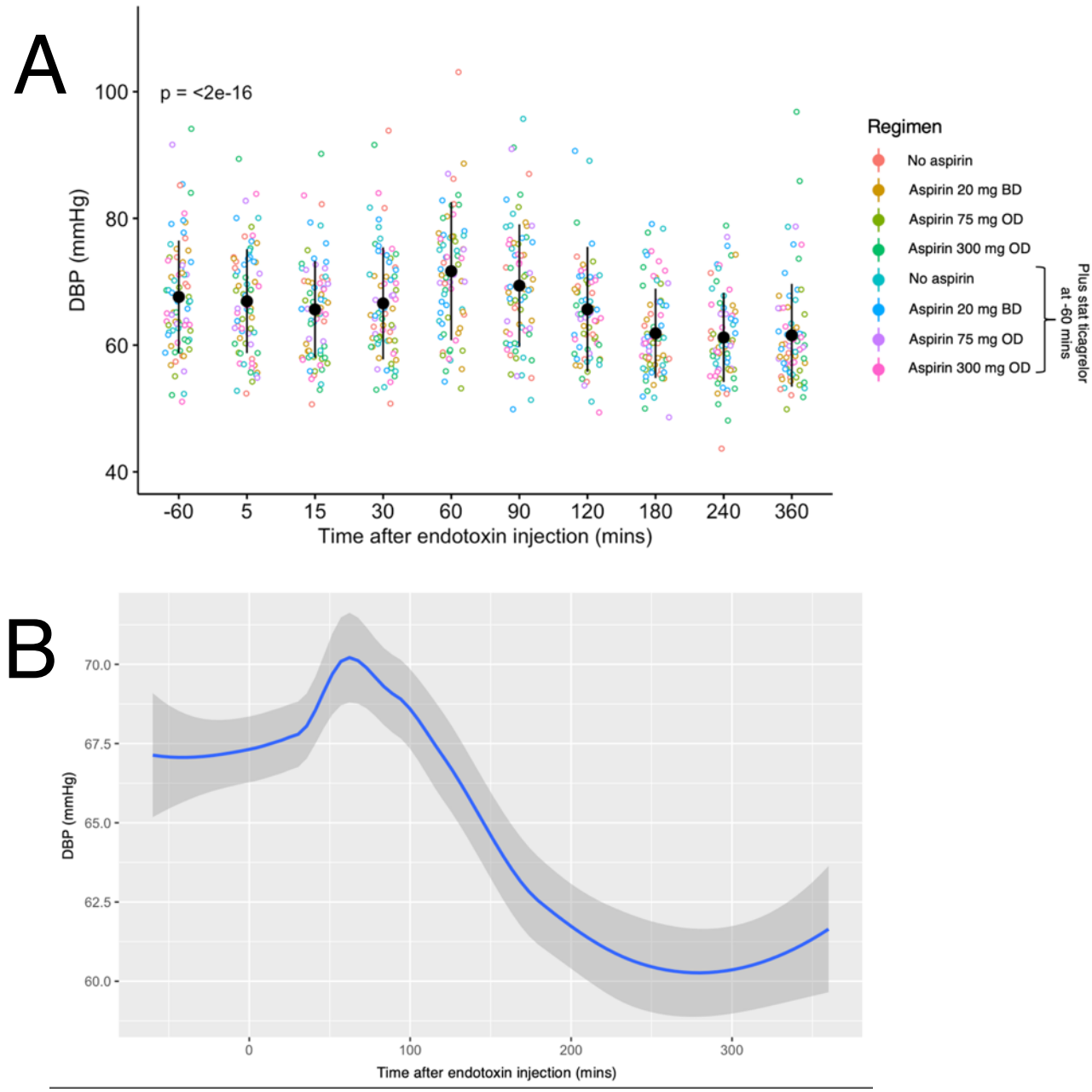

Figure 7.6 Diastolic blood pressure (DBP) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

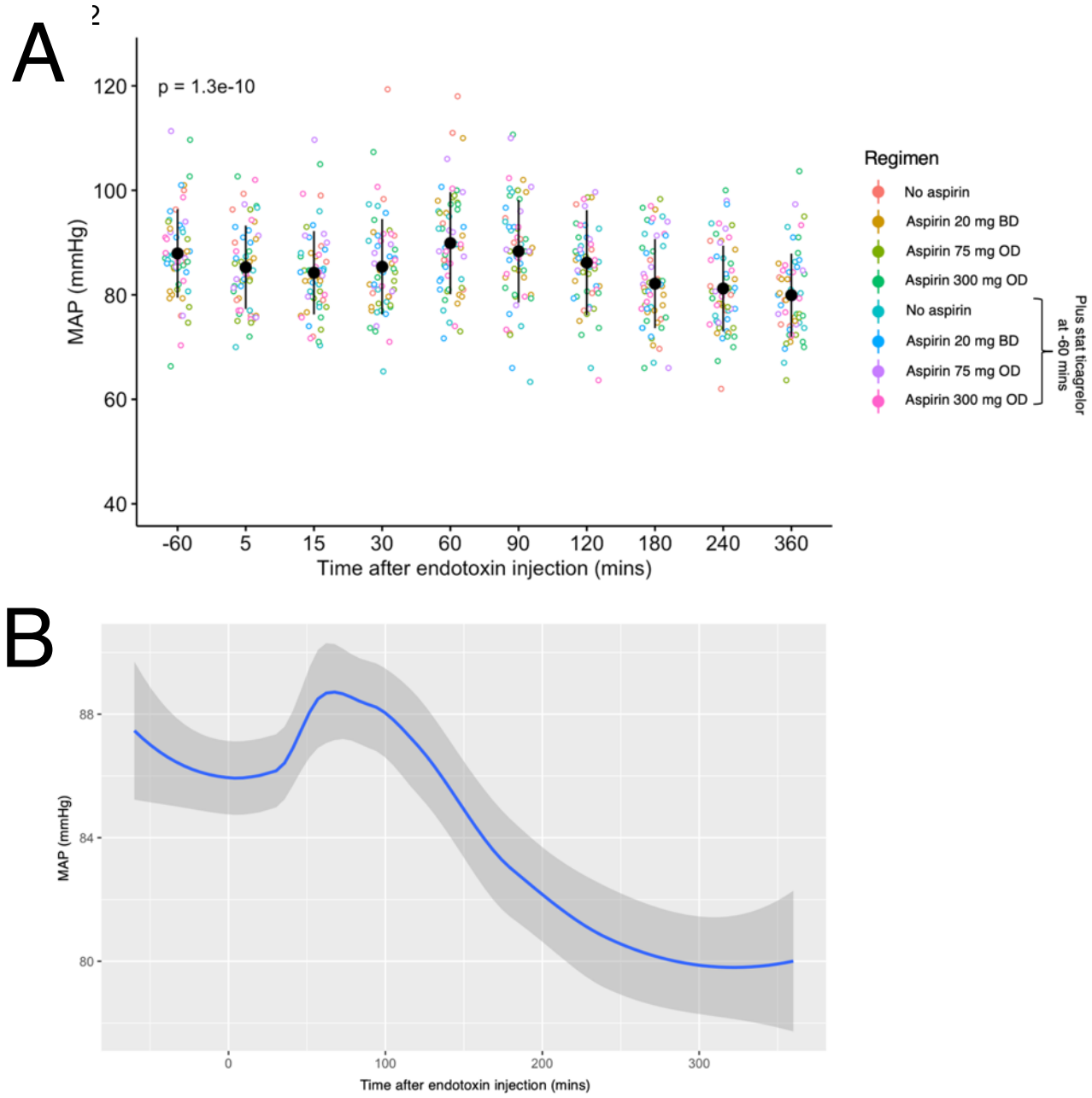

Figure 7.7 Mean arterial pressure (MAP) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

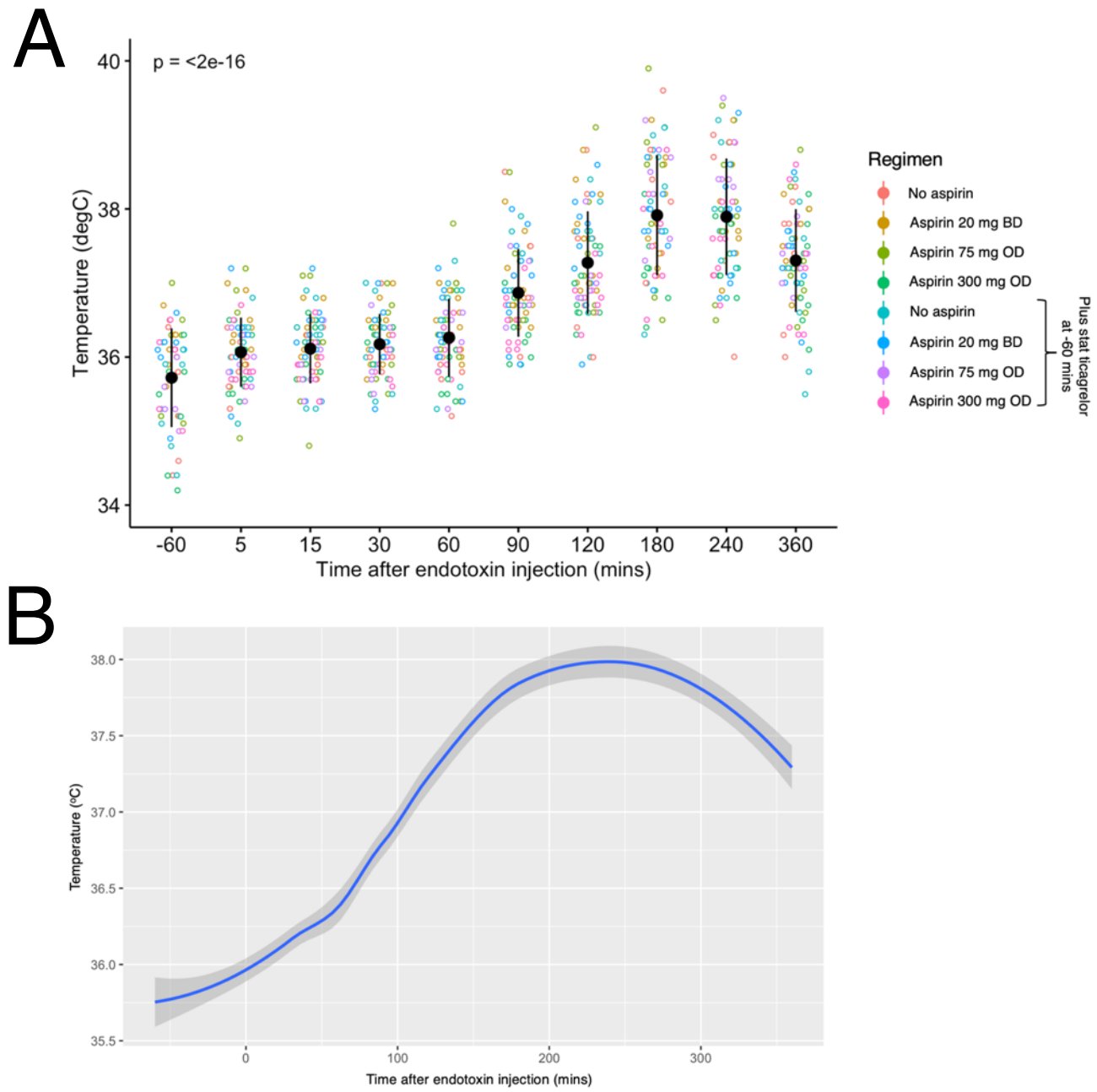

Figure 7.8 Core body temperature before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

II. Effects on leukocyte counts and activation markers

There were significant changes in total leukocyte, neutrophil, lymphocyte and mixed cell counts over time after endotoxin injection (all $p<0.0001$). Total leukocyte count fell by 60 minutes post-injection before rising again by 90 minutes and continued to climb until the last sampling point at 6 hours (**Figure 7.9**). Neutrophil count followed a similar pattern (**Figure 7.10**). Lymphocyte count fell significantly from baseline by 60 minutes post-injection and continued to decrease until stabilising after 4 hours (**Figure 7.11**). Mixed cell count fell rapidly after injection (significantly decreasing by 30 minutes) before stabilising between 1 and 3 hours then rising by 4 hours and normalising by 6 hours.

Cell surface expression of CD11b on monocytes and neutrophils in whole blood was measured as median fluorescence intensity by flow cytometry before and after endotoxin injection (**Figures 7.13 and 7.14**). For both cell types, CD11b expression was significantly affected over time, both increasing significantly by 1 hour after injection. Whereas monocyte CD11b appeared to have a single, sustained peak from 1 to 3 hours before normalising by 6 hours, neutrophil CD11b demonstrated two distinct peaks, the first at 1 hour and the second at 3 hours.

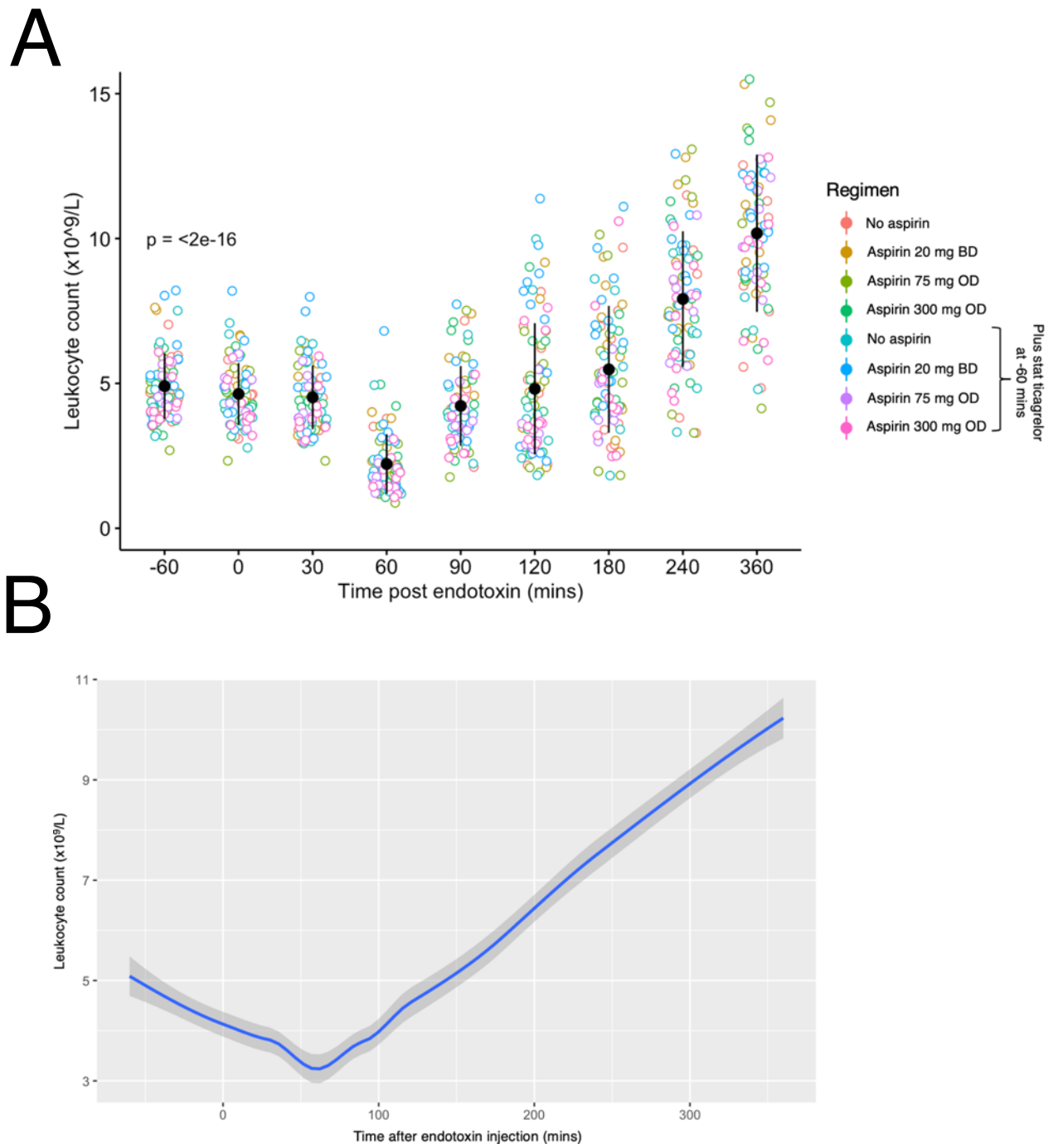

Figure 7.9 Circulating leukocyte count before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

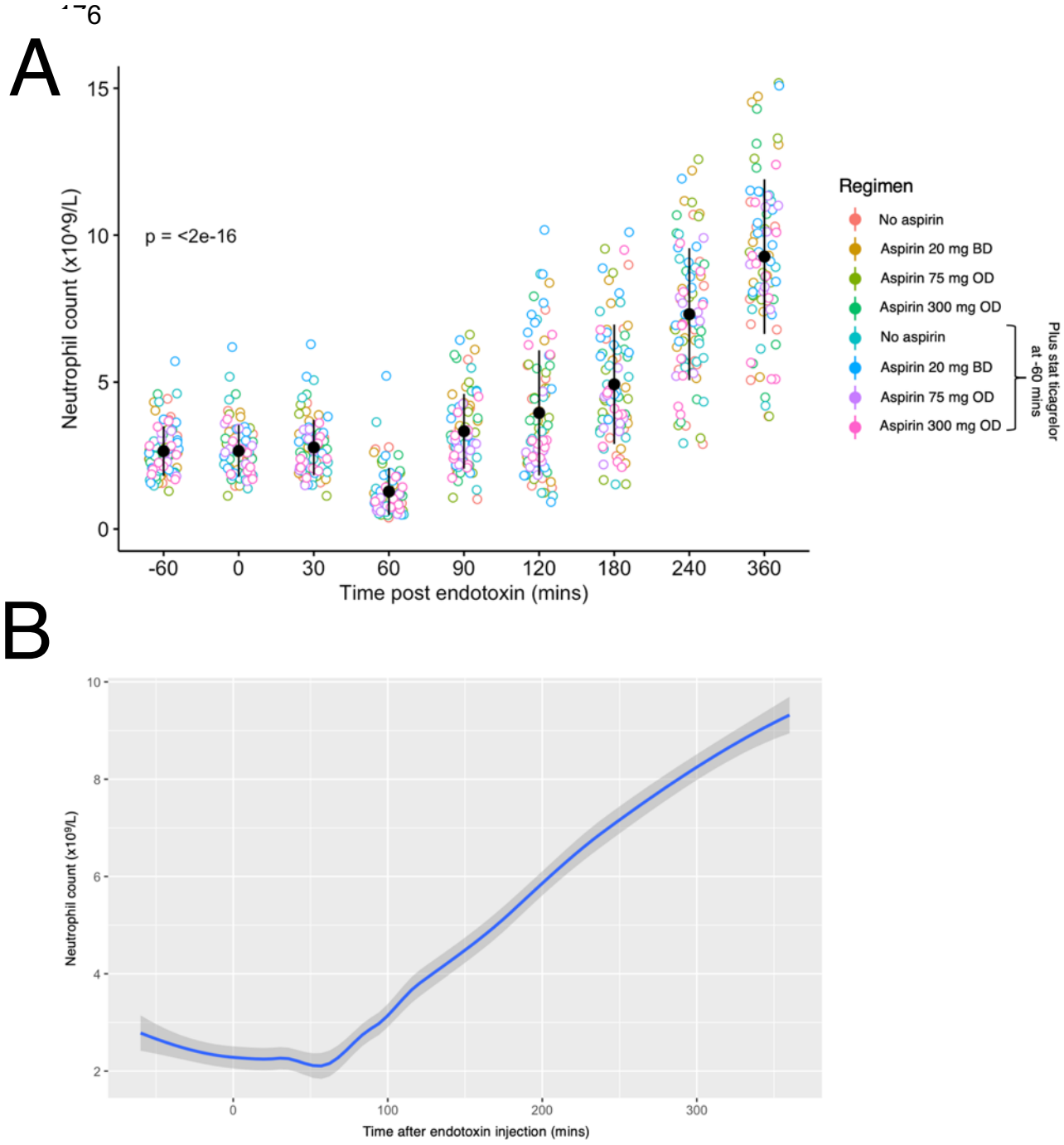

Figure 7.10 Circulating neutrophil count before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

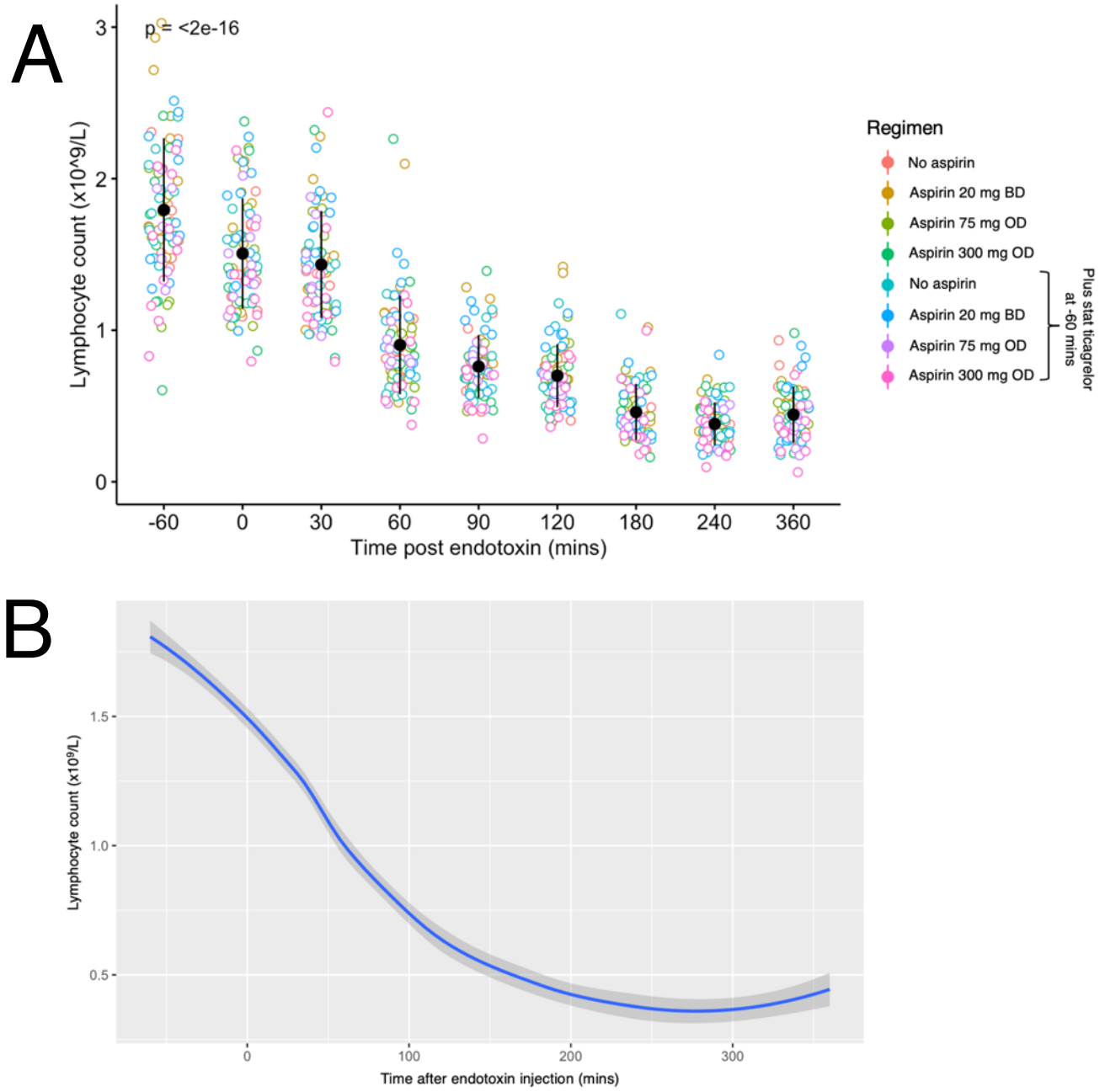

Figure 7.11 Circulating lymphocyte count before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

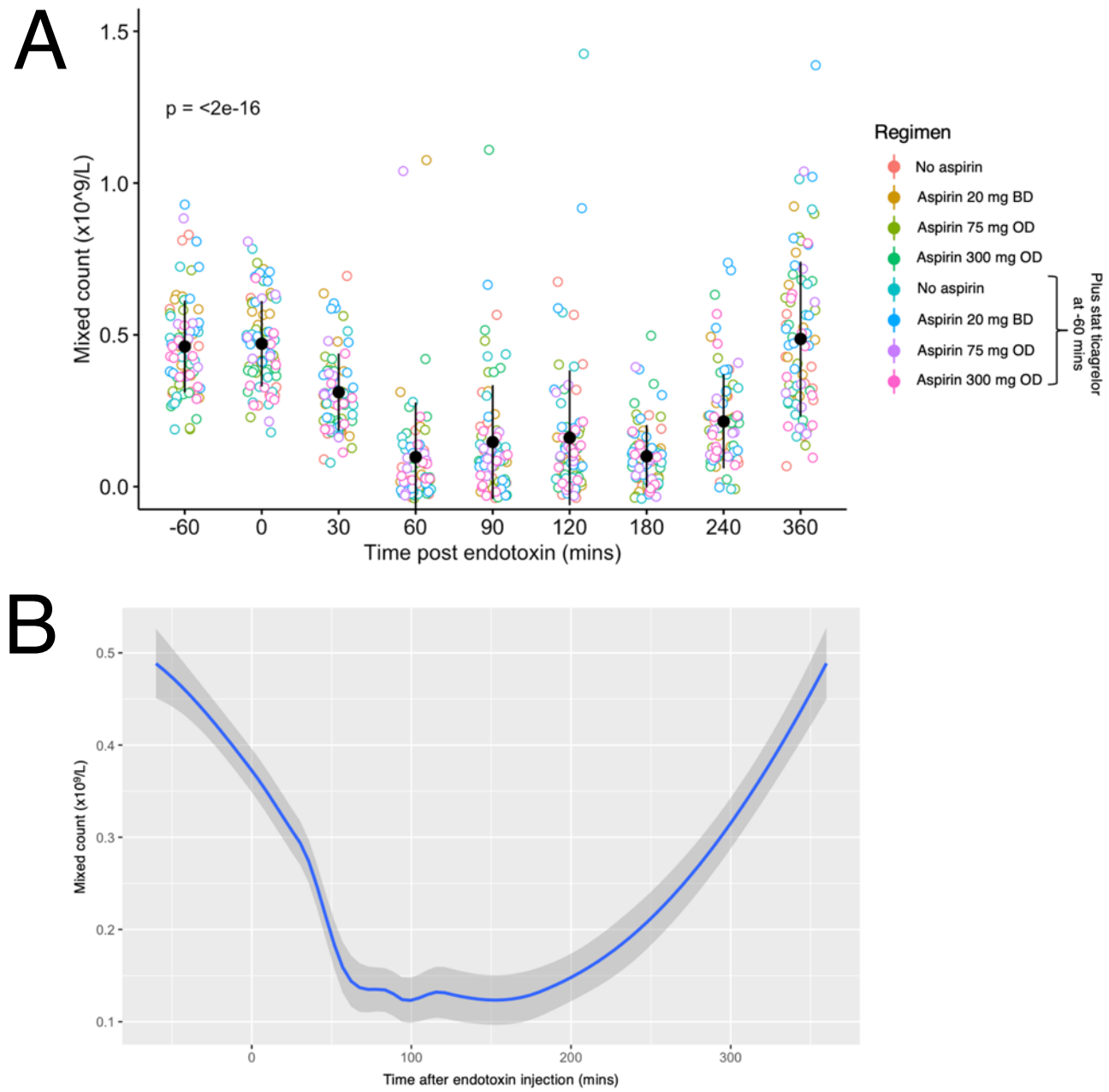

Figure 7.12 Circulating mixed cell count (sum of monocyte, eosinophil and basophil counts) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

A

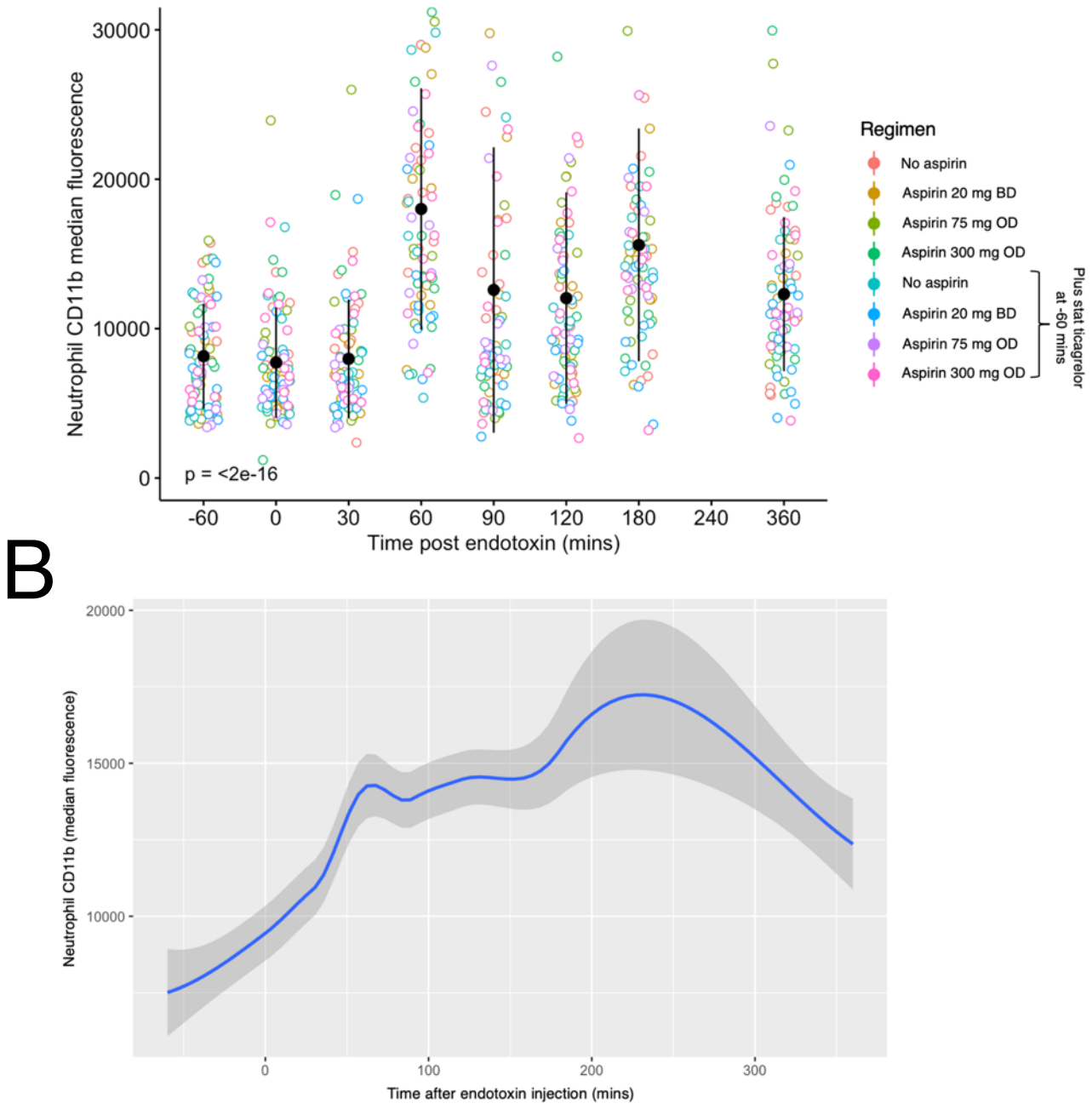

Figure 7.13 Cell surface expression of CD11b (measured as median fluorescence intensity) of circulating neutrophils before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

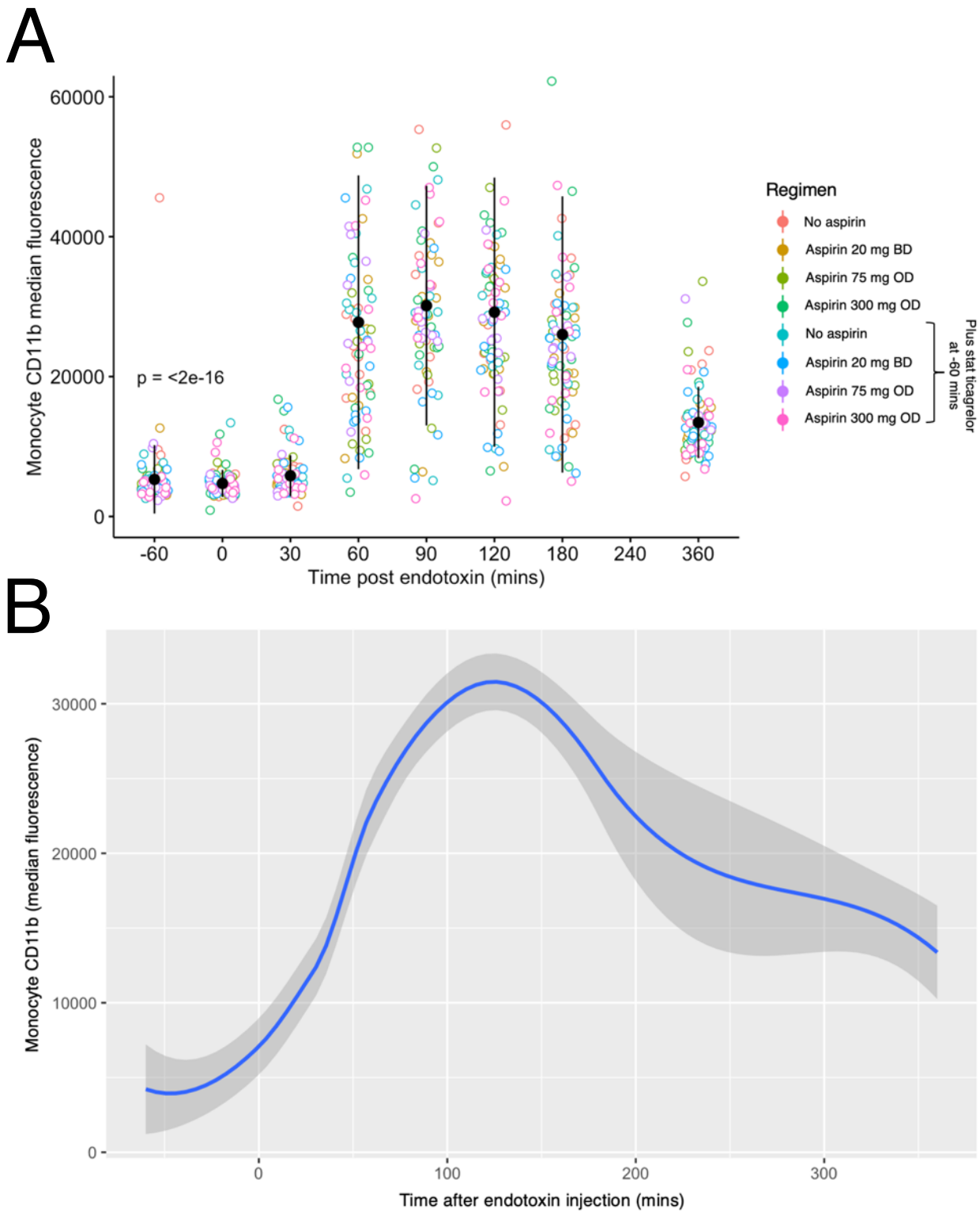

Figure 7.14 Cell surface expression of CD11b (measured as median fluorescence intensity) of circulating monocytes (non-segregated) before and after intravenous administration of 2 ng/kg endotoxin shown using (A) bars representing mean ± SD for each timepoint (p value for one-way ANOVA with timepoint as factor) and (B) locally weighted polynomial regression (blue line represents smoothed mean, shaded area represents 95% CI).

D. Discussion

Bacterial endotoxin stimulates an innate immune response by acting on TLR4, found on many cell types including leukocytes and platelets (Andonegui et al. 2005). IV injection of sterile bacterial endotoxin in healthy volunteers is a well-established, experimental model of inflammation (Suffredini et al. 1999). Though safe, transient and predictable, it effects significant physiological changes that are similar to those seen in systemic bacterial infection. Devised primarily to study sepsis, the model also has direct applicability for studying atheroinflammation as TLR4 also acts as a damage-associated molecular pattern receptor and is thus stimulated during atherogenesis.

At the time of the study being halted, recruitment had been progressing well and 83 IV endotoxin challenges had been performed, exceeding the previous sum total performed in prior studies at our centre. There was a roughly even spread of participants receiving each of the 8 treatment regimens, although there was a lower number in the aspirin 75 mg OD plus ticagrelor group than others. The investigators are blinded to the randomisation block size, which was set by the sponsor at the time of study set up as per a study-specific standard operating procedure, but anticipate any inequality in the groups present at the stage of this interim analysis will be evened out by the time all randomisations and challenges are performed.

It is important to reflect on differences between this study and those previously performed at our centre. One obvious difference with the previous study of Iqbal et al (2018) was the dose of endotoxin used: 0.5 ng/kg compared to 2 ng/kg in this study. The response to endotoxin is dose-dependent. Up to 4 ng/kg, either as a bolus or infusion, has been used in challenge studies and found to be safe (Suffredini et al. 1999). The study of Thomas et al. (2015) also used a 2 ng/kg bolus dose and thus is most similar to the present study. However, one difference between this study and the two previous undertaken in Sheffield is the specific batch of endotoxin used. Periodically, the National Institutes of Health arrange for a new batch to be manufactured, either on exhaustion of the previous supply or significant loss of potency detected on serial testing. They have only released 3 lots in the last 15 years. The two previous studies at our centre used lot #1188844, and there is good evidence for efficacy and safety of this batch. Conversely, the present study utilises stock from lot #94332B1, which is of recent manufacture. There is evidence that this lot may induce a more potent cytokine response than #1188844 and, whilst this has not been associated with any safety concerns, there are relatively few data on its physiological effects (Kiers et al. 2019). Similarly, there are differences between this study and the study of Kiers et al (2017), which investigated the effects of aspirin 75 mg OD and/or ticagrelor on the response to

endotoxin. That study administered a dose of 2 ng/kg using lot #1188844, but administered it as a bolus of 1 ng/kg followed by an infusion of another 1 ng/kg over an hour, in contrast to the 2 ng/kg bolus that was used by Thomas et al. (2015) and in the present study. To the candidate's knowledge, after initial validation in 4 subjects for regulatory purposes, the only published study to include intravenous injection using a dose of 2 ng/kg of this lot included just 8 participants (Hassani et al. 2020; Kiers et al. 2019).

The data in this chapter, therefore, confirm and characterise the physiological effects of a bolus intravenous injection of 2 ng/kg endotoxin from lot #94332B1, providing valuable data supporting its use as a challenge agent for the study of inflammation.

Chapter 8: Dose-dependent effects of aspirin, with or without ticagrelor, on safety parameters, haemodynamics, thrombosis and haemostasis before and during experimental human endotoxaemia

A. Laboratory safety parameters

Venous blood samples were obtained from participants after 10-14 days of each medication period and analysed for safety parameters (full blood count, serum urea and electrolytes, liver function tests, clotting screen) (**Table 8.1**). These were drawn at the time of inserting the first intravenous cannula, and therefore were prior to the last dose of aspirin (if applicable), the single dose of ticagrelor (if applicable), IV fluid administration and endotoxin injection.

I. Serum potassium

After 10-14 days of treatment with study medication, there was a significant difference in serum potassium levels between the groups (p=0.0003). Pairwise comparison revealed that serum potassium was significantly greater when receiving aspirin 300 mg OD compared to 20 mg BD (p=0.019) or 75 mg OD (p=0.0027) with no other comparisons significant (**Figure 8.1**). The effect was modest and is not felt to represent a safety concern.

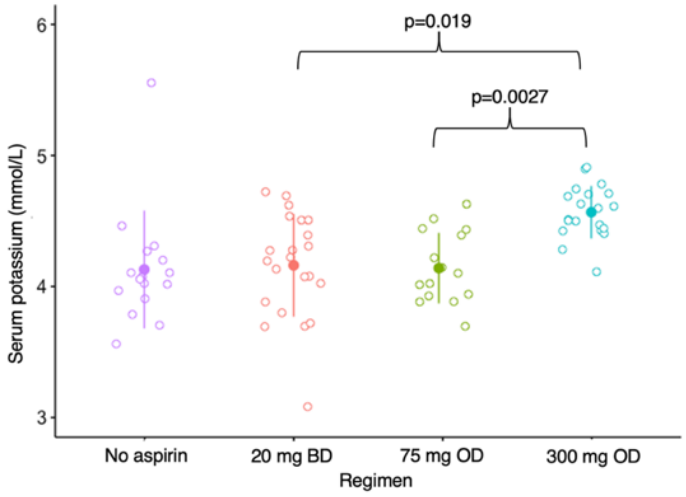

Figure 8.1 Serum potassium measured after 10-14 days of study medication by regimen. Bars represent mean ± SD.

Table 8.1 Laboratory safety parameters measured after 10-14 days of study medication, 1 hour before endotoxin administration, grouped by aspirin regimen. P-values generated by one-way ANOVA with regimen as factor. ALP, alkaline phosphatase; ALT, alanine transferase; APTT, activated partial thromboplastin time.

Parameter	Regimen				
	No aspirin	20 mg BD	75 mg OD	300 mg OD	
	Mean +/- SD				p
Haemoglobin (g/L)	145.9 +/- 6.7	151.9 +/- 9.6	145.1 +/- 8.7	142.6 +/- 10	0.0094
Leukocyte count (x10^9/L)	5.5 +/- 1.4	5.9 +/- 1.7	5.2 +/- 1.1	5.1 +/- 1.1	0.28
Platelet count (x10^9/L)	209.1 +/- 41.5	228.1 +/- 40.1	240.2 +/- 29	224.3 +/- 49.1	0.46
Sodium (mmol/L)	141.2 +/- 1.3	140.6 +/- 1.4	140.9 +/- 1.3	141.3 +/- 1.7	0.46
Potassium (mmol/L)	4.1 +/- 0.5	4.2 +/- 0.4	4.1 +/- 0.3	4.6 +/- 0.2	0.0003
Urea (mmol/L)	5.2 +/- 1.1	5.4 +/- 1.6	5.1 +/- 1.0	5.5 +/- 1.5	0.8
Creatinine (μmol/L)	84.9 +/- 9.4	81.7 +/- 12	83.1 +/- 14.7	76.2 +/- 10.5	0.13
Bilirubin (μmol/L)	10.6 +/- 3.4	10.6 +/- 5.1	11.2 +/- 7.3	10.2 +/- 4.0	0.95
ALP (IU/L)	66.3 +/- 17.4	65.1 +/- 11.9	72.2 +/- 19.3	60 +/- 10.4	0.12
ALT (IU/L)	17 +/- 3.5	20.9 +/- 18.6	21.8 +/- 7.5	24.7 +/- 26.5	0.64
Albumin (g/L)	48.6 +/- 2.2	48 +/- 2.5	46.9 +/- 1.7	46.2 +/- 2.5	0.01
Prothrombin time (s)	11.1 +/- 0.8	11.3 +/- 0.6	11 +/- 0.8	11.4 +/- 0.8	0.35
APTT (s)	25.7 +/- 2	26.2 +/- 2.2	25.4 +/- 1.8	25.8 +/- 1.3	0.62
Fibrinogen (g/L)	2.6 +/- 0.5	2.6 +/- 0.5	2.5 +/- 0.6	2.4 +/- 0.4	0.77

II. Serum albumin

There was a significant difference between the groups in serum albumin (p=0.01). On pairwise comparison, serum albumin was significantly lower compared to no aspirin when receiving aspirin 75 mg OD (p=0.0056) and 300 mg OD (p=0.0048) (**Figure 8.2**)

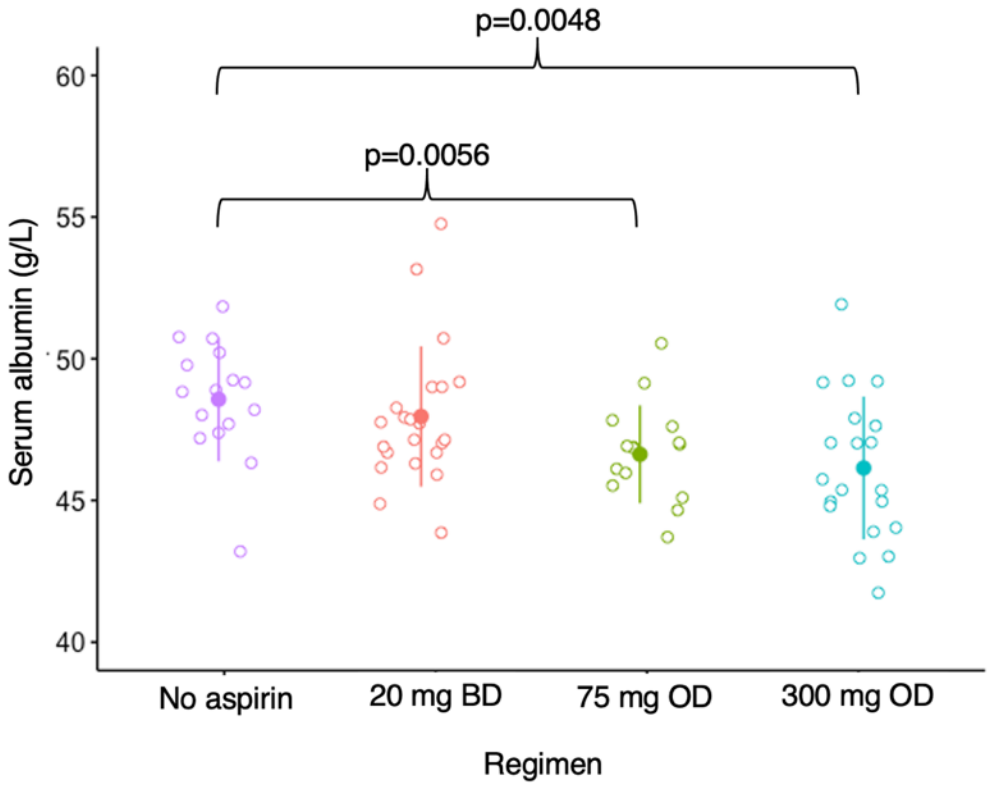

Figure 8.2 Serum albumin measured after 10-14 days of study medication by regimen. Bars represent mean ± SD.

III. Haemoglobin

There was a significant difference in haemoglobin levels between the regimens (p=0.0094). On pairwise comparison, the only significant difference was between the aspirin 20 mg BD and 300 mg OD groups (p=0.019) (**Figure 8.3**).

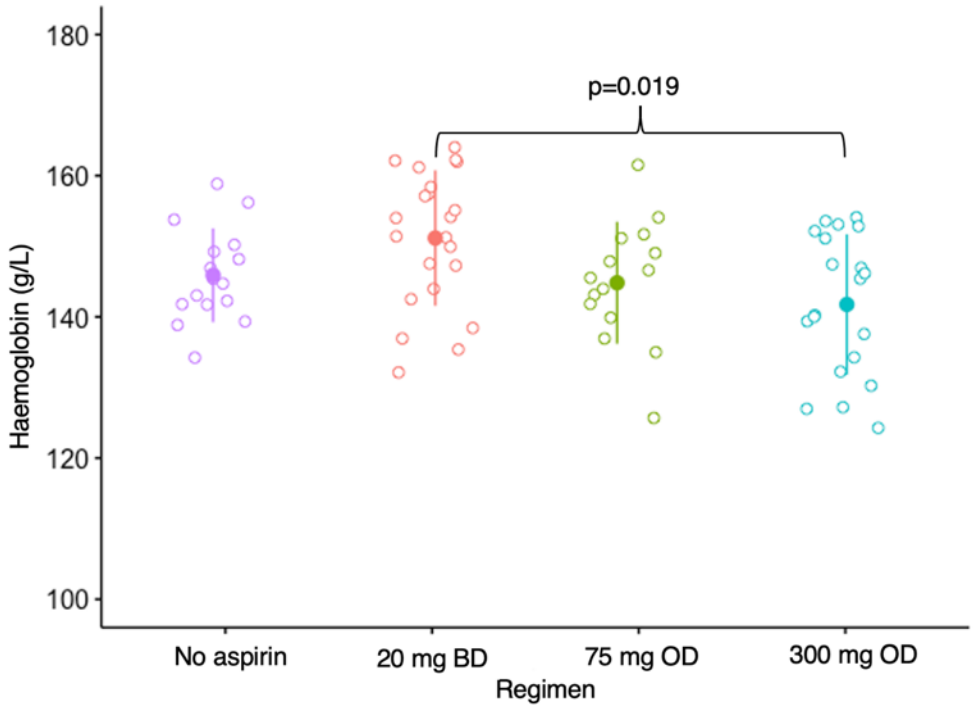

Figure 8.3 Haemoglobin measured after 10-14 days of study medication by regimen. Bars represent mean ± SD.

B. Haemodynamic response to endotoxaemia

I. Blood pressure

Both SBP and DBP significantly varied with timepoint. Treatment had no significant effect on blood pressure measurements and there was no evidence of timepoint*treatment interaction when assessed using two-way repeated measures ANOVA (**Table 8.2**). Ticagrelor had no significant effect on blood pressure (**Figures 8.4, 8.5**).

II. Heart rate

There was evidence of a difference in heart rate over the time course between the treatment regimens when receiving ticagrelor (p=0.033) but not when ticagrelor was not given (p=0.31) (**Table 8.2**). Pairwise comparison revealed a significant difference between the aspirin 300 mg OD group and the 20 mg BD and 75 mg OD (**Table 8.3**). Aspirin 300 mg OD appeared to be associated with a lower heart rate during endotoxaemia compared to the other regimens (**Figure 8.6**)

Table 8.2 P values generated by two-way repeated-measures ANOVA to compare haemodynamics (systolic blood pressure, SBP; diastolic blood pressure, DBP; heart rate, HR) between all regimens, regimens not including ticagrelor, and regimens including ticagrelor by timepoint, treatment and timepoint*treatment interaction. P values <0.05 are shown in bold.

	All regimens			No ticagrelor			Ticagrelor		
Factor ➔	Timepoint	Treatment	Timepoint*Treatment	Timepoint	Treatment	Timepoint*Treatment	Timepoint	Treatment	Timepoint*Treatment
SBP	**<0.0001**	0.51	0.31	**0.0008**	0.94	0.75	**0.002**	0.12	0.12
DBP	**<0.0001**	0.88	0.66	**<0.0001**	0.79	0.70	**<0.0001**	0.85	0.24
HR	**<0.0001**	0.065	0.85	**<0.0001**	0.31	0.62	**<0.0001**	**0.033**	0.64

Table 8.3 Pairwise comparison of treatment effect on heart rate over time by regimen (ticagrelor-containing regimens only) using repeated-measures ANOVA. P values <0.05 are shown in bold. ^{T}=loading dose of ticagrelor given 1 hour before endotoxin injection.

Aspirin 20 mg BD^{T} vs. no aspirin^{T}	Aspirin 75 mg OD^{T} vs. no aspirin^{T}	Aspirin 300 mg OD^{T} vs. no aspirin^{T}	Aspirin 75 mg OD^{T} vs. 20 mg BD^{T}	Aspirin 300 mg OD^{T} vs. 20 mg BD^{T}	Aspirin 300 mg OD^{T} vs. 75 mg OD^{T}
0.16	0.082	0.26	0.93	**0.028**	**0.008**

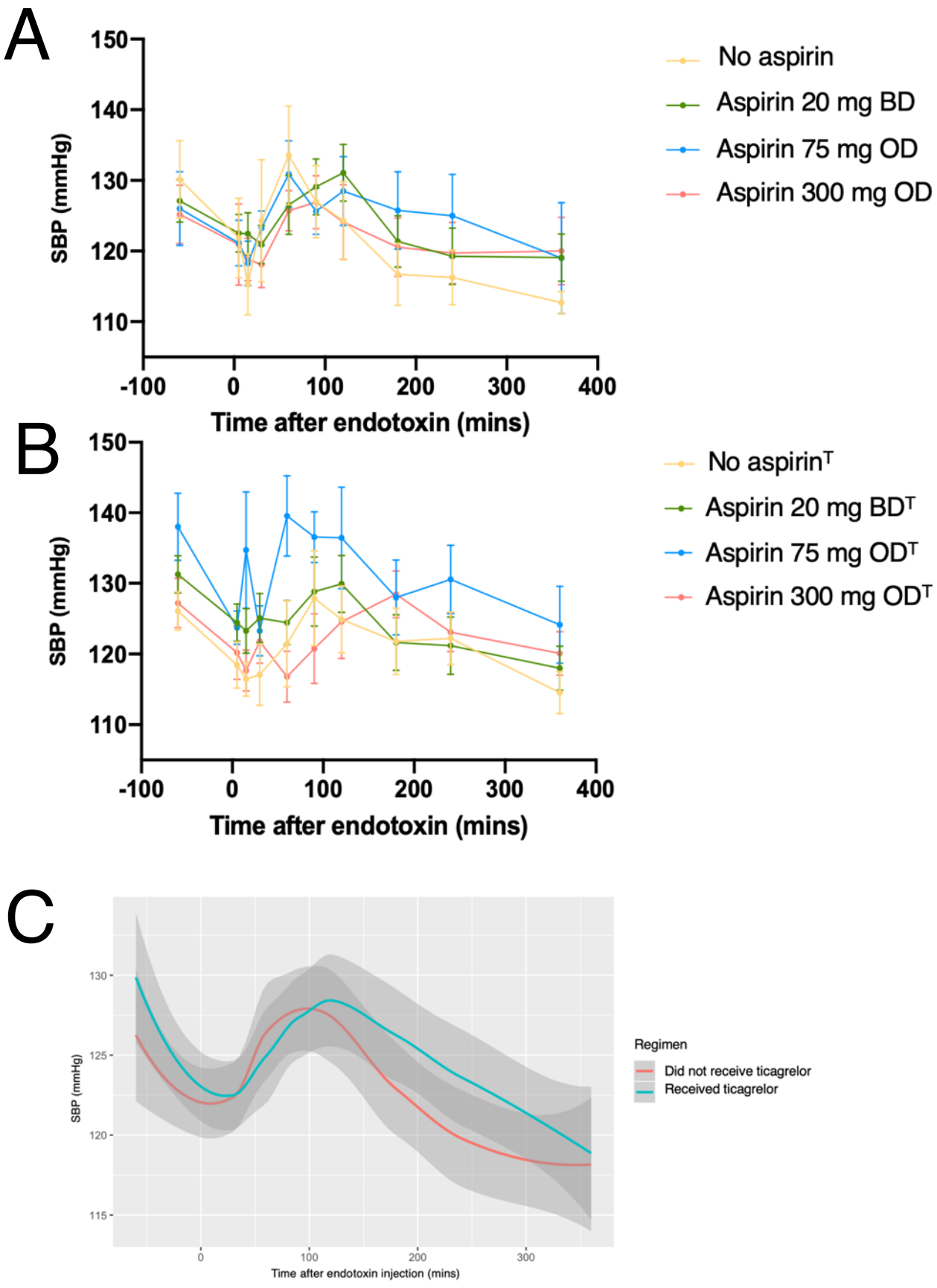

Figure 8.4 Systolic blood pressure (SBP) before and during endotoxaemia in participants receiving (A) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (without ticagrelor), (B) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (^{T} = with a loading dose of ticagrelor) or (C) ticagrelor (and any regimen of aspirin or no aspirin) vs. no ticagrelor (and any regimen of aspirin or no aspirin). In (A) and (B), bars indicate mean ± SEM. In (C), solid lines represent smoothed means using locally weighted polynomial regression and shaded areas 95% CI.

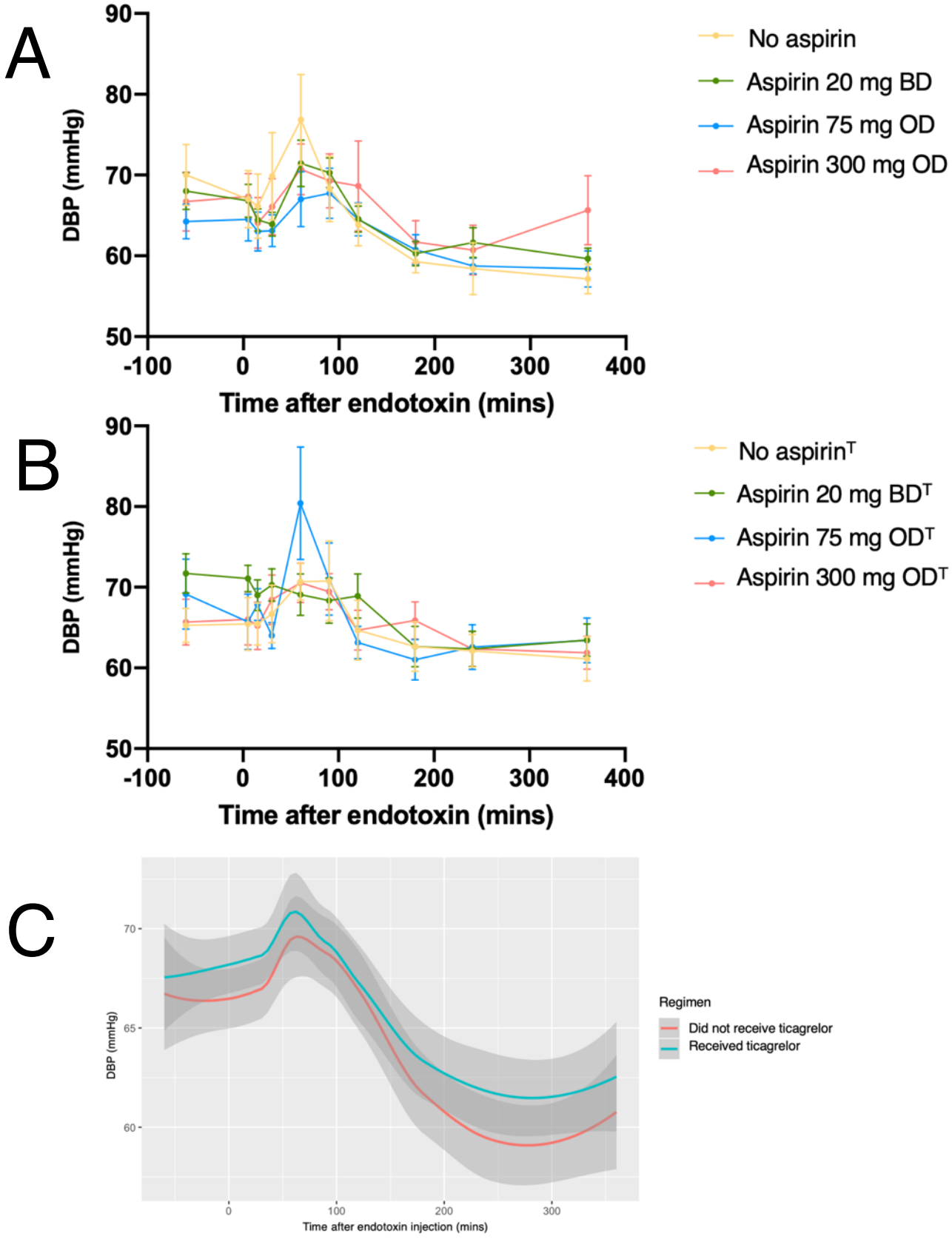

Figure 8.5 Diastolic blood pressure (DBP) before and during endotoxaemia in participants receiving (A) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (without ticagrelor), (B) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (^{T} = with a loading dose of ticagrelor) or (C) ticagrelor (and any regimen of aspirin or no aspirin) vs. no ticagrelor (and any regimen of aspirin or no aspirin). In (A) and (B), bars indicate mean ± SEM. In (C), solid lines represent smoothed means using locally weighted polynomial regression and shaded areas 95% CI.

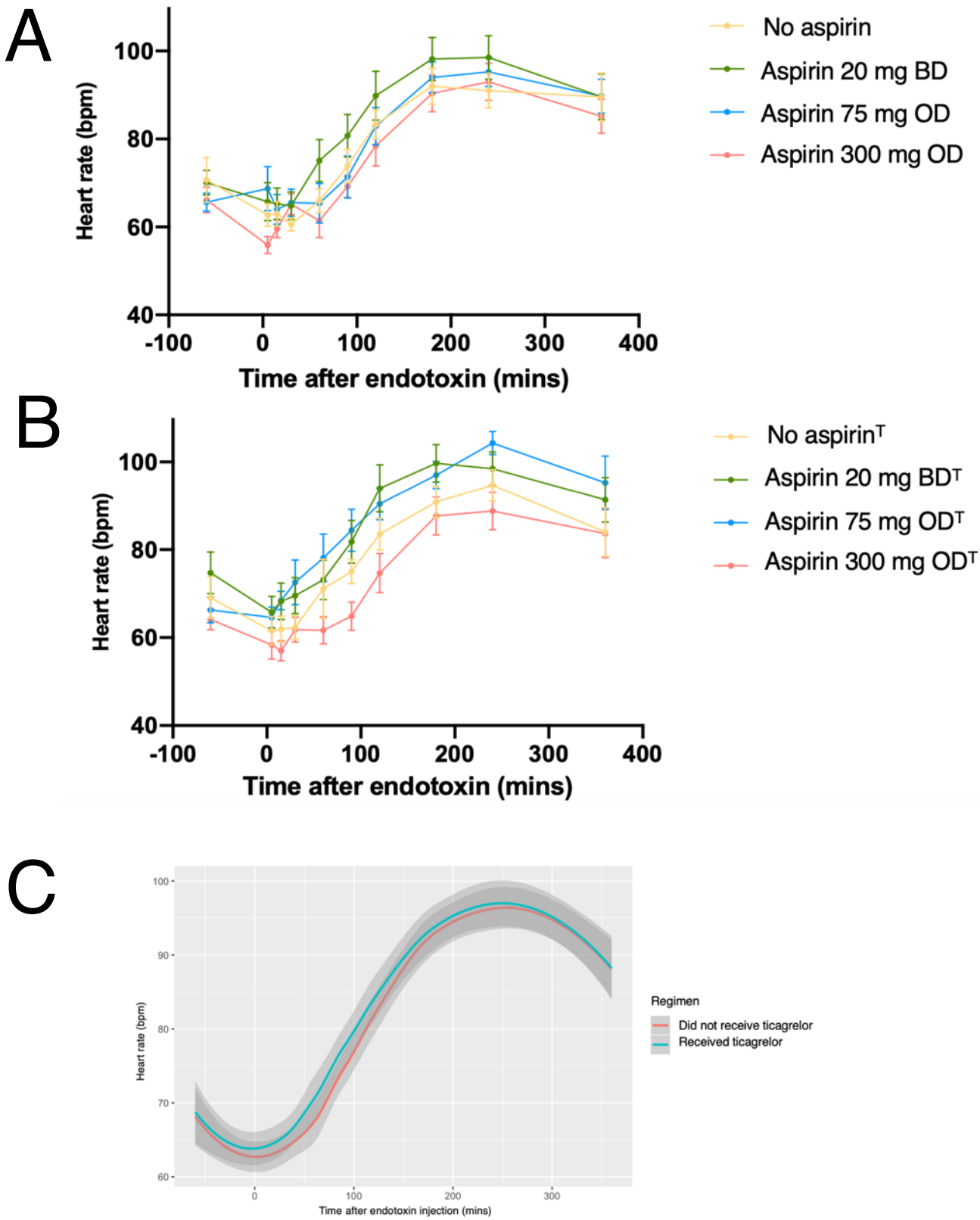

Figure 8.6 Heart rate before and during endotoxaemia in participants receiving (A) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (without ticagrelor), (B) no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD (each with a loading dose of ticagrelor) or (C) ticagrelor (and any regimen of aspirin or no aspirin) vs. no ticagrelor (and any regimen of aspirin or no aspirin). In (A) and (B), bars indicate mean ± SEM. In (C), solid lines represent smoothed means using locally weighted polynomial regression and shaded areas 95% CI.

III. Intravenous fluid requirement

Whilst a standard regimen of IV fluid was administered as per the study protocol (total 1000 mL), additional fluid boluses were given at the discretion of the investigators depending on vital signs and symptoms of hypotension/hypovolaemia. Mean fluid requirement per endotoxin challenge was similar for all treatment regimens, whether these were treated individually (p=0.99, **Figure 8.7A**) grouped by aspirin dose (p=0.94, **Figure 8.7B**) or according to receipt of ticagrelor (^{T}) or not (p=0.84, **Figure 8.7C**).

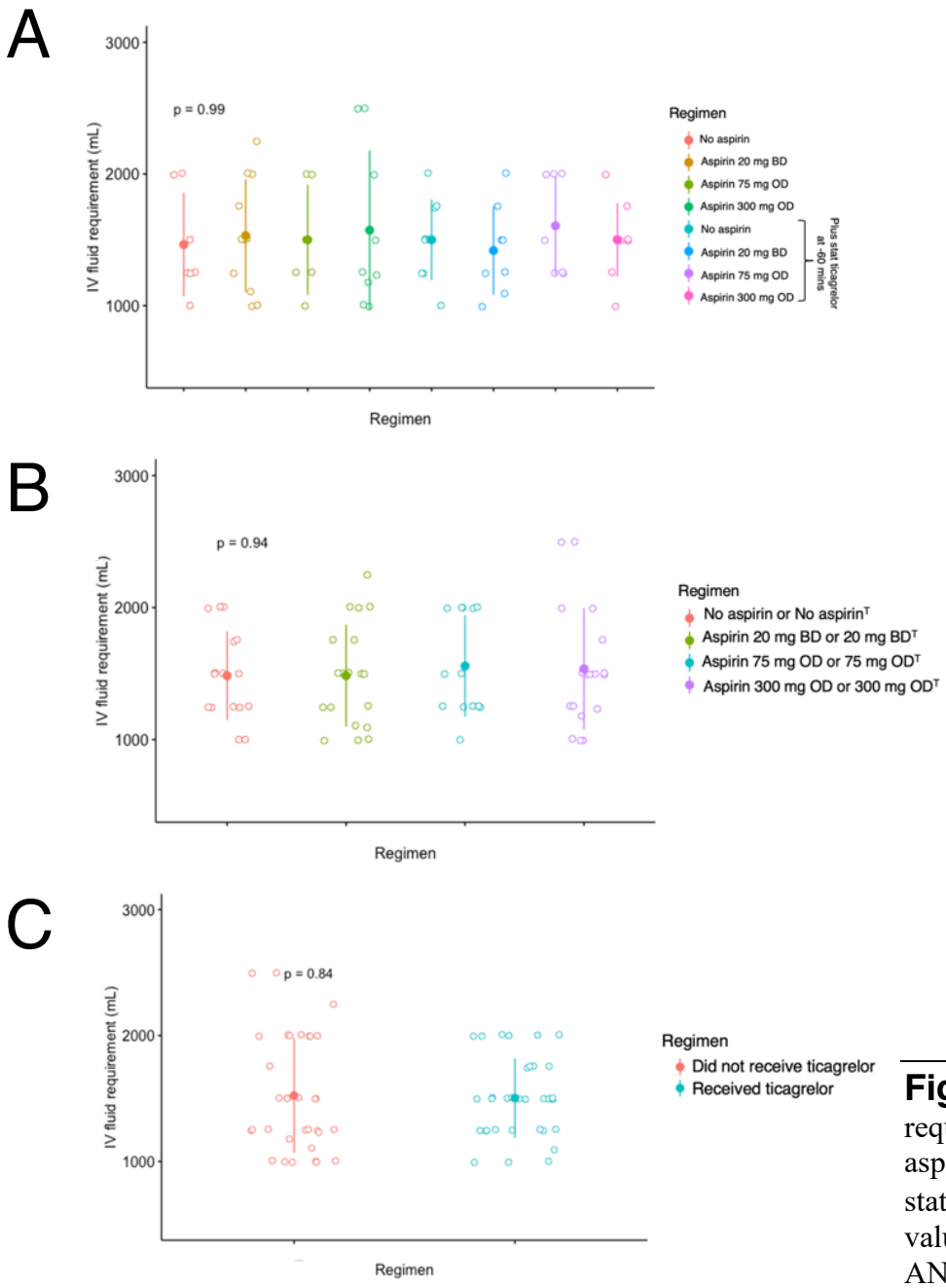

Figure 8.7 Intravenous (IV) fluid requirement by (A) regimen (B) aspirin dose and (C) ticagrelor status. Bars represent mean ± SD. P values generated by one-way ANOVA (A/B) and paired t-test (C).

C. Anti-thrombotic effects

I. Suppression of thromboxane A_2 generation

At 1 hour after endotoxin injection (2 hours after aspirin), there was evidence of a dose-dependent effect of aspirin on levels of serum TXB_2 (the major metabolite of TXA_2), both in the presence and absence of ticagrelor ($p<0.0001$, **Table 8.4, Figure 8.8**). When participants did not receive a loading dose of ticagrelor 1 hour prior to endotoxin injection, aspirin 300 mg OD significantly reduced serum TXB_2 compared to 75 mg OD ($p=0.02$), which reduced serum TXB_2 compared to 20 mg BD ($p=0.003$), which again in turn reduced serum TXB_2 compared to no aspirin ($p<0.0001$). When participants had received ticagrelor, aspirin 300 mg OD significantly reduced TXB_2 compared to 75 mg OD, but there was no significant difference between TXB_2 levels when receiving 20 mg BD compared to 75 mg OD ($p=0.97$), which both significantly reduced TXB_2 compared to no aspirin. Furthermore, mean serum TXB_2 was significantly lower when receiving aspirin 20 mg BD after a loading dose of ticagrelor compared to no ticagrelor ($p=0.02$). Alone or when receiving aspirin 75 mg OD or 300 mg OD, ticagrelor did not significantly influence TXB_2 levels (**Figure 8.8**).

Table 8.4 Serum thromboxane B_2 measured 1 hour after endotoxin injection by treatment group. ^{T} = Loading dose of ticagrelor 1 hour prior to endotoxin injection.

Treatment group	**Serum thromboxane B_2 (pg/mL) (Mean +/- SD)**
No aspirin	160638 +/- 60934
Aspirin 20 mg BD	1335 +/- 901.1
Aspirin 75 mg OD	502 +/- 332.8
Aspirin 300 mg OD	164.6 +/- 207
No aspirin^T	132173 +/- 80946
Aspirin 20 mg BD^T	645.7 +/- 474.6
Aspirin 75 mg OD^T	616.9 +/- 324.5
Aspirin 300 mg OD^T	130.5 +/- 220.1

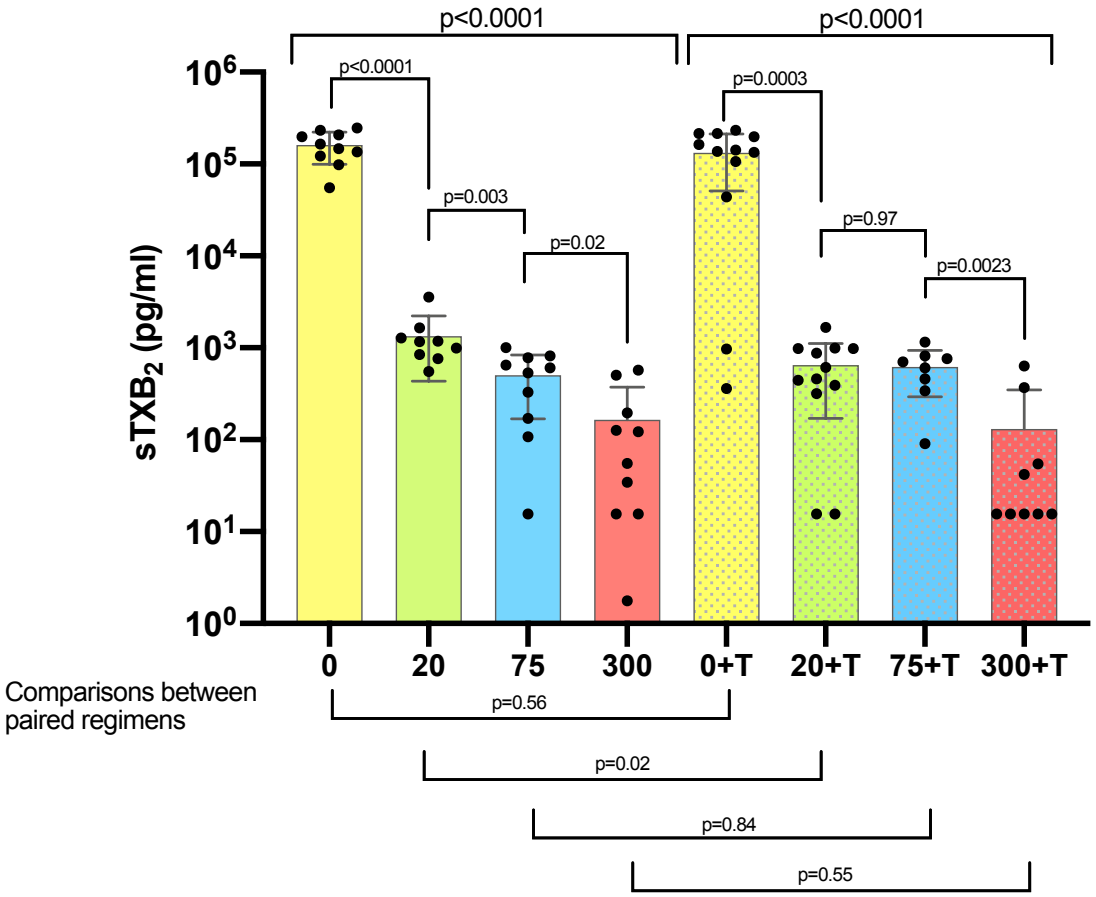

Figure 8.8 Serum thromboxane B_2 measured 1 hour after endotoxin injection by treatment group. Bars represent mean ± SD. ^{T} = Loading dose of ticagrelor 1 hour prior to endotoxin injection.

II. Effects on platelet aggregation responses

A summary of maximum aggregation responses, measured by LTA using AA, ADP and collagen as agonists, is shown in **Figures 8.12 to 8.17, Figure A.1 and Tables A.1 to A.5 (Appendix)**.

When not receiving ticagrelor, there was no significant variation over time in MA response to ADP 20 μmol/L, collagen 4 or 16 μg/mL, or AA 1 mmol/L in the no aspirin and aspirin 20 mg BD groups (**Figures 8.13 and 8.14, Table A.1[Appendix]**). When receiving aspirin 75 mg OD or 300 mg OD, though there was no significant variation over time in responses to AA or ADP, responses to collagen did significantly reduce between trough and peak effect (aspirin 75 mg OD: collagen 4 μg/mL, p=0.037; 300 mg OD : collagen 4 μg/mL, p=0.014; collagen 16 μg/mL, p=0.044).

When receiving a loading dose of ticagrelor at the -1 hour timepoint, maximum aggregation responses significantly reduced, over time, to all agonists in all treatment groups, with the exception of the responses to AA 1 mmol/L, which did not significantly change in those receiving any of the three regimens containing aspirin (**Figure 8.13**). Whilst effects of ticagrelor without aspirin on collagen-induced platelet aggregation were modest, in the presence of any dose of aspirin these appeared stronger, providing evidence of an additive effect (**Figures 8.16 and 8.17**).

MA responses were also compared at each timepoint within the ticagrelor-free and ticagrelor-receiving regimens (**Figure 8.12 and 8.15**). When not receiving ticagrelor, there was evidence of dose-dependency with regards to inhibition of collagen-induced aggregation, which was significant for comparisons between 300 mg OD and the other two aspirin regimens at -1 and +3 hours (when 4 μg/mL collagen was used as agonist). However, when receiving ticagrelor, there were no significant differences between the aspirin regimens at the 0 or +3 hour timepoints, suggesting the dose-dependency of aspirin's effect was to an extent ameliorated during DAPT, although numerical trends were still observed. Final aggregation responses followed similar patterns (**Tables A.6 and A.7 [Appendix]**).

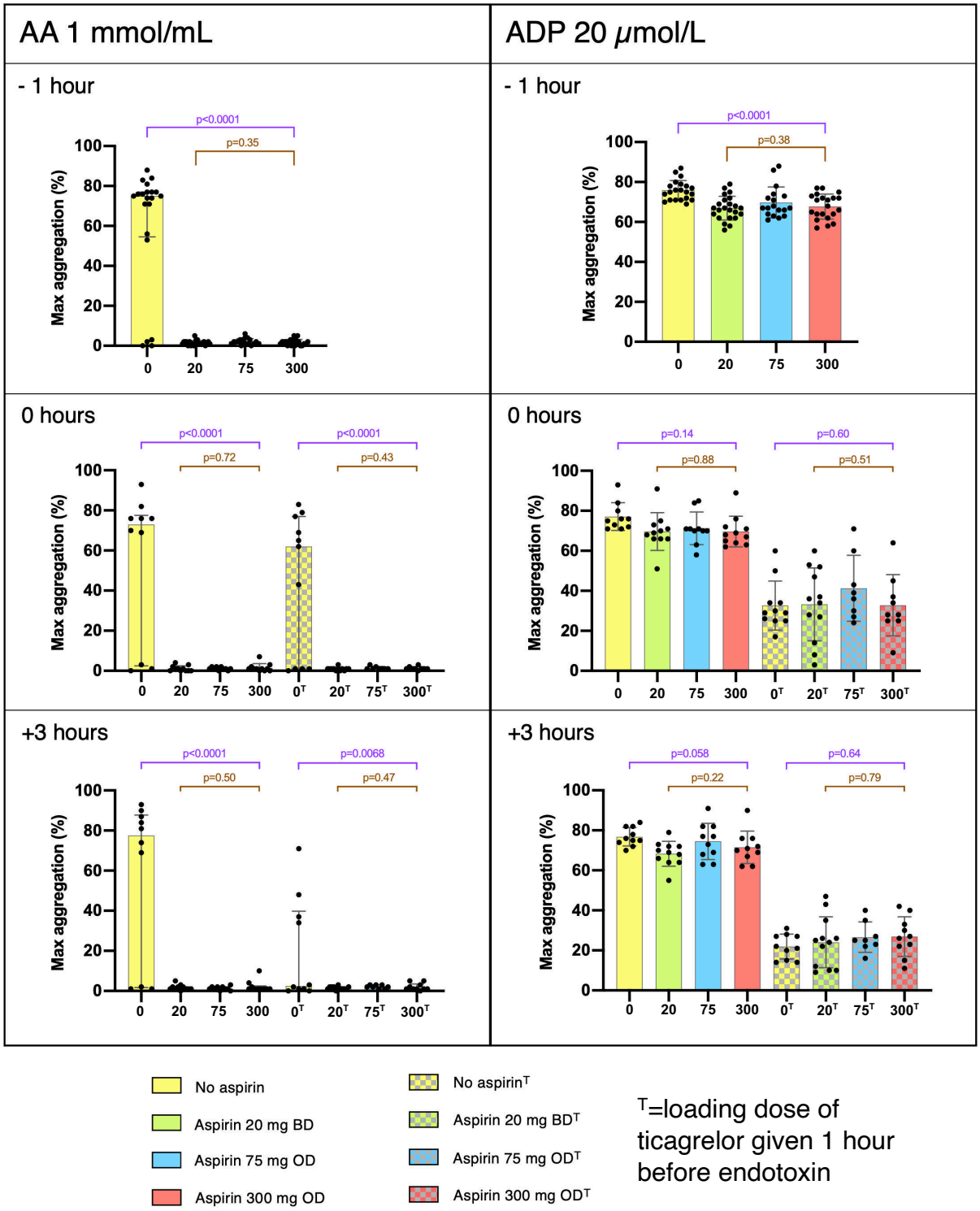

Figure 8.12 Maximum aggregation responses to AA and ADP during treatment with each regimen measured -1, 0 and 3 hours after endotoxin injection. P values generated by one-way ANOVA with treatment as factor.

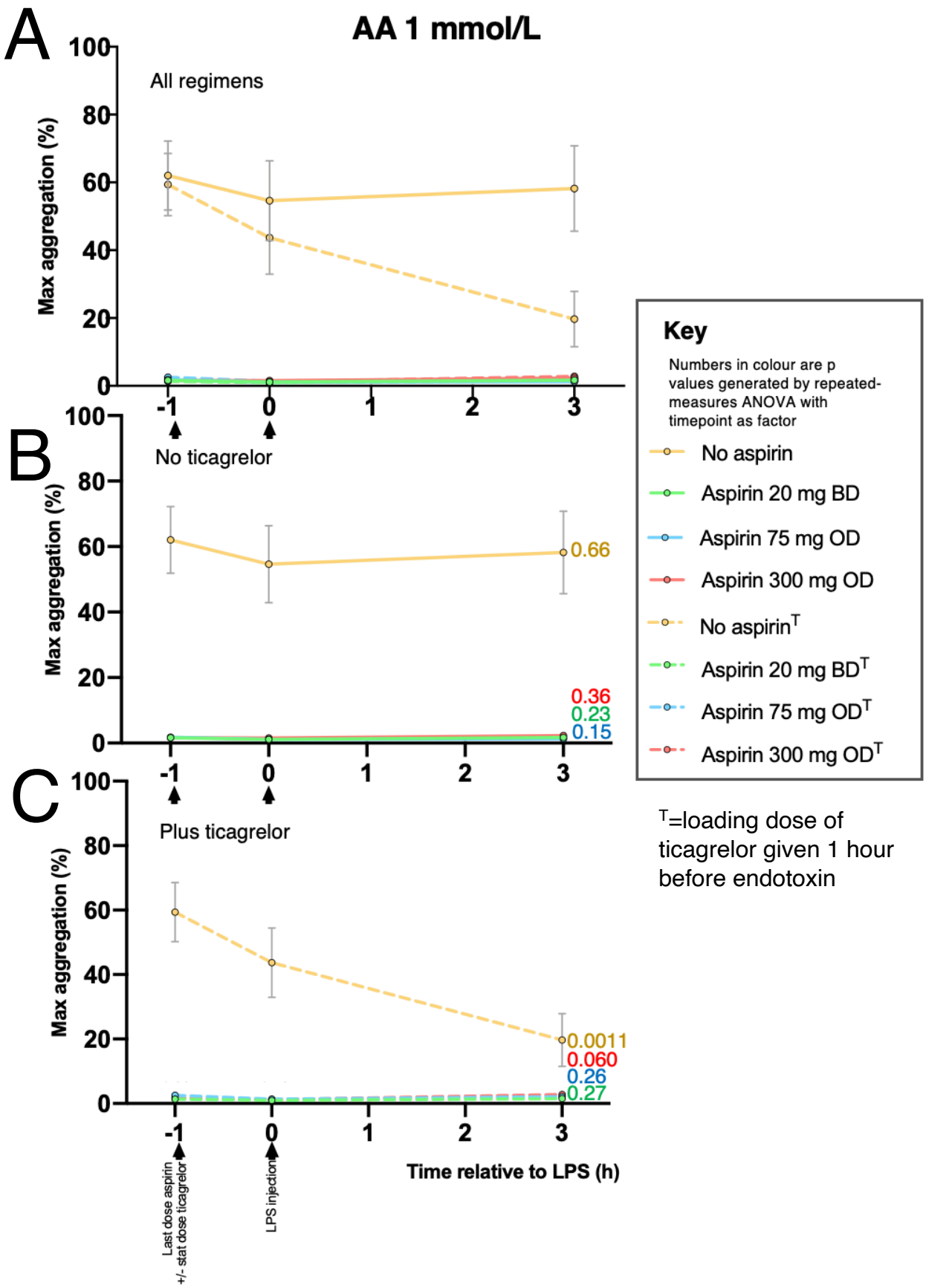

Figure 8.13 Maximum aggregation responses to AA over time for (A) all treatment groups (B) treatment groups not including ticagrelor and (C) treatment groups including ticagrelor. P values generated by repeated measures ANOVA with treatment as factor.

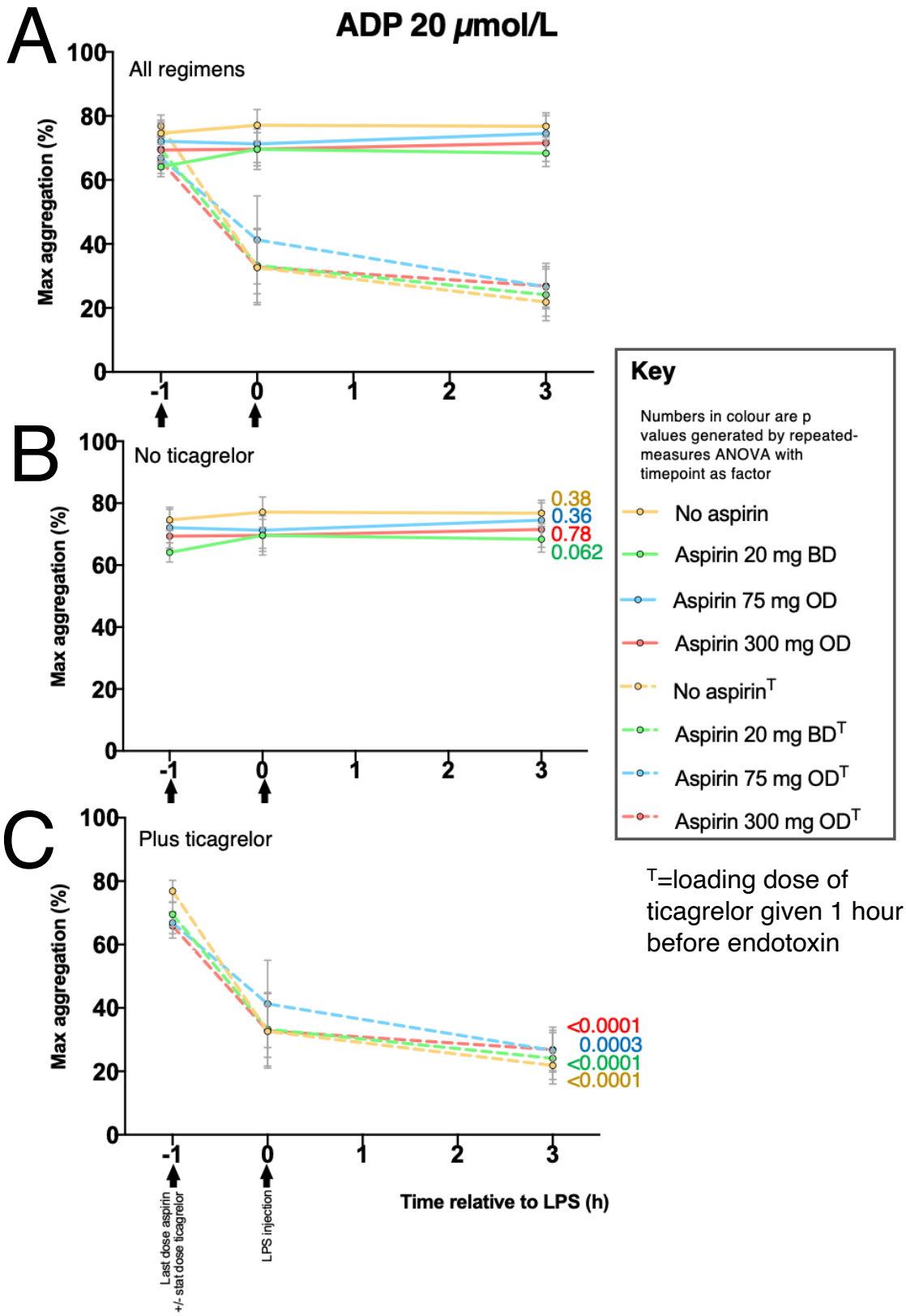

Figure 8.14 Maximum aggregation responses to ADP over time for (A) all treatment groups (B) treatment groups not including ticagrelor and (C) treatment groups including ticagrelor. P values generated by repeated measures ANOVA with treatment as factor.

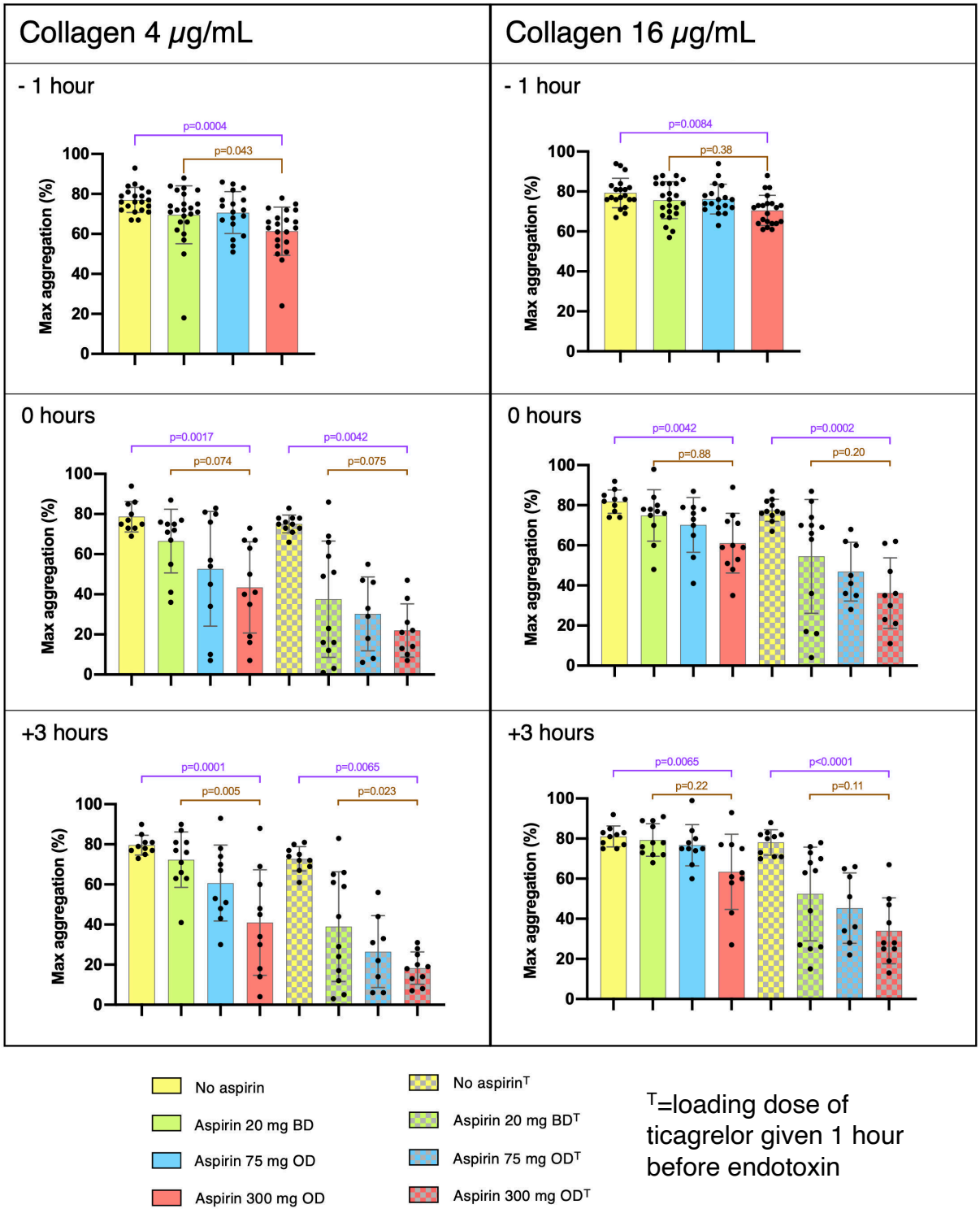

Figure 8.15 Maximum aggregation responses to collagen 4 and 8 µg/mL during treatment with each regimen measured -1, 0 and 3 hours after endotoxin injection. P values generated by one-way ANOVA with treatment as factor.

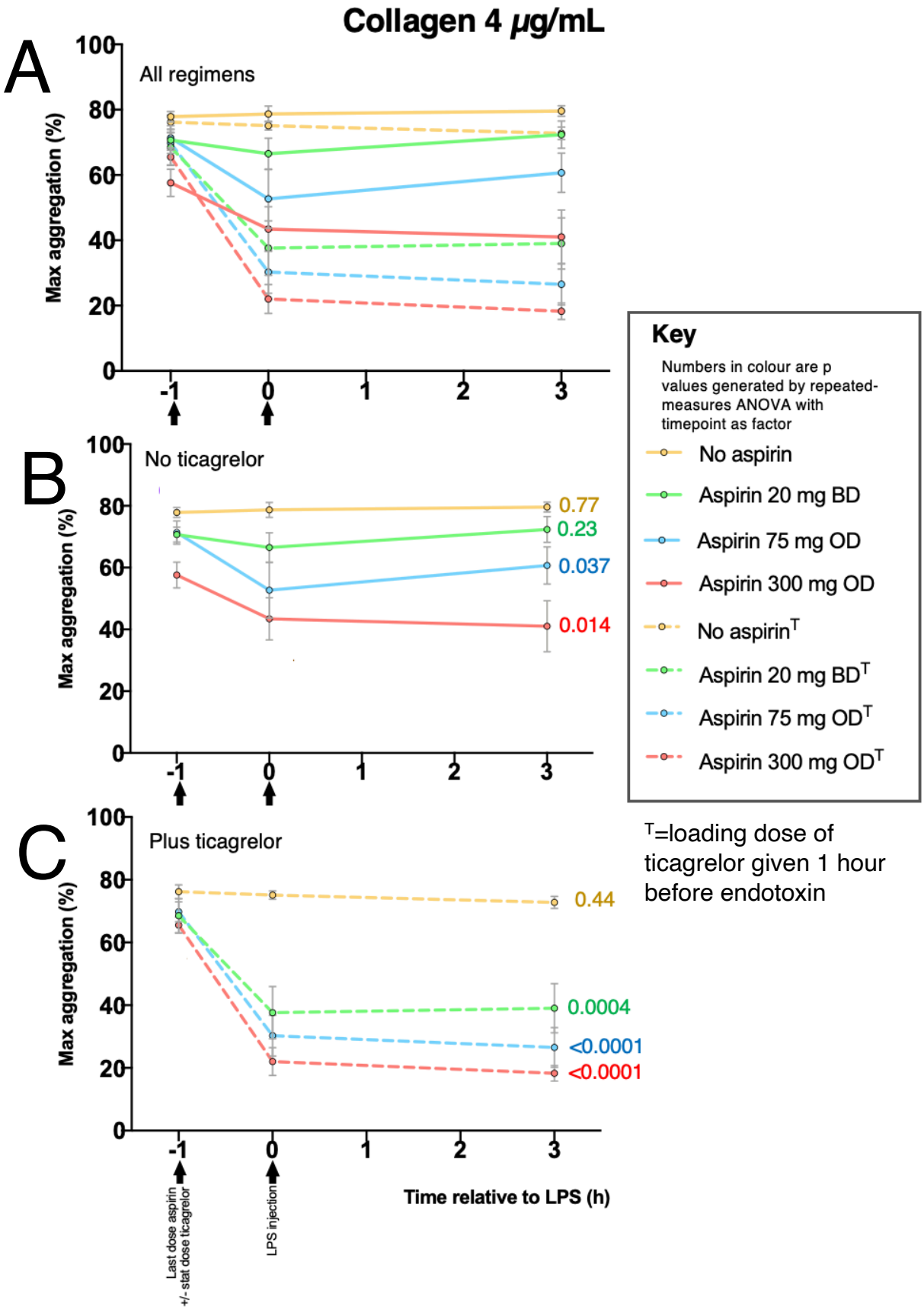

Figure 8.16 Maximum aggregation responses to collagen (4 μg/mL) over time for (A) all treatment groups (B) treatment groups not including ticagrelor and (C) treatment groups including ticagrelor. P values generated by repeated measures ANOVA with treatment as factor.

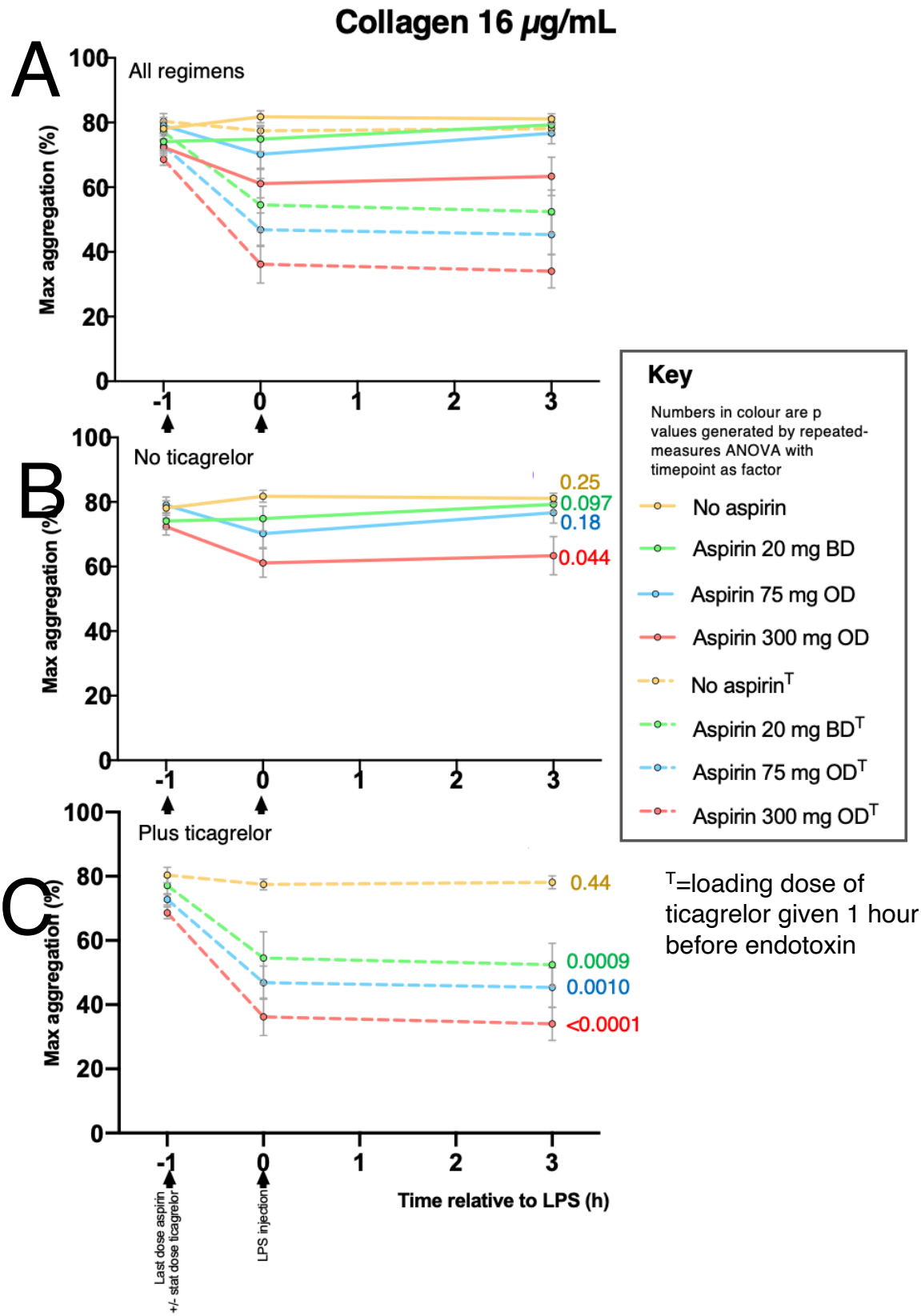

Figure 8.17 Maximum aggregation responses to collagen (16 µg/mL) over time for (A) all treatment groups (B) treatment groups not including ticagrelor and (C) treatment groups including ticagrelor. P values generated by repeated measures ANOVA with treatment as factor.

III. Platelet P-selectin expression

Platelet P-selectin expression after stimulation of whole blood with ADP (final concentration 30 μmol/L) was measured as % of population positive and median fluorescence intensity (MFI), one hour before and six hours after endotoxin injection.

Whether measuring as % of population or MFI, there was a significant reduction in platelet P-selectin expression between the -1 and +6 hour timepoints when receiving any treatment regimen, including no antiplatelet therapy (**Table A.8 [Appendix], Figures 8.18 and 8.19**).

Aspirin had no effect on platelet P-selectin expression at either timepoint (**Table 8.5**).

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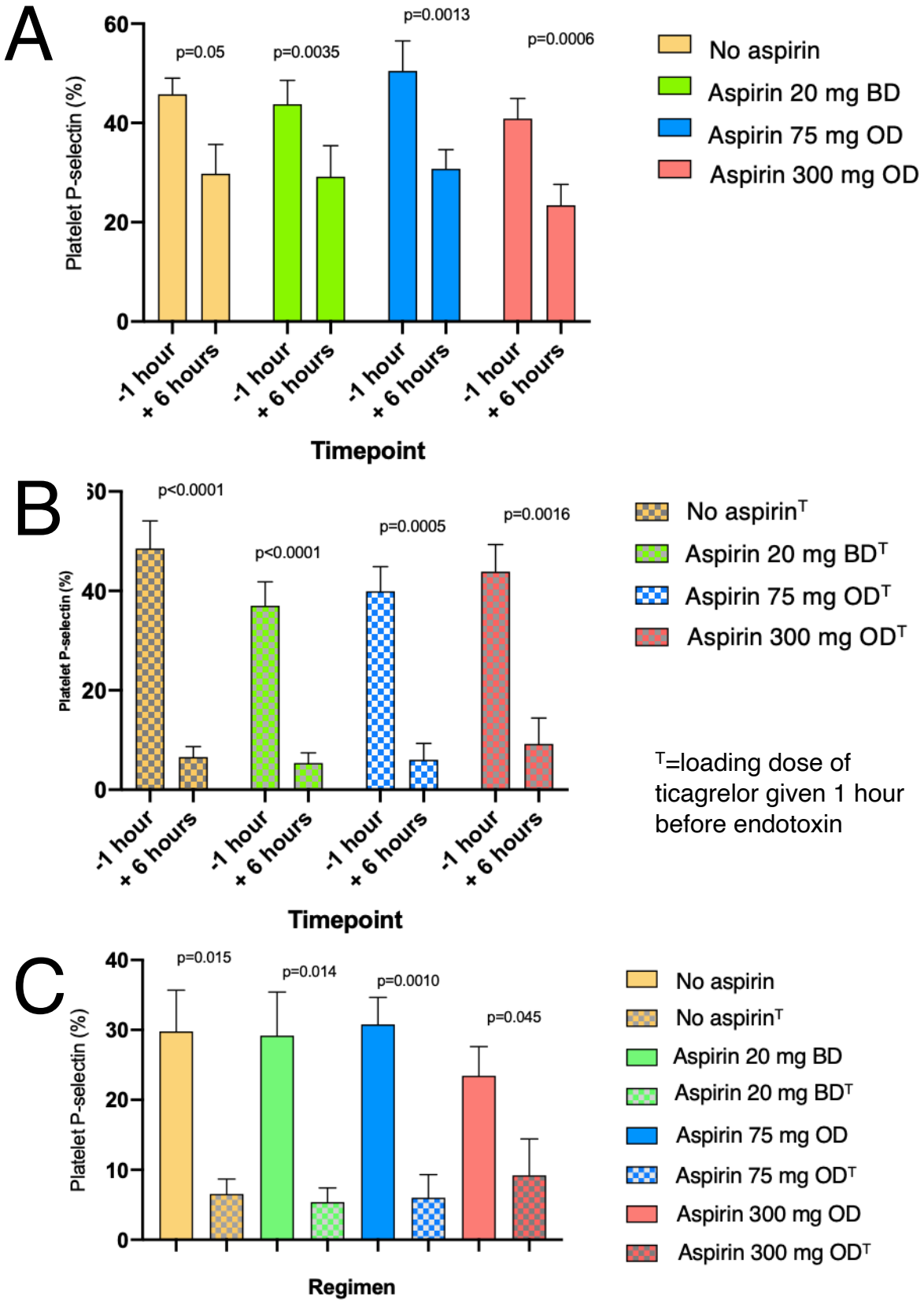

Figure 8.18 Platelet P selectin (% of population) compared (A) between timepoints when not receiving ticagrelor, (B) between timepoints when received ticagrelor and (C) at 6 hours after endotoxin injection compared between paired ticagrelor-no ticagrelor regimens. P values generated by unpaired (A, B) and paired (C) t-tests. Bars show mean + SD.

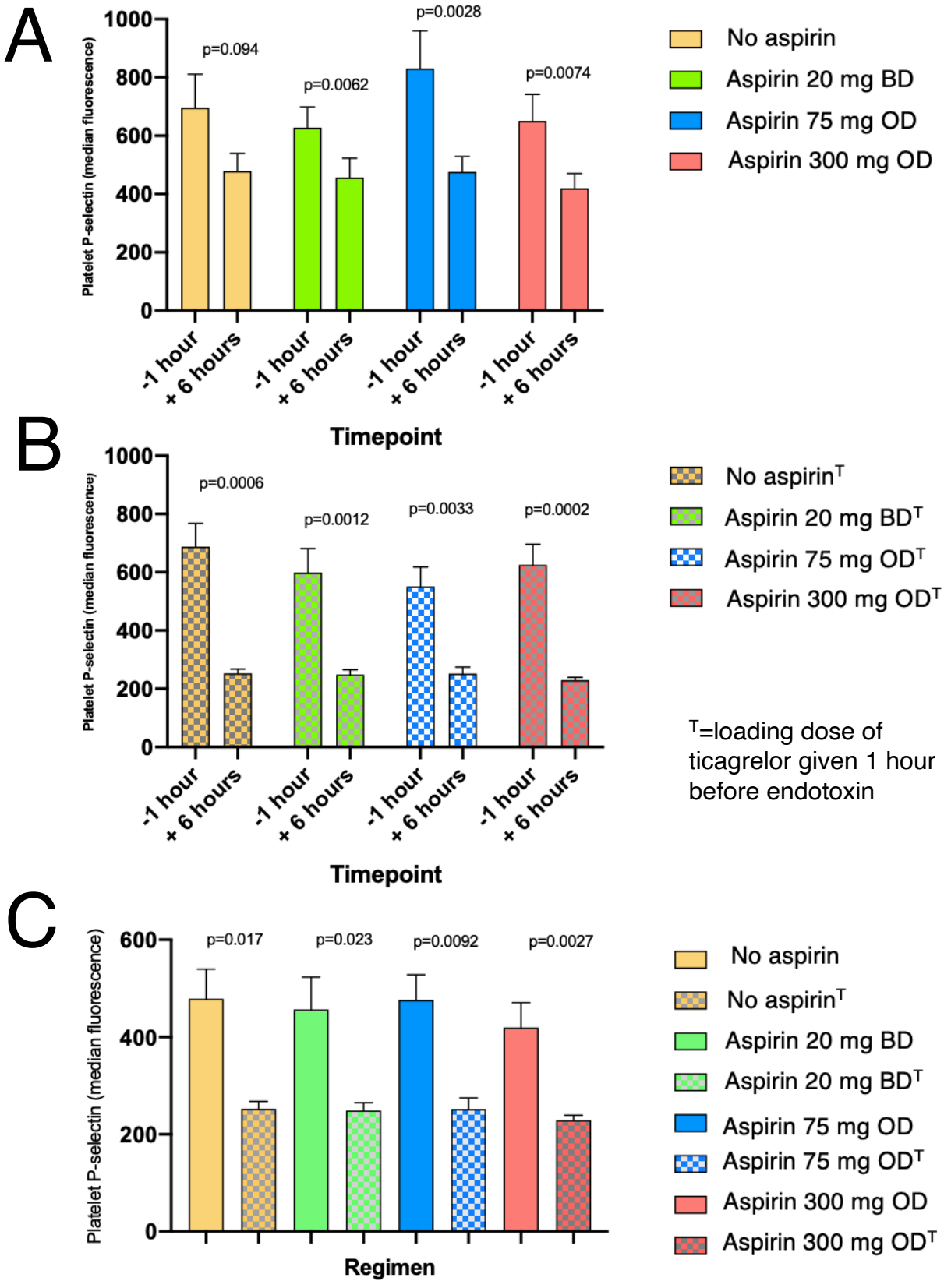

Figure 8.19 Platelet P selectin (median fluorescence) compared (A) between timepoints when not receiving ticagrelor, (B) between timepoints when received ticagrelor and (C) at 6 hours after endotoxin injection compared between paired ticagrelor-no ticagrelor regimens. P values generated by unpaired (A, B) and paired (C) t-tests. Bars show mean + SD.

Table 8.5 P values for comparisons of platelet P-selectin expression between regimens at each time point (one-way ANOVA)

	P value	
	-1 hour	**+6 hours**
Platelet P-selectin expression (%)		
All regimens	0.56	<0.0001
Did not receive ticagrelor	0.53	0.72
Received ticagrelor	0.42	0.85
Platelet P-selectin expression (median fluorescence)		
All regimens	0.59	<0.0001
Did not receive ticagrelor	0.51	0.87
Received ticagrelor	0.68	0.70

D. Haemostasis

Forearm bleeding time was measured at randomisation (before any study medication), 1 hour before endotoxin injection (just before the last dose of aspirin), and 3 hours after endotoxin injection (4 hours after the last dose of aspirin +/- a single dose 180 mg of ticagrelor) (**Figures 8.20 and 8.21, Table A.9 [Appendix]**).

At trough aspirin effect and before endotoxin, though mean bleeding time was numerically longer when receiving aspirin 20 mg BD compared to no aspirin, this was not statistically significantly different (p=0.18 compared to no aspirin group at same timepoint, p=0.17 compared with paired value at randomisation [data not shown]). Both aspirin 75 mg OD and 300 mg OD prolonged bleeding time compared to either no aspirin (p=0.0096 & p=0.024 respectively) or 20 mg BD (p=0.015 & p=0.034). There was no significant difference in bleeding time between those receiving aspirin 75 mg OD and 300 mg OD (p=0.34).

At 3 hours after endotoxin/4 hours after last dose of study medication, ticagrelor prolonged mean bleeding time regardless of aspirin regimen. Though there was a similar numerical pattern in the dose-dependent effect of aspirin on bleeding time, there were no significant differences between aspirin dosing regimens (including no aspirin) either when receiving ticagrelor (Kruskal-Wallis test, p=0.31) or not (p=0.60).

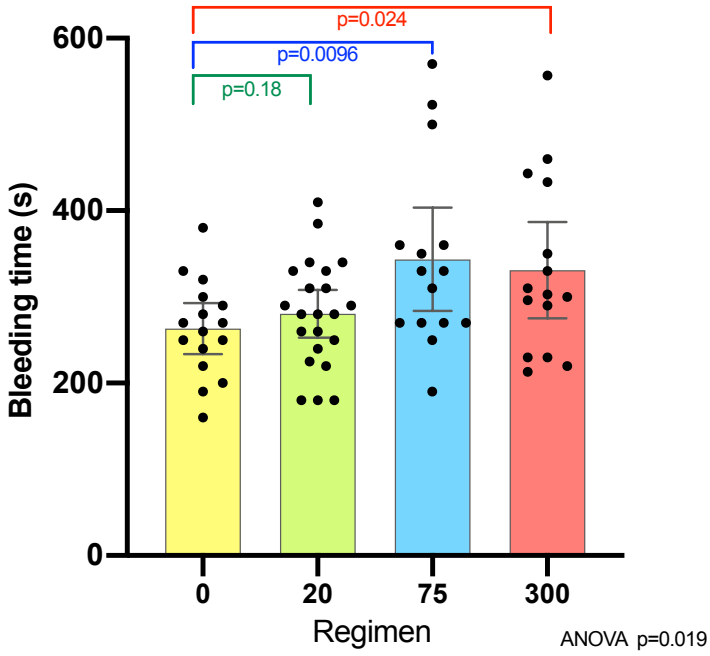

Figure 8.20 Forearm bleeding time measured immediately before the last dose of aspirin (trough effect), 1 hour before endotoxin injection in participants receiving no aspirin ('0'), aspirin 20 mg BD ('20'), aspirin 75 mg OD ('75') or aspirin 300 mg OD ('300').

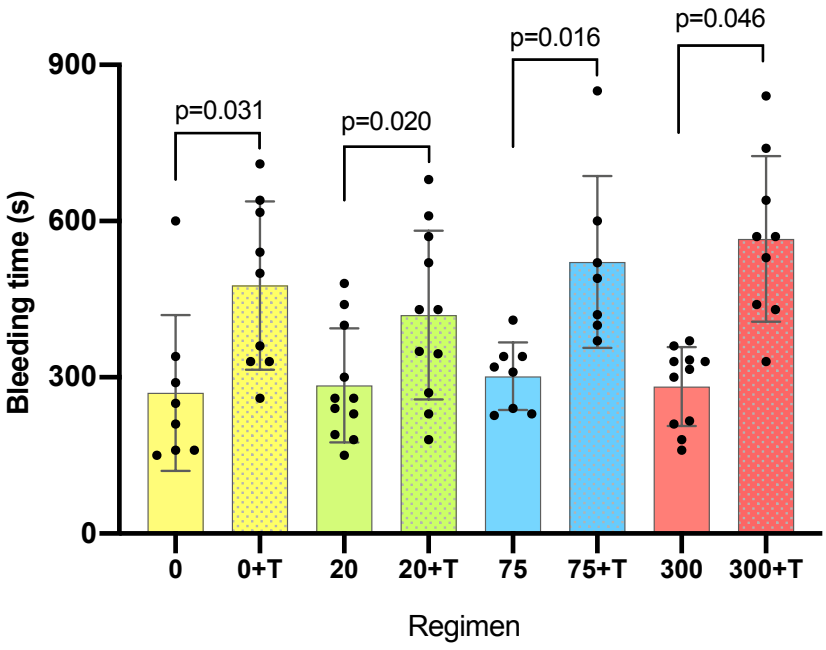

Figure 8.21 Forearm bleeding time measured 3 hours after endotoxin injection (4 hours after the last dose of aspirin) in participants receiving no aspirin ('0'), aspirin 20 mg BD ('20'), aspirin 75 mg OD ('75') or aspirin 300 mg OD ('300'), with ('+T') or without a single 180 mg dose of ticagrelor 1 hour prior to endotoxin administration.

E. Discussion

The data in this chapter derive from an interim analysis, performed after the trial was halted due to the COVID-19 pandemic. Formal criteria for determining if the study had either reached its endpoint or was futile were not met (see Chapter 9). As such, a formal hierarchical analysis was not performed and any analyses were deemed exploratory. Clearly the power of any statistical test is compromised compared to at full completion of the study. This becomes even more the case if correcting for multiple tests when, for example, following up the result of a significant ANOVA result. For this reason, the decision was made not to correct for multiple testing in the analyses. As such, there is a risk of either over-interpreting apparently significant findings due to type I error or not recognising the importance of observed trends because of a lack of power resulting in type II error.

Nevertheless, the data obtained during the study so far can provide some valuable insights into what the eventual final study analysis may more robustly demonstrate. Moreover, they have considerable novelty as the first comparison of the effects of different aspirin regimens (and the effect of combining ticagrelor with these) during experimental endotoxaemia.

Firstly, the data suggest that clinically used regimens of aspirin, 75 mg OD and 300 mg, at peak effect and during endotoxaemia, inhibit TXA_2 generation in a dose-dependent manner, whether ticagrelor is given alongside or not. Aspirin 20 mg BD, on the other hand, provided reduced inhibition compared to 75 mg OD when given alone, but similar levels when given with ticagrelor. This is in contrast to the WILLOW ACS study, which demonstrated significantly reduced inhibition by 20 mg BD compared to 75 mg OD in ticagrelor-treated patients at an equivalent timepoint. Whilst obtaining more data will help to confirm or refute this more robustly, reasons for this discrepancy might include the fact that aspirin in healthy volunteers, who by definition were free of co-medications and co-morbidities but were also on average younger and lighter, may have greater bioavailability than in the ACS patients included in the previous study. Similarly, it is possible that the kinetics and/or dynamics of ticagrelor were not comparable between the maintenance regimen studied in WILLOW ACS and the single loading dose given in the present study. Whilst ticagrelor is known to have no effect on serum TXB_2 levels either alone or in combination with, for example, aspirin 75 mg OD (because levels are maximally suppressed by the aspirin already), hypothetically there may be an additive effect between doses of aspirin leading to submaximal COX1 inhibition (e.g. 20 mg BD) and ticagrelor. Furthermore, it is plausible that if 75 mg OD potentiates the inflammatory response to endotoxin, as pointed to

by the inflammatory data, this might include a more intense underlying surge of TXA_2 generation during endotoxaemia that might balance out any greater potency of inhibition.

What are not assessed here are any countereffects on PGI_2 release from the endothelium. For example, though aspirin 300 mg OD led to more inhibition of TXA_2 generation and collagen-induced platelet aggregation than 75 mg OD, it would be expected to also lead to significantly greater PGI_2 inhibition. It is planned to measure urinary PGI_2 metabolites in the final analysis of the study and this will help to address this point. Similarly, this interim analysis has only included samples from 1 hour after endotoxin, chosen because animal studies suggest this is the time that inflammation-induced TXA_2 generation peaks after endotoxin injection. It is planned to measure serum TXB_2 at multiple timepoints in the final study analysis, as well as urinary thromboxane metabolites.

Though there was less difference in peak TXA_2 inhibition between ticagrelor-treated individuals receiving aspirin 20 mg BD or 75 mg OD than might have been suggested by the WILLOW ACS study, a more familiar pattern was observed with respect to collagen-induced platelet aggregation, which is a useful marker because it is typically neither maximally inhibited by aspirin nor $P2Y_{12}$ inhibitors, allowing more nuanced differences in strength of effect to be determined. At trough aspirin effect, aspirin 20 mg BD and 75 mg OD offered similar levels of inhibition whilst, at peak effect, 75 mg OD provided stronger inhibition, following the pattern observed in WILLOW ACS. Reassuringly, despite any differential effects on inhibition of TXA_2 generation, AA-induced platelet aggregation remained fully suppressed by all of the aspirin-containing regimens tested. Adding ticagrelor only had a significant effect on AA-induced aggregation when given alone, but was not as potent as any dose of aspirin in its effect on this pathway. Whereas maximal ADP-induced aggregation was achieved by 1 hour after antiplatelet dosing (timepoint 0 in relation to endotoxin), AA-induced aggregation was significantly more inhibited by ticagrelor (in the absence of aspirin) at 4 hours after dosing compared to 1 hour (3 hours after endotoxin compared to 0 hours). On the one hand, this might suggest that, although by the time of endotoxin injection adequate ticagrelor had been absorbed to inhibit ADP-induced responses, plasma levels rose further over the next 3 hours, reflected in more potent inhibition of AA-induced aggregation by this point. On the other hand, given findings regarding platelet P-selectin (discussed below), it may be evidence of platelet exhaustion. In this study, it was decided to include a single loading dose rather than maintenance regimens of ticagrelor, in order to minimise participant risk (particularly when receiving the higher doses of aspirin) and to avoid any issues with intolerance.

The finding that platelet P-selectin was reduced significantly between 1 hour before and 6 hours after endotoxin injection regardless of drug regimen was not observed in the previous study of Thomas et al (2015), which found no significant effect when receiving no antiplatelet medication. This may hypothetically be related to a degree of platelet exhaustion, which has been observed to occur during and after intense inflammatory states (Pareti et al. 1980; Margraf and Zarbock 2019). Platelet P-selectin expression was not assessed in other studies of platelet function during endotoxaemia (Spiel et al. 2012; Kiers et al. 2017). Endotoxaemia is known to increase levels of soluble P-selectin (Jilma-Stohlawetz et al. 2001), but this does not appear to correlate with surface expression (McCabe et al. 2004). Furthermore, there is evidence that human P-selectin expression reduces on stimulation with cytokines such as TNF-α (Liu et al. 2010). There were no significant methodological differences between this study and that of Thomas et al (2015), other than a different batch of endotoxin being used. The present lot may be associated with higher peak levels of cytokines such as TNF-α (Kiers et al. 2019) and is a possible explanation. The finding that ticagrelor, but not aspirin, caused a (further) significant reduction in P-selectin expression concurs with previous work (Storey et al. 2002; Thomas et al. 2015; Parker et al. 2020).

At trough drug effect, bleeding time was significantly shorter when receiving aspirin 20 mg BD compared to 75 mg OD or 300 mg OD. Trough effect measures of bleeding time in those receiving ticagrelor were not available due to the design of the study. Ticagrelor lengthened bleeding time regardless of presence or dose of aspirin, consistent with healthy volunteer studies in the non-endotoxaemic state (Teng et al. 2013). However, there were no differences between the aspirin regimens at peak drug effect during endotoxaemia, which is in contrast to the findings of the WILLOW ACS study that showed significantly shorter bleeding time when receiving aspirin 20 mg BD compared to 75 mg OD in ticagrelor-treated ACS patients at 2 hours post-dose. As well as the fact that the post-dose timepoint in this study was 4 hours, there are other factors that may hypothetically influence bleeding time in the endotoxaemic compared to non-endotoxaemic state. These may include pyrexia, the vasomotor response to endotoxin (which can include both peripheral vasoconstriction or vasodilatation at different phases) and proinflammatory changes in fibrin clot dynamics (Suffredini et al. 1999; Thomas et al. 2015). These may explain why aspirin alone did not appear to prolong bleeding time, though obtaining more data during the next phase of the study will help to define this better.

Heart rate followed a lower trend when receiving aspirin 300 mg OD and this was nominally significant. Although there is some evidence that aspirin may reduce heart rate during an experimental animal model of myocardial infarction (Schoemaker et al. 1998), it may be more

plausible that in the present study this was related to antipyretic effects, which are mediated via COX2 inhibition and therefore greater when receiving aspirin 300 mg OD than lower doses (Vane and Botting 2003). Heart rate certainly correlated with temperature (**Figure 8.22**). Similarly, it is feasible that this finding was due to chance alone as there was a trend towards variation in heart rate at randomisation (p=0.1 between the groups) with higher heart rate in those allocated to aspirin 20 mg BD and lower in those allocated to 300 mg (**Table 7.2**).

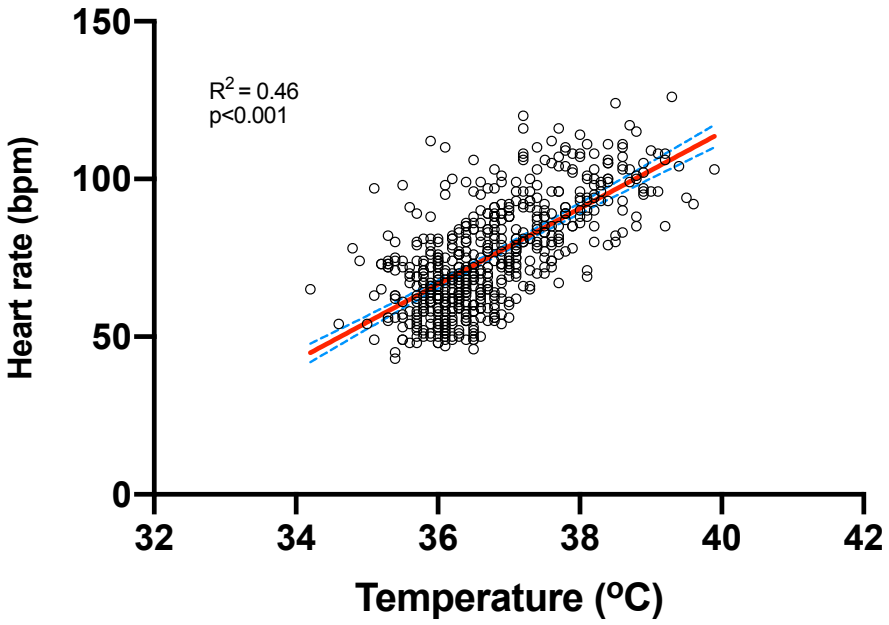

Figure 8.22 Correlation of heart rate with temperature, analysed using simple linear regression. Solid red line indicates line of regression, blue dashed lines indicate 95% confidence intervals.

Regarding the differences seen in laboratory safety blood tests between the regimens at the end of the treatment period, the finding that serum albumin was lower when receiving aspirin 300 mg OD may be consistent with evidence of a higher baseline state of inflammation (Don and Kaysen 2004), discussed further in Chapter 9. Serum potassium was also greater when receiving aspirin 300 mg OD than other regimens. Although some inorganic ions do bind to albumin, with regards to potassium this is minimal and therefore any differences in albumin levels would not account for this observation (van Os and Koopman-van Eupen 1957). Similarly, the excipients of the aspirin lysine preparation used in the study do not include any potassium-containing compounds. Feasible explanations may include that this effect is due to the modestly greater oral acid load

when receiving the higher dose of aspirin (Bovée et al. 2020), the inhibition of renal PGI_2 resulting in reduced secretion of potassium in the distal convoluted tubule (Aljadhey et al. 2010), or related to chance as a result of interindividual variation and multiple testing. Measuring PGI_2 metabolite and free potassium levels in the urine may help to explore this further. Finally, haemoglobin appeared to be higher when receiving aspirin 20 mg BD compared to 300 mg OD. Whilst there is some evidence that factors inhibited by aspirin, for example PGE_2, may play a role in haematopoesis (North et al. 2007), it is plausible that this was a chance finding as, although there were no significant differences between haemoglobin at enrolment, there was a trend towards higher levels in those randomised to aspirin 20 mg BD (see **Table 7.2**).

Chapter 9: Dose-dependent effects of aspirin, with or without ticagrelor, on markers of inflammation during experimental human endotoxaemia

The main objective of the WILLOW TREE study is to determine effects of aspirin dosing on the inflammatory response to endotoxaemia. When completed, the trial is expected to have good power to assess these endpoints but at the point of the interim analysis this is less the case. Nevertheless, data obtained thus far during the study can provide early insights into important effects and are therefore included in this thesis to provoke discussion.

A. Core body temperature

Mean core body temperature significantly increased over time after endotoxin injection in all treatment groups (all $p<0.0001$) (**Table 9.1, Figure 9.1**). When assessed by two-way repeated measures ANOVA with timepoint as the within-subject factor and treatment as the between-subject factor, there were no significant relationships with treatment identified, although graphically there was a trend towards increased body temperature when receiving aspirin 75 mg OD and ticagrelor, compared to the other aspirin (or no aspirin) plus ticagrelor groups (**Figure 9.1**). Given the small sample size and exploratory nature of this analysis, an alternative method of trend analysis was deployed. Locally weighted polynomial regression ('LOWESS') can help to identify trends in data with more complex non-linear relationships through data modelling. This was carried out for body temperature data using RStudio version 1.1.456 using the software's default settings for LOWESS within the 'geom_smooth' function of the 'ggplot2' package. There was evidence, denoted by a lack of overlapping 95% confidence intervals, of a significantly higher peak temperature in those receiving aspirin 75 mg OD, either with ticagrelor or when grouped with those not receiving ticagrelor, compared to other aspirin regimens or no aspirin. There was also a trend towards a flatter temperature curve when receiving aspirin 300 mg OD (with or without ticagrelor, **Figure 9.1**) but this did not appear significant on two-way ANOVA or polynomial regression (**Figure 9.2**). Overall, ticagrelor status did not appear to influence temperature (**Figure 9.2**).

Table 9.1 Results of comparisons using two-way repeated measures ANOVA (with timepoint as within-subject factor and treatment as between-subject factor) of body temperature. Data shown are p values.

All regimens			No ticagrelor			Ticagrelor		
Timepoint	Treatment group	Timepoint* Treatment group	Timepoint	Treatment group	Timepoint* Treatment group	Timepoint	Treatment group	Timepoint* Treatment group
<0.0001	0.42	0.32	<0.0001	0.27	0.43	<0.0001	0.44	0.11

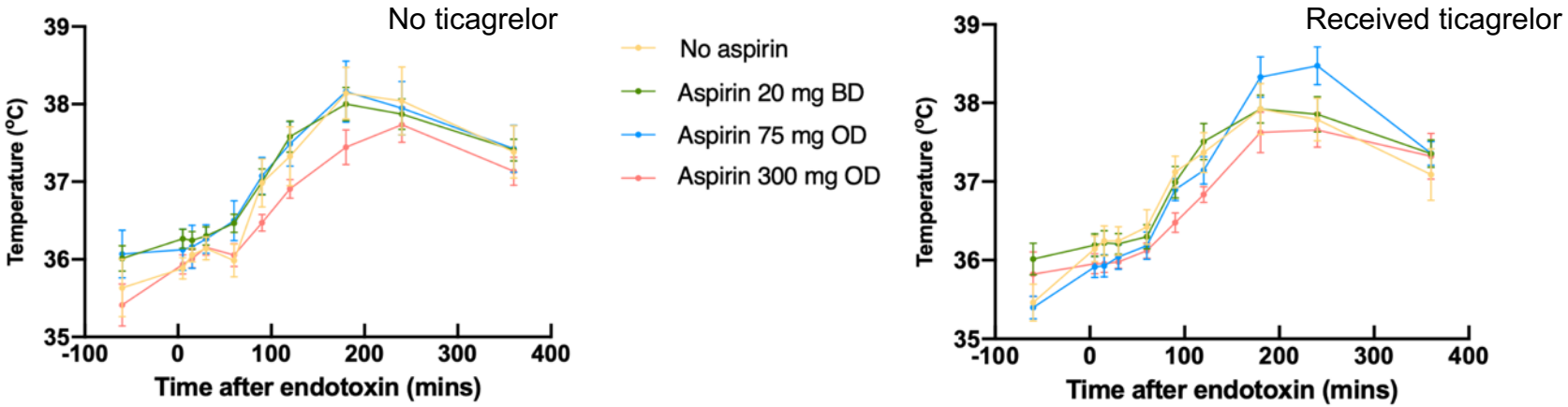

Figure 9.1 Body temperature before and after endotoxin injection when receiving (right) and not receiving (left) ticagrelor. Bars represent mean ± SD.

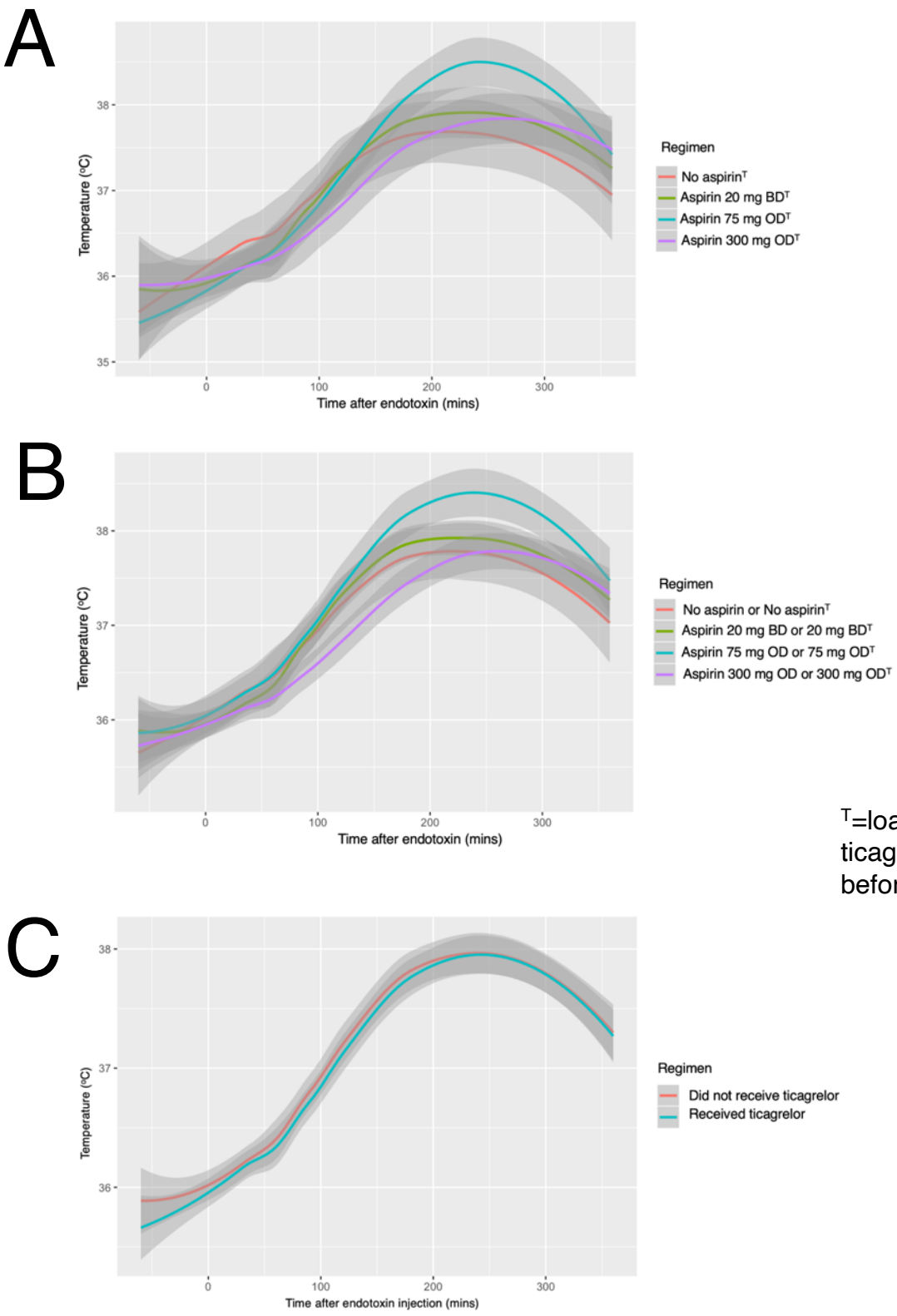

Figure 9.2 Locally weighted polynomial regression plots of temperature when receiving ticagrelor-containing regimens (A), all regimens (grouped by aspirin dose) (B), or all regimens (grouped by ticagrelor status) (C). Solid lines indicate smoothed mean and shaded area 95 % CI.

B. Changes in leukocyte counts and activation markers

Total leukocyte count and that of all subsets significantly changed over time (**Table 9.2, Figure 9.3**). When receiving ticagrelor, there was evidence of a significant effect of treatment group on leukocyte count (p=0.048) and mixed cell count (p=0.03). Treatment group had no significant effect on counts when not receiving ticagrelor, but there was a significant interaction with regards to mixed cell count between timepoint and treatment (p=0.0023).

Monocyte and neutrophil CD11b and TLR4 expression changed significantly with time, with the exception of monocyte (p=0.22) and neutrophil (0.063) TLR4 (measured as MFI) when not receiving ticagrelor, and neutrophil TLR4 (measured as % positive) either when receiving (p=0.23) or not receiving (p=0.19) ticagrelor (**Table 9.2, Figures 9.4 and 9.5**). Treatment group had no significant effect on CD11b or TLR4 expression, although there was close to a significant effect on monocyte TLR4 expression (% positive, p=0.061). Furthermore, there was a significant interaction between timepoint and treatment with reference to monocyte TLR4 expression (% positive, p=0.049) when not receiving ticagrelor, and monocyte and neutrophil TLR4 (MFI, p<0.0001 and p=0.012 respectively) when receiving it.

To gain more insight into these complex findings, further repeated-measures ANOVAs were performed for the variables found to show significant relationships with treatment or timepoint*treatment, but only including two regimens at a time, thus allowing pairwise comparisons to be made. This identified a number of pairwise comparisons with significant differences (**Tables 9.3 and 9.4**).

Table 9.2 Results of comparisons between regimens of leukocyte counts, and monocyte and neutrophil CD11b and TLR4 expression using two-way repeated-measures ANOVA (timepoint as within-subject factor and treatment as between-subject factor). Values shown are p values.

	Did not receive ticagrelor			Received ticagrelor		
	Timepoint	Treatment group	Timepoint* Treatment group	Timepoint	Treatment group	Timepoint* Treatment group
Leukocyte count	**<0.0001**	0.46	0.97	**<0.0001**	**0.048**	**0.041**
Neutrophil count	**<0.0001**	0.59	0.98	**<0.0001**	0.078	**0.027**
Mixed cell count	**<0.0001**	0.73	**0.0023**	**<0.0001**	**0.03**	0.35
Lymphocyte count	**<0.0001**	0.24	0.14	**<0.0001**	0.12	**0.034**
Monocyte CD11b median fluorescence	**<0.0001**	0.73	0.78	**<0.0001**	0.31	0.64
Monocyte TLR4 expression (% positive)	**<0.0001**	0.061	**0.049**	**<0.0001**	0.33	0.72
Monocyte TLR4 median fluorescence	0.22	0.55	0.85	**0.019**	0.18	**<0.0001**
Neutrophil CD11b median fluorescence	**<0.0001**	0.55	0.19	**<0.0001**	0.16	0.68
Neutrophil TLR4 expression (% positive)	0.19	0.84	0.85	0.23	0.87	0.32
Neutrophil TLR4 median fluorescence	0.063	0.6	0.82	**0.0017**	0.85	**0.012**

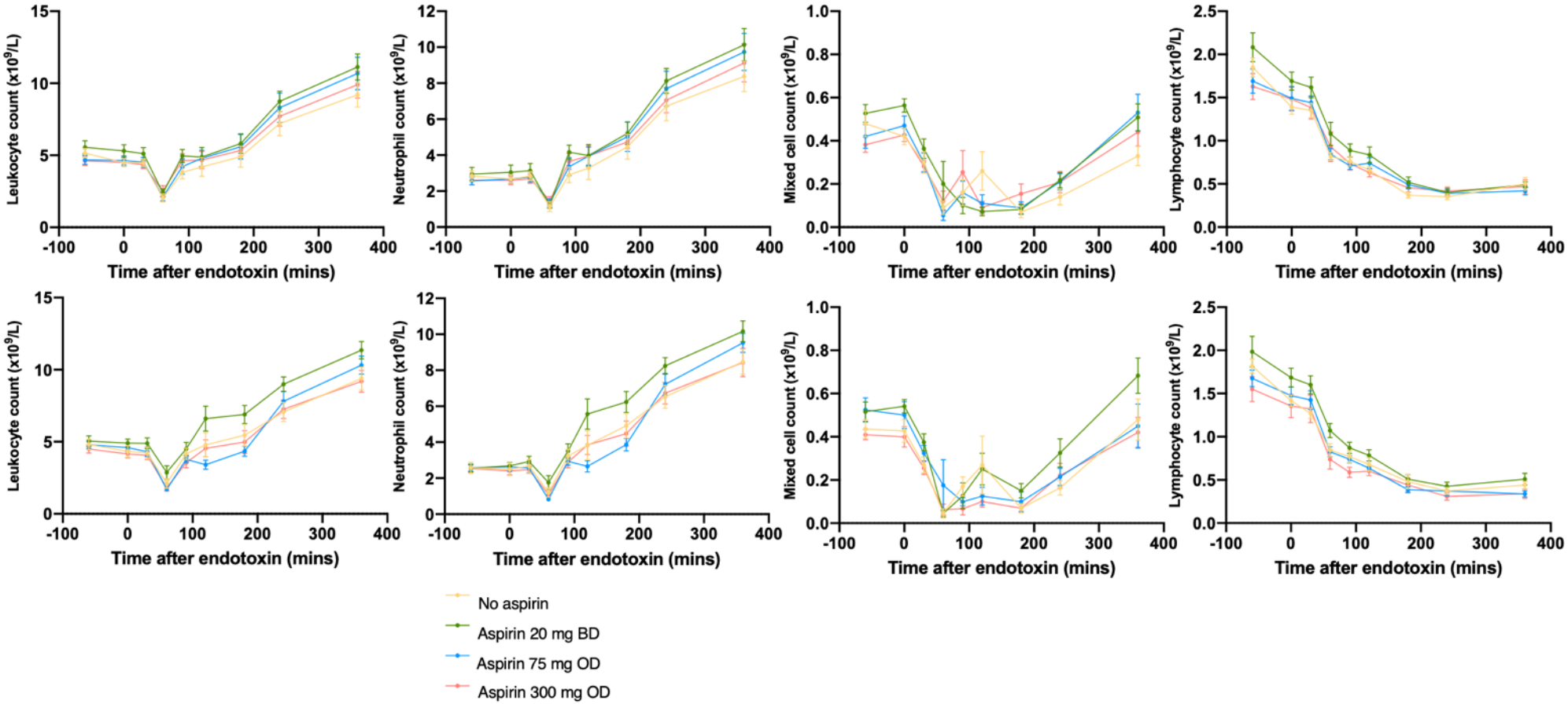

Figure 9.3 Leukocyte count and subsets before and after endotoxin injection in participants receiving no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD with (bottom panel) or without (top panel) a loading dose of ticagrelor one hour before endotoxin. Bars represent mean ± SEM.

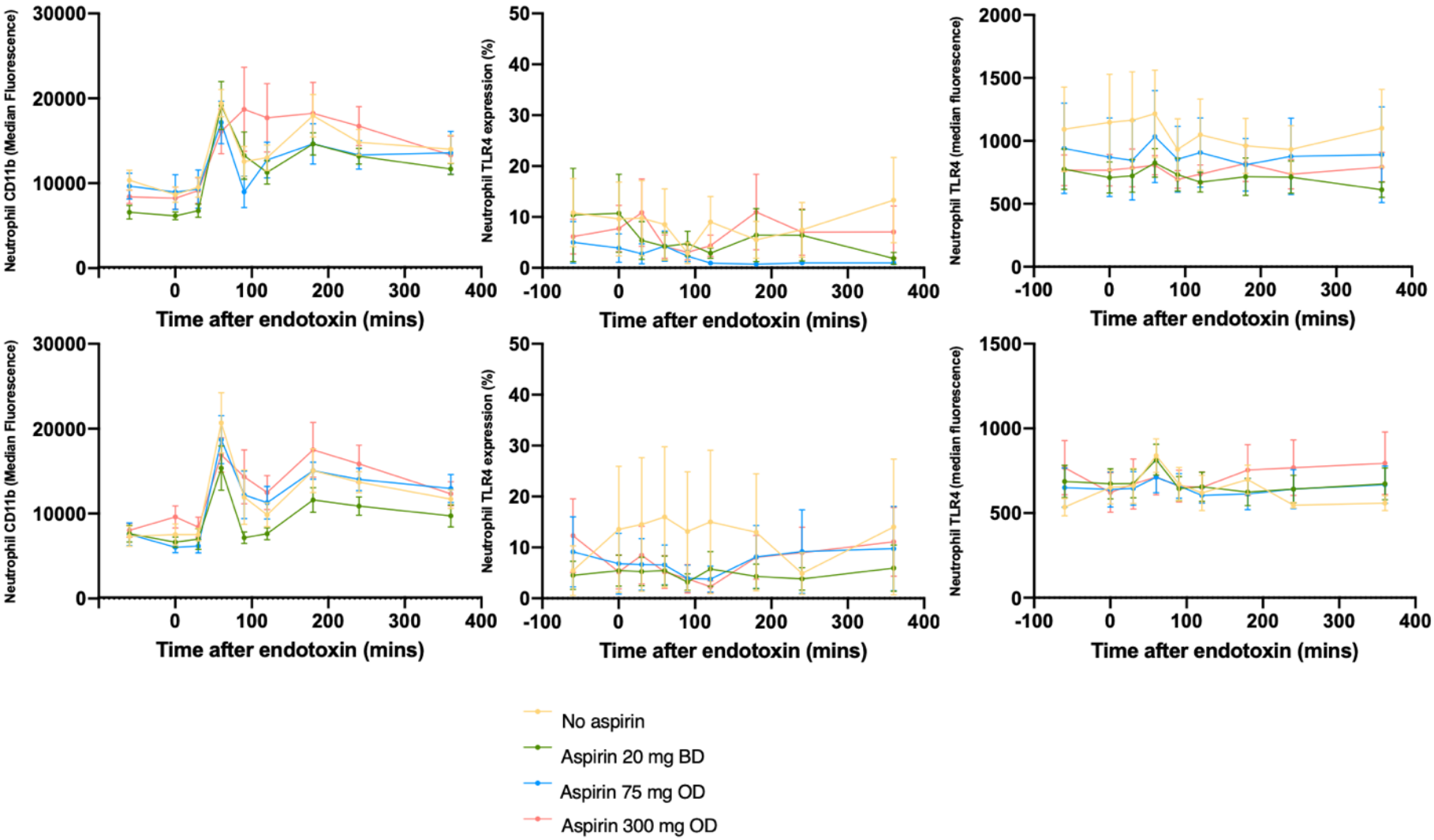

Figure 9.4 Neutrophil activation markers before and after endotoxin injection in participants receiving no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD with (bottom panel) or without (top panel) a loading dose of ticagrelor one hour before endotoxin. Bars represent mean ± SEM.

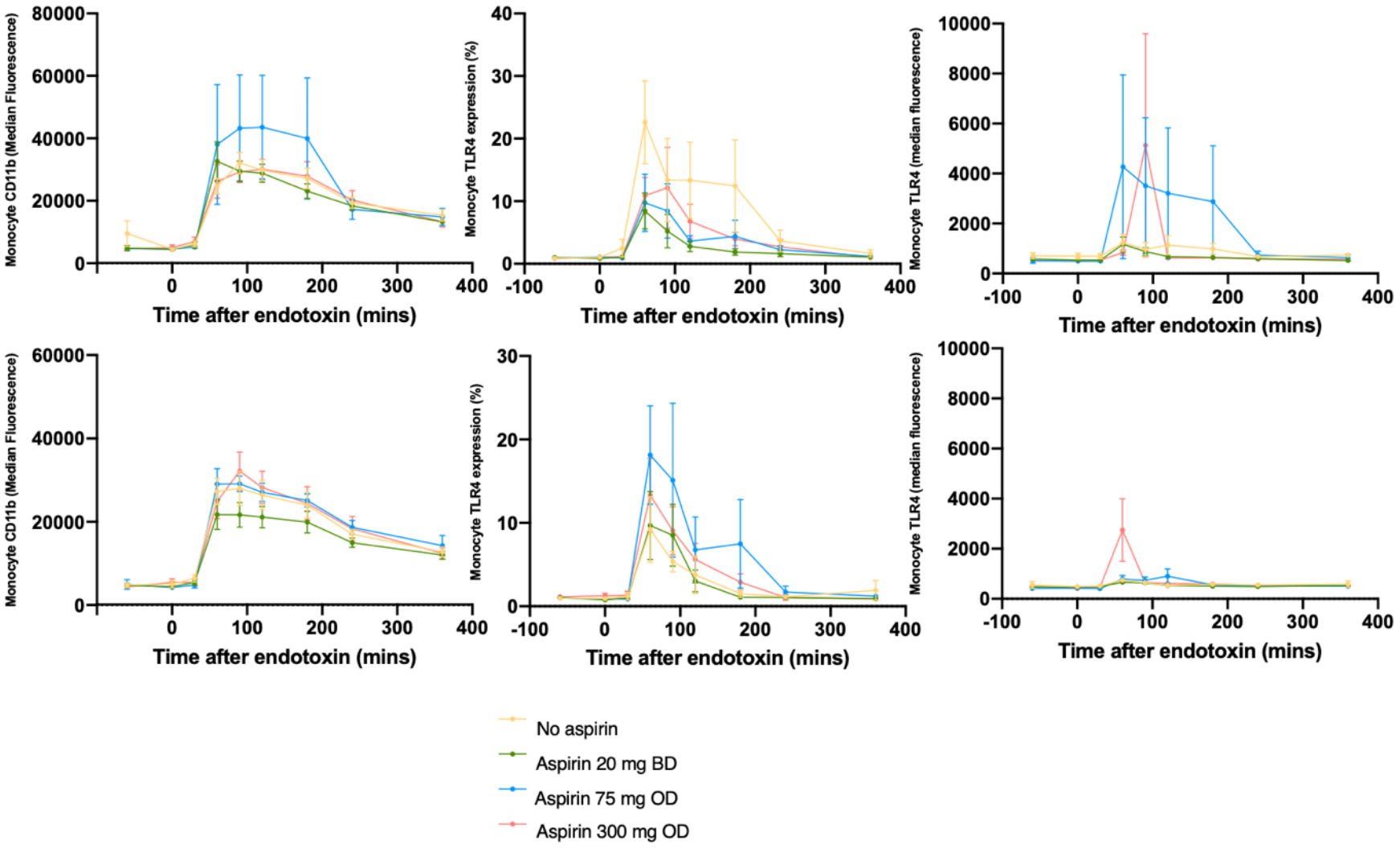

Figure 9.5 Monocyte activation markers before and after endotoxin injection in participants receiving no aspirin, aspirin 20 mg BD, 75 mg OD or 300 mg OD with (bottom panel) or without (top panel) a loading dose of ticagrelor one hour before endotoxin. Bars represent mean ± SEM.

Table 9.3 Pairwise comparisons of regimens for endpoints identified as having significant treatment or timepoint*treatment effect in participants undergoing endotoxin stimulation who had **not** received ticagrelor. Values represent p values.

Parameter	Aspirin regimens in comparison ➔	20 mg BD vs No aspirin	75 mg OD vs No aspirin	300 mg OD vs No aspirin	300 mg OD vs 20 mg BD	300 mg OD vs 75 mg OD	75 mg OD vs 20 mg BD
Monocyte TLR expression %	Timepoint	**0.0009**	**0.0052**	**0.0005**	**0.0069**	**0.013**	**0.0056**
	Treatment	**0.023**	0.15	0.17	0.18	0.64	0.37
	Timepoint* Treatment	**0.0007**	0.14	0.19	0.33	0.98	0.84
Mixed cell count	Timepoint	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	Treatment	0.26	0.85	0.77	0.4	0.94	0.41
	Timepoint* Treatment	**0.0042**	**0.0064**	**0.042**	**0.038**	0.3	0.15

Table 9.4 Pairwise comparisons of regimens for endpoints identified as having significant treatment or timepoint*treatment effect in participants undergoing endotoxin stimulation who **had** received ticagrelor. Values represent p values.

Parameter	Aspirin regimens in comparison ➔	20 mg BD^T vs No aspirin^T	75 mg OD^T vs No aspirin^T	300 mg OD^T vs No aspirin^T	300 mg OD^T vs 20 mg BD^T	300 mg OD^T vs 75 mg OD^T	75 mg OD^T vs 20 mg BD^T
Leukocyte count	Timepoint	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	Treatment	0.058	0.83	0.78	**0.025**	0.92	**0.026**
	Timepoint* Treatment	0.17	**0.038**	<0.99	0.22	0.13	**0.002**
Neutrophil count	Timepoint	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	Treatment	0.069	0.82	0.92	0.04	0.88	**0.025**
	Timepoint* Treatment	0.11	**0.0498**	>0.99	0.13	0.13	**0.0009**
Mixed cell count	Timepoint	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Treatment	0.069	0.57	0.38	**0.0083**	0.095	0.18
	Timepoint* Treatment	0.3	0.49	0.51	0.19	0.94	0.16
Lymphocyte count	Timepoint	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	Treatment	0.15	0.82	0.39	0.054	0.051	0.13
	Timepoint* Treatment	**0.021**	**0.037**	0.063	0.055	0.79	0.49
Monocyte TLR 4 (MFI)	Timepoint	**0.0094**	0.067	0.074	**0.044**	0.062	**0.016**
	Treatment	0.5	0.92	0.24	0.092	0.26	0.23
	Timepoint* Treatment	0.99	0.28	**0.01**	**0.0009**	**0.015**	0.092
Neutrophil TLR4 (MFI)	Timepoint	**0.0011**	**0.0093**	**0.048**	**0.045**	0.14	**0.012**
	Treatment	0.79	0.96	0.51	0.6	0.55	0.85
	Timepoint* Treatment	**0.0055**	**0.017**	**0.02**	0.22	0.94	0.59

Those variables/regimens identified as exhibiting a significant treatment or treatment*timepoint effect were yet further investigated by plotting locally weighted polynomial regression curves as described earlier in this thesis in order to identify the nature of significant relationships.

Whilst in some cases there were no clear differences between the regimens (**Figure 9.6**), ten comparisons did show clear differences (denoted by the lack of overlap between 95% CI) (**Figures 9.7 to 9.9**). When participants received ticagrelor, treatment with aspirin 20 mg BD was associated with a more steep return from nadir in total leukocyte or neutrophil count, the traces separating significantly between the 60 and 300 minute timepoints, before converging with overlap in the 95% CI. Similarly, in ticagrelor-treated individuals, there appeared to be a slower fall in lymphocyte count after endotoxin injection when receiving aspirin 20 mg BD compared to no aspirin.

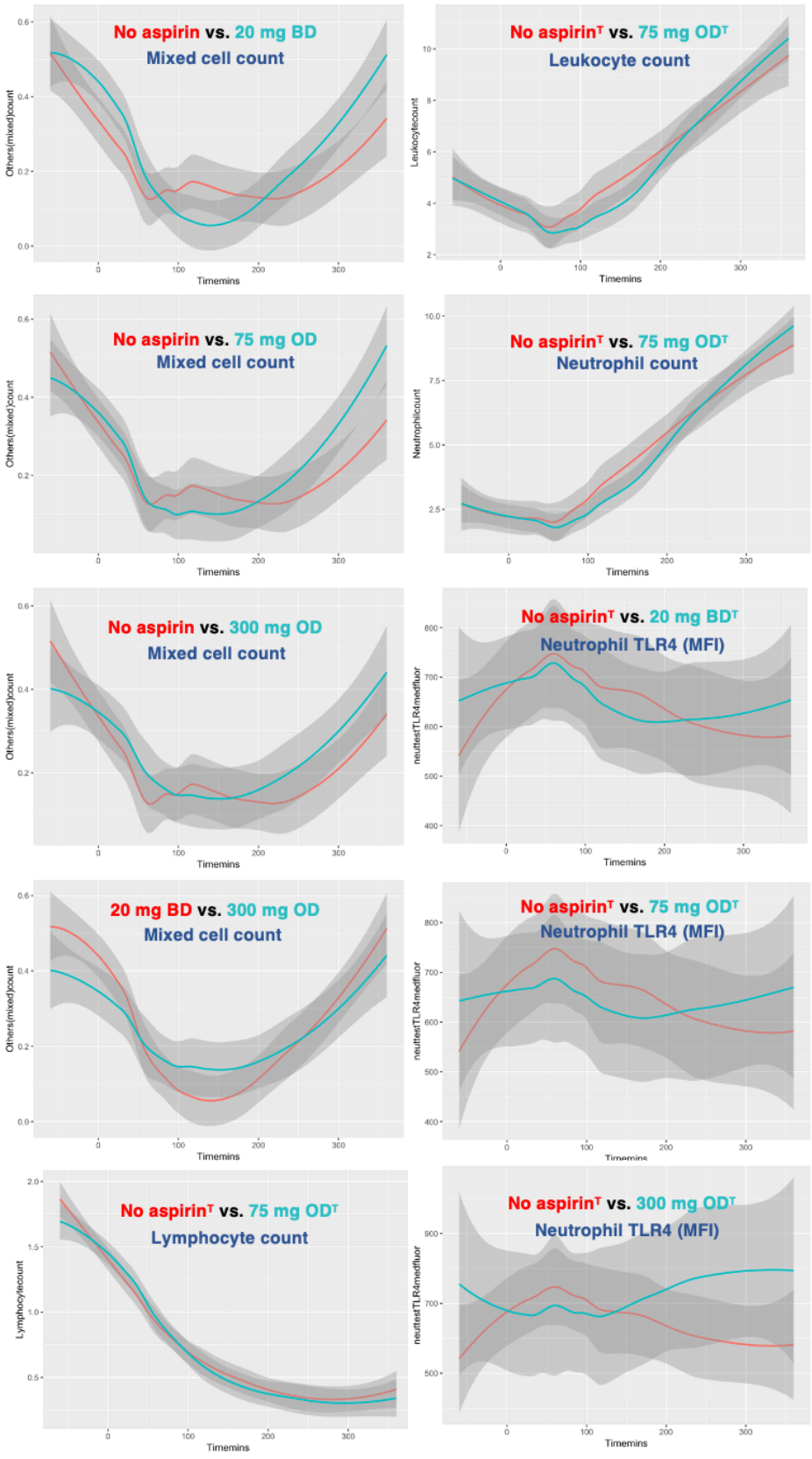

Figure 9.6 Locally weighted polynomial regression plots for variable/regimen combinations on which treatment or treatment*timepoint exhibited a significant effect but for which there was no clear graphical differences between the regimens. Solid line indicates smoothed mean, shaded area 95% CI. ^{T}=received loading dose of ticagrelor 1 hour before endotoxin injection. Time in minutes is on the x axis.

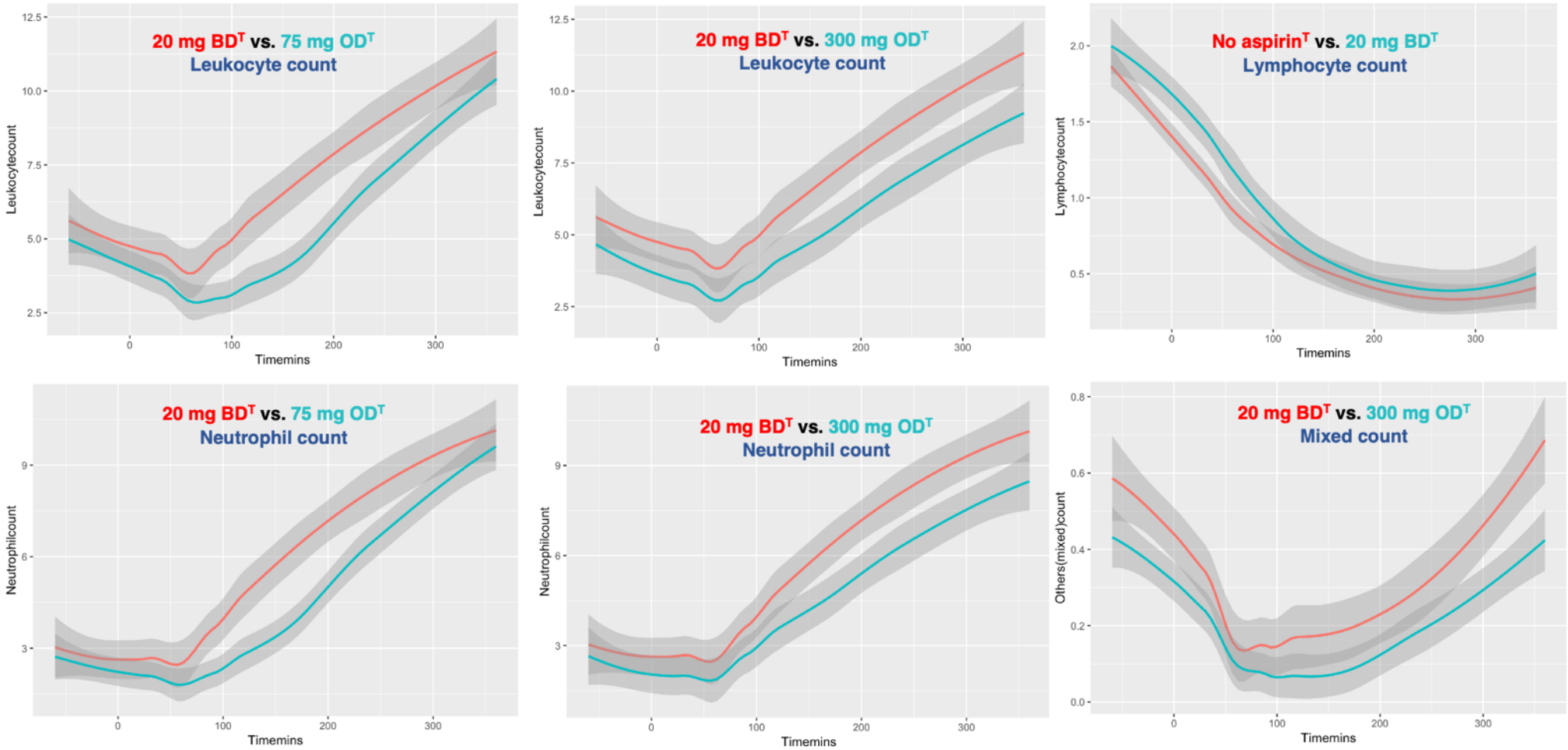

Figure 9.7 Locally weighted polynomial regression plots for leukocyte count (or subset)/regimen combinations on which treatment or treatment*timepoint exhibited a significant effect and for which there were clear graphical differences between the regimens. Solid line indicates smoothed mean, shaded area 95% CI. ^{T}=received loading dose of ticagrelor 1 hour before endotoxin injection. Time in minutes is on the x axis.

C. Effects on toll-like receptor 4 expression

There was evidence of regimen-specific effects on TLR4 expression. When receiving aspirin 20 mg BD (but not 75 mg or 300 mg OD), monocyte TLR4 expression (%) was significantly lower than when receiving no aspirin, in individuals who did not receive ticagrelor (**Figure 9.8**).

Finally, during endotoxaemia, participants who received ticagrelor (but not those who did not) exhibited significantly greater peak TLR4 expression (MFI) when receiving aspirin 300 mg OD compared to 20 mg BD, 75 mg OD or no aspirin (**Figure 9.9**).

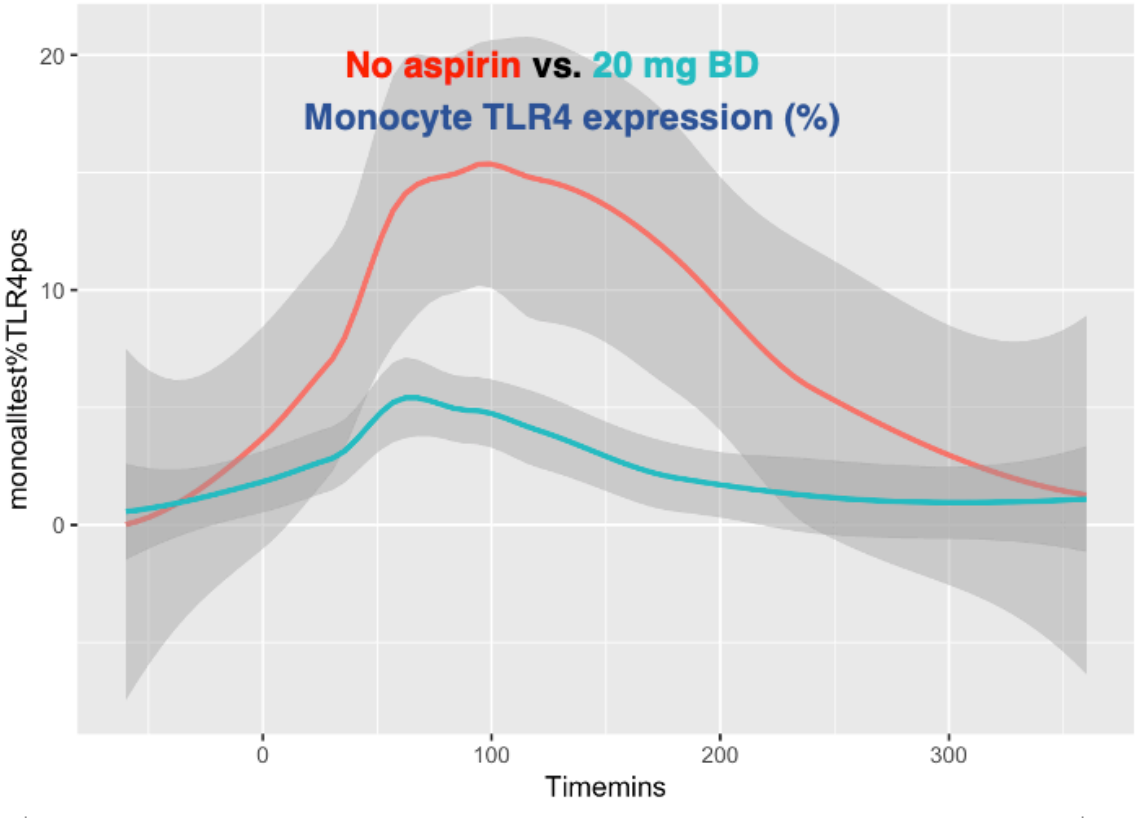

Figure 9.8 Locally weighted polynomial regression plots for monocyte TLR4 expression (%) when receiving no aspirin or aspirin 20 mg BD. Solid line indicates smoothed mean, shaded area 95% CI. Time in minutes is on the x axis.

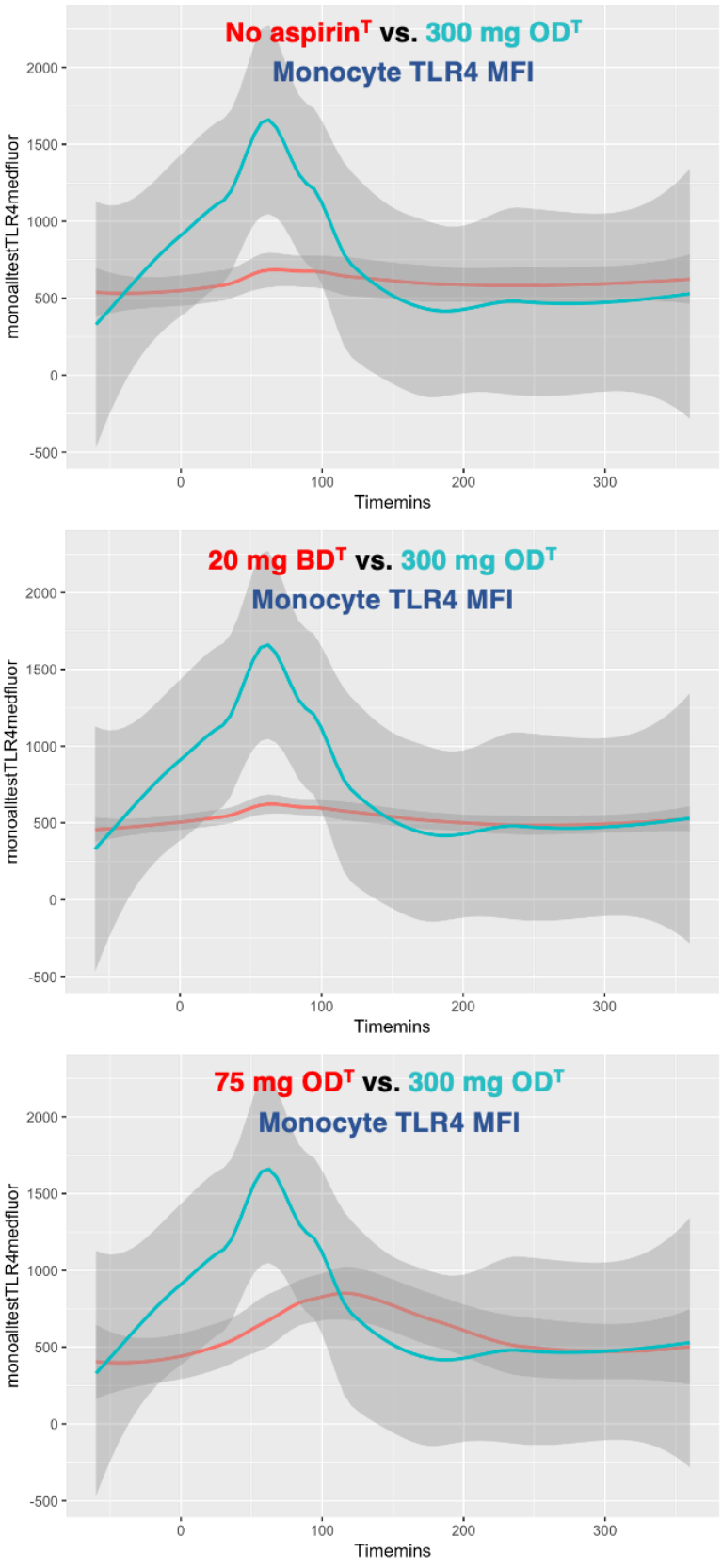

Figure 9.9 Locally weighted polynomial regression plots for monocyte TLR4 median fluorescence/regimen combinations on which treatment or treatment*timepoint exhibited a significant effect and for which there were clear graphical differences between the regimens. Solid line indicates smoothed mean, shaded area 95% CI. [T]=received loading dose of ticagrelor 1 hour before endotoxin injection. Time in minutes is on the x axis.

D. Cytokine response

I. Pre-specified premature study discontinuation criteria

At two hours after endotoxin injection, plasma TNF-α was not significantly different between the no aspirin, aspirin 20 mg BD, 75 mg OD and 300 mg OD groups (p=0.81) when assessed using one-way ANOVA (treatment as factor). Accordingly, the interim analysis did not meet the criteria for premature discontinuation.

Similarly, as the mean difference between plasma TNF-α at 2 hours after endotoxin injection when receiving aspirin 300 mg OD vs. no aspirin was +124 pg/mL with a 95% confidence interval of -316 to 563 (i.e. the lower bound was not more negative than -2000 pg/mL), premature discontinuation on the grounds of futility was not indicated either.

Therefore, the decision from the interim analysis was to continue the trial, restarting when feasible and safe to do so.

There were no significant differences in plasma IL-6 levels, two hours after endotoxin, between the regimens (**Table 9.5**).

Table 9.5 Plasma levels of tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) measured 2 hours after endotoxin injection. P values generated by one-way ANOVA. Data are shown as mean ± SD. ^{T}=received loading dose of ticagrelor 1 hour prior to endotoxin.

	No aspirin	Aspirin 20 mg BD	Aspirin 75 mg OD	Aspirin 300 mg OD	p	No aspirin^{T}	Aspirin 20 mg BD^{T}	Aspirin 75 mg OD^{T}	Aspirin 300 mg OD^{T}	p
TNF-α (pg/mL)	404.5 +/- 356.1	641.2 +/- 593.4	509.4 +/- 645.8	528.4 +/- 569.8	0.81	317.8 +/- 248.5	326.3 +/- 309.6	505.6 +/- 522.8	549.7 +/- 659.6	0.74
IL-6 (pg/mL)	2119 +/- 1501	2114 +/- 1017	1998 +/- 1316	2664 +/- 1914	0.60	1878 +/- 991	1514 +/- 1186	2715 +/- 1495	2154 +/- 1511	0.26

II. Plasma tumour necrosis factor α levels over time

Though there were no significant differences in plasma TNF-α between the regimens, the study was likely underpowered to detect this at the interim analysis stage. Plotting mean levels measured at 1, 1.5, 2 and 3 hours after endotoxin injection suggested a trend towards higher levels when receiving aspirin compared to no aspirin (**Figure 9.10**).

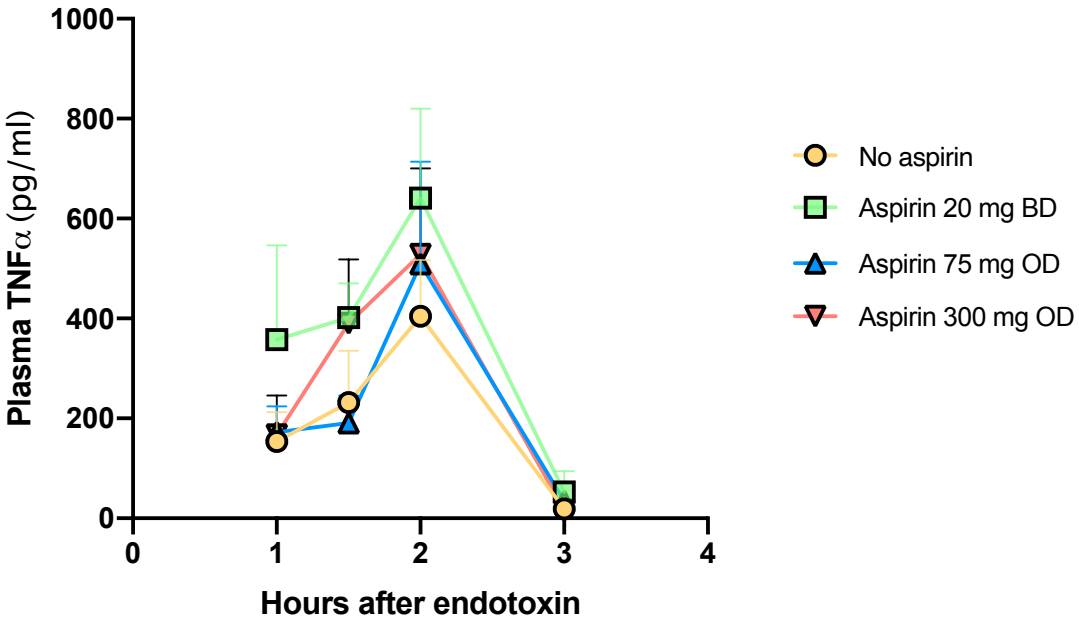

Figure 9.10 Plasma TNF-α levels measured at timepoints between 1 and 3 hours after endotoxin injection in participants receiving no aspirin or one of the three aspirin regimens (did not receive ticagrelor). Bars indicate mean + SEM.

Plotting mean levels of TNF-α from 1 to 3 hours after endotoxin injection in those participants who had received ticagrelor suggested a trend towards lower peak levels when receiving no aspirin or 20 mg BD compared to aspirin 75 mg OD or 300 mg OD (**Figure 9.11**).

Furthermore, a paired comparison of plasma TNF-α between each ticagrelor-free and ticagrelor-receiving regimen showed that ticagrelor significantly reduced peak levels when participants were taking aspirin 20 mg BD (326 ± 310 pg/ml vs. 641±593 pg/ml, p=0.024) (**Figure 9.12**) but not 75 mg OD (506 ± 523 pg/ml vs. 509 ± 646 pg/mL, p=0.40), 300 mg OD (550 ± 660 pg/mL vs. 528 ± 570 pg/mL, p=0.48) or no aspirin (317.8 ± 249 pg/mL vs. 405 ±356 pg/mL, p=0.32) though there was a notable trend in the latter comparison and more data are needed to evaluate relationships robustly.

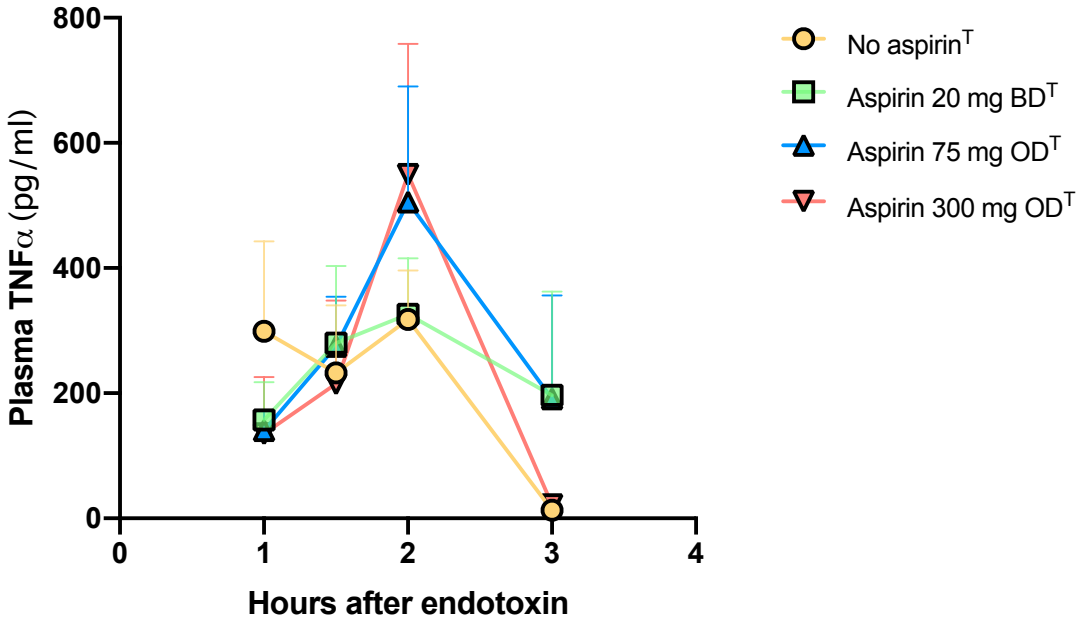

Figure 9.11 Plasma TNF-α levels measured at timepoints between 1 and 3 hours after endotoxin injection in participants receiving no aspirin or one of the three aspirin regimens plus a loading dose of ticagrelor. Bars indicate mean + SEM.

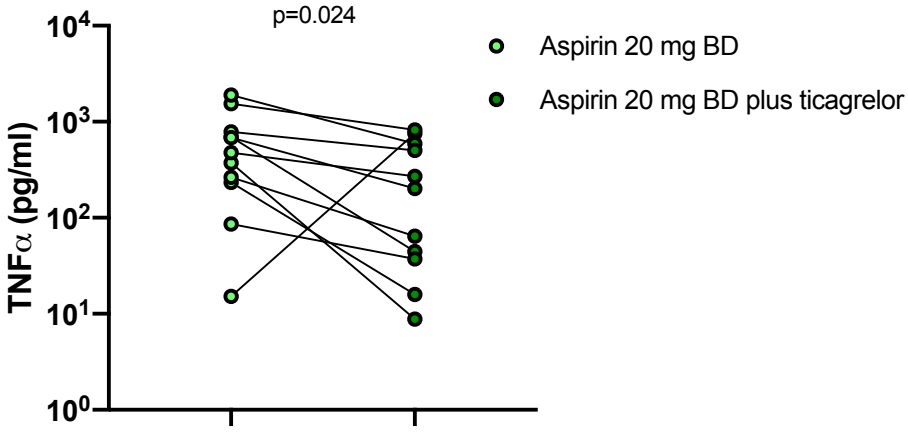

Figure 9.12 Plasma TNF-α levels measured at 2 hours after endotoxin injection in participants receiving aspirin 20 mg BD, with or without a loading dose of ticagrelor. P value generated using a paired t-test.

E. Discussion

Aspirin, at a dose of around 75 mg OD, is known to potentiate the increase in plasma levels of proinflammatory cytokines such as TNF-α and IL-6 observed during endotoxaemia (Kiers et al. 2017), but it is unknown how this effect varies with aspirin dose. Similarly, it is established that ticagrelor inhibits the same cytokine responses (Thomas et al. 2015), but any interactions between this effect and aspirin dose have not been characterised.

The present study aims to utilise a mixture of parallel group and crossover elements in order to comprehensively study the dose-dependent effects of aspirin, when given with or without ticagrelor. This is an issue that has direct clinical relevance to patients with IHD as inflammation drives atherogenesis and therefore any pro-inflammatory effects of aspirin are likely to be counterproductive, compromising any benefit conferred by its antiplatelet properties. The WILLOW ACS study showed that modulating aspirin dose during DAPT with ticagrelor can improve the temporal pharmacodynamic profile of aspirin's effects, maintaining 24-hour suppression of TXA_2 release whilst minimising effects on haemostasis, findings that are broadly reproduced in the data obtained in this study thus far (Chapter 8). If this approach can also reduce any pro-inflammatory effects of aspirin it would greatly enhance the case for conducting outcome-based studies.

Whilst the primary endpoint of the study is based on plasma levels of TNF-α, the interim analysis was clearly underpowered to assess this, demonstrated by the fact that neither criteria for termination on the grounds of efficacy or futility were met. Nevertheless, there was a trend towards increased peak TNF-α during therapy with aspirin compared to no aspirin, supporting a previous study suggesting the same (Kiers et al. 2017). Whilst this did not appear to be dose-dependent when receiving aspirin only, when receiving a loading dose of ticagrelor, aspirin 75 mg OD or 300 mg OD, but not 20 mg BD, appeared to potentiate the response to endotoxin, though this was not statistically significant. Furthermore, ticagrelor only significantly reduced plasma TNF-α compared to the corresponding ticagrelor-free regimen when participants were receiving aspirin 20 mg BD. Clearly, further data are needed to make these comparisons robustly, but the trends support continuing the study and suggest a potential interaction between aspirin 20 mg BD and ticagrelor that reduces cytokine release during endotoxaemia, and therefore by association in the setting of atheroinflammation, compared to standard aspirin regimens.

There was also evidence of regimen-specific effects on the monocyte cell surface expression of

the endotoxin receptor, TLR4. When participants received a loading dose of ticagrelor, aspirin 300 mg OD, compared to the other regimens, was associated with a significantly higher peak TLR4 MFI. Clearly more data are needed, but this supports the hypothesis that aspirin may increase monocyte TLR4 expression. The monocyte data presented here relate to the total cell population. Further exploration of any differential effects on monocyte subpopulations (classical, non-classical and intermediate) is planned in the final analysis of the study.

The mechanism by which aspirin might reduce TLR4 expression remains unconfirmed, but related observations suggest that inhibition of platelet-derived PGE_2, a prostanoid known to downregulate monocyte TLR4 expression, may be implicated (Kiers et al. 2017; Degraaf, Zasłona, et al. 2014). Whether this represents an important mediator of any pro-inflammatory effects of aspirin or is merely a marker of them remains to be determined.

In the PLATO study, higher (≥300 mg OD) vs. lower (<300 mg) doses of aspirin were associated with worse outcomes when receiving ticagrelor but not clopidogrel. If a combination of aspirin 300 mg OD and ticagrelor leads to higher levels of TLR4 expression than seen with lower doses of aspirin, this may hypothetically contribute to this observation (Mahaffey et al. 2011). Why the same relationship was not observed in those receiving aspirin and clopidogrel remains unclear. Speculatively, it is possible that off-target immunosuppressive effects of clopidogrel may mitigate any pro-inflammatory effects of aspirin (Storey et al. 2014). Similarly, it is feasible that a mechanistic interaction occurs between ticagrelor and higher doses of aspirin, for example, increasing levels of nitric oxide generation from the anti-inflammatory to pro-inflammatory ranges (Hetzel et al. 2013; Nanhwan et al. 2014; Tripathi et al. 2007). This, however, clearly requires further study before a definitive explanation can be stated.

Chapter 10: General discussion

A. Overview of the project's aims and influences

Broadly, the objectives of this project were two-fold. First, to rationally design and test an aspirin regimen that optimised the pharmacodynamic profile of aspirin during ticagrelor-based DAPT. Second, to gain insight into the 'North American Paradox' by using a process of reverse translation to generate hypotheses and explore mechanisms by which aspirin dose-dependently interacts with ticagrelor.

The impetus for this work was principally the emerging evidence that prolonged DAPT conferred significant anti-ischaemic benefits in high-risk groups, but at a not unsubstantial counter-increase in bleeding, sometimes dissuading clinicians and patients alike from pursuing this clinical strategy (Bonaca, Bhatt, Oude Ophuis, et al. 2016).

Looking at the experimental arms of recently published studies of antiplatelet therapy in patients with IHD, there has been a noticeable change in the direction of the sought intensity, particularly with regards to the post-PCI patient. The first 15 years of the 21st century saw a chain of important clinical trials underlining the fact that increasing the potency and reliability of antiplatelet therapy in a stepwise fashion reduced the risk of ischaemic events, with net clinical benefit even when bleeding was taken into account (Parker and Storey 2016a). With the realisation of improvements in stent design such as ultrathin struts and more biocompatible, degradable or absent polymers, the risk of stent thrombosis, the process that DAPT was originally conceived to prevent, has become very low (Iantorno et al. 2018). Complementing this is the fact that oral $P2Y_{12}$ inhibitors have increased in potency and reliability as newer agents prasugrel and ticagrelor have become available (Joshi et al. 2014), meaning worries about interindividual variability seen with clopidogrel no longer need to be a significant factor. Ticagrelor monotherapy after a short period of DAPT is, in particular, an emerging post-PCI strategy with promise to offer lower bleeding risk but maintained anti-ischaemic benefits when compared to aspirin and ticagrelor, though this latter outcome is yet to be robustly demonstrated in trials large enough to give complete confidence (Parker 2020b). Nevertheless, there remains good evidence that in individuals at high-risk of ongoing ischaemic events, in particular those with prior MI and additional risk factors, such as those meeting the inclusion criteria for the PEGASUS TIMI 54 study, prolonged DAPT

offers net clinical benefit (Bonaca et al. 2015).

Currently, we are therefore experiencing the rational emergence of a divergent strategy based on an individual's ischaemic and bleeding risk, on the one hand de-escalating intensity of antiplatelet therapy where bleeding risk outweighs ischaemic risk and intensifying therapy where ischaemic risk outweighs bleeding risk (Storey 2020).

Furthermore, the clinical relevance of the inflammatory hypothesis of atherogenesis, which has gradually achieved prominence within the field (Libby et al. 2019), has recently been robustly demonstrated. Targeting components of the atheroinflammatory response through drug or biologic therapy has now been proven to reduce significantly the incidence of cardiovascular events. However, just as an increase in the intensity of antithrombotic therapy is typically associated with a penalty in bleeding risk, targeted anti-inflammatory therapy can, for example, increase the risk of fatal infections in the case of canakinumab and may increase non-cardiovascular death in the case of colchicine, though this remains to be definitively assessed. Strategies that reduce levels of atheroinflammation when compared to standard-of-care without leading to an increase in adverse events clearly may offer significant benefits to patients with atherosclerotic disease.

B. Relevance to current practice

The work presented in thesis advances and informs the current state of knowledge in three main ways.

First, it provides further evidence that aspirin's antithrombotic effects remain significant even in the presence of potent $P2Y_{12}$ inhibition. This was gained through in vitro studies using cangrelor alongside a range of aspirin concentrations, through demonstrating that modulation of aspirin dose leads to changes in pharmacodynamic parameters in patients receiving DAPT for ACS and through the conduct of a healthy volunteer study that allowed comparison with drug-free, aspirin-only and ticagrelor-only groups.

Second, it provides clear evidence that a novel dosing regimen of aspirin, 20 mg BD, offers a range of pharmacodynamic benefits over current standard regimens when given alongside ticagrelor. These may include improved haemostasis, reduced peak-trough variation in antithrombotic effect, improved glomerular filtration and evidence of improvement in inflammatory parameters.

Third, mechanistic insights into aspirin's effects and how these interact with ticagrelor have been gained. For example, the finding that peak monocyte TLR4 expression during endotoxaemia appeared to be greater when receiving ticagrelor and aspirin 300 mg OD compared to other doses generates the hypothesis that this is a mechanism by which inflammation is potentiated, leading to the significantly reduced/inverted benefit of ticagrelor over clopidogrel in patients receiving ≥300 mg aspirin OD (mostly 300-325 mg) during ACS treatment in the PLATO study (Mahaffey et al. 2011).

C. Plans for future work

I. Restarting the WILLOW TREE study

The interim analysis of data from the WILLOW TREE study, detailed in this thesis, has proven valuable in guiding further progress of the study. Prior to any analysis, robust a priori criteria for premature termination, either on the grounds of certainty or futility, were drawn up with the assistance of a Chartered Statistician and received approval from the relevant regulatory bodies. The criteria were not met but the study was shown to be non-futile and promising trends were seen. Clearly, a present priority is to restart and complete the planned study in order to gain well-powered, high-quality data and confirm or refute the original, pre-specified hypotheses. This seems particularly important with regards to data on inflammatory parameters. Furthermore, completing the study will enable the other protocol-defined endpoints to be addressed, including other prostanoids to assess effects on prostacyclin and PGE_2 release, monocyte subset data to gain further insights into changes in TLR4 expression, and fibrin clot turbidimetry to assess effects on the pro-coagulant changes associated with endotoxaemia.

II. Further investigation of the effects of aspirin dosing on the inflammatory response

Whilst an interim analysis was not planned when the study was begun, it was deemed necessary in order to prioritise resource allocation at a critical time for clinical practice and clinical research. An advantage, however, is that insights have been gained into modulation of the response to

endotoxin by aspirin and/or ticagrelor that may have not been available until the end of the study.

These interim data can therefore help to focus further mechanistic work on areas such as this, and these could even be built into the WILLOW TREE study when it restarts. For example, determining changes in monocyte signalling pathways relating to TLR4 would help to both confirm and further understand regimen-dependent effects. This might be achieved by separating and storing monocytes from study blood samples for work using techniques such as Western blot or quantitative polymerase chain reaction.

III. Exploring the wider applicability of the 'WILLOW' principle

a) Characterisation of a novel regimen of very low-dose aspirin combined with rivaroxaban in patients with chronic coronary syndromes: the WILLOW CCS study

The current baseline antiplatelet regimen for secondary prevention of cardiovascular events for many patients with CCS is aspirin 75-100 mg OD (Montalescot et al. 2013; Knuuti et al. 2019). The Cardiovascular OutcoMes for People using Anticoagulation StrategieS (COMPASS) study showed that, in high-risk individuals, adding rivaroxaban, a non-vitamin-K-antagonist oral anticoagulant (NOAC), at a dose of 2.5 mg BD to standard aspirin treatment leads to a significantly lower risk of major adverse cardiovascular events (MACE) (Eikelboom et al. 2017), representing an alternative to long-term DAPT where appropriate (Knuuti et al. 2019). This may be because this combination, known as low-dose dual antithrombotic therapy (DATT), provides not only antiplatelet effects but also anticoagulation. Anticoagulation might affect parameters of fibrin clot dynamics that are an emerging risk factor for MACE (Sumaya et al. 2018; Konigsbrugge et al. 2018). However, this approach also leads to increased bleeding (Eikelboom et al. 2017). Whilst this may apply broadly across patient groups, there may be those in whom the risk-benefit profile is particularly difficult to balance, for example those over the age of 75 years or those with a history of CCS but at the lower end of the ischaemic-risk spectrum. Accordingly, those patients with CCS in whom DATT is currently not recommended may hypothetically derive benefit, over standard aspirin monotherapy, from a combination regimen with a better balance of anti-ischaemic benefit and harm from bleeding. Improving the safety profile of combination therapy with aspirin and a NOAC by applying the principle of very low-

dose BD aspirin could lead to wider applicability of the approach and substantial improvement in clinical outcomes for patients at significant risk of ischaemic events.

The WILL lOWer dose aspirin be better with rivaroxaban in patients with Chronic Coronary Syndromes? (WILLOW CCS) study, planned to begin recruitment in January 2021, is a pharmacodynamic study to determine the effect of aspirin 20 mg BD plus rivaroxaban 2.5 mg BD on haemostasis, fibrin clot dynamics, inflammatory markers, platelet function and arachidonic acid metabolites when compared to standard regimens of aspirin 75 mg OD and aspirin 75 mg OD plus rivaroxaban 2.5 mg BD.

In a randomised open-label three-period crossover design, patient participants receiving aspirin 75 mg OD for secondary prevention of IHD will be randomised 1:1 to receive one of two sequences of aspirin: aspirin 75 mg OD, then aspirin 20 mg BD plus rivaroxaban 2.5 mg BD, then aspirin 75 mg OD plus rivaroxaban 2.5 mg BD; or aspirin 75 mg OD, then aspirin 75 mg OD plus rivaroxaban 2.5 mg BD, then aspirin 20 mg BD plus rivaroxaban 2.5 mg BD (**Figure 10.1**).

At the end of each 14 day medication period, they will attend a study visit at which blood and urine samples will be obtained, and bleeding time measured, before and 2 hours after the last dose of IMP of the treatment period. The samples will be tested for fibrin clot dynamics; inflammatory markers; prostanoids; and platelet function.

The principal hypothesis is that the difference in bleeding time when receiving aspirin 75 mg OD alone vs. aspirin 20 mg BD plus rivaroxaban 2.5 mg BD will be significantly less than the difference in bleeding time between aspirin 75 mg OD alone vs. aspirin 75 mg OD plus rivaroxaban 2.5 mg BD. Secondary hypotheses include that fibrin clot parameters will be improved (lysis time shortened) by treatment with either rivaroxaban-containing regimen compared to aspirin 75 mg OD alone, that treatment with aspirin 20 mg BD plus rivaroxaban 2.5 mg BD will lead to reduced peak suppression of TXA_2 release but maintained trough effect, and that post-dose levels of TNF-α and IL-6 will be lower when receiving aspirin 20 mg BD plus rivaroxaban 2.5 mg BD when compared to either regimen containing aspirin 75 mg OD.

b) Further expansion of the target clinical population

Whilst aspirin 20 mg BD given alone appeared to offer significant and consistent antiplatelet effect when compared to other regimens in healthy volunteers, this has not been studied in patients with IHD, and some care should be taken when making assumptions about the applicability of findings in one group to another. Nevertheless, it does generate the hypothesis that aspirin 20 mg BD may offer benefits over standard aspirin regimens in those patients requiring single antiplatelet therapy. This could be explored in future pharmacodynamic studies in the first instance. The applicability of this approach could also potentially be extended for investigation in primary prevention settings, in which recent studies of aspirin have shown a lack of net clinical benefit in any reduction in ischaemic risk when balanced against increases in bleeding risk (Bowman et al. 2018). Finally, long-term aspirin therapy is also indicated for other conditions such as essential thrombocythaemia and emerging as a strategy for chemoprevention of colorectal cancer. In the former case, because of very rapid platelet turnover, BD aspirin dosing is known to offer particular benefits in consistency over once-daily (Larsen et al. 2018). In the latter, the belief that COX2 inhibition may be more important than COX1 inhibition may mean this might be a less appropriate application (Langley et al. 2011).

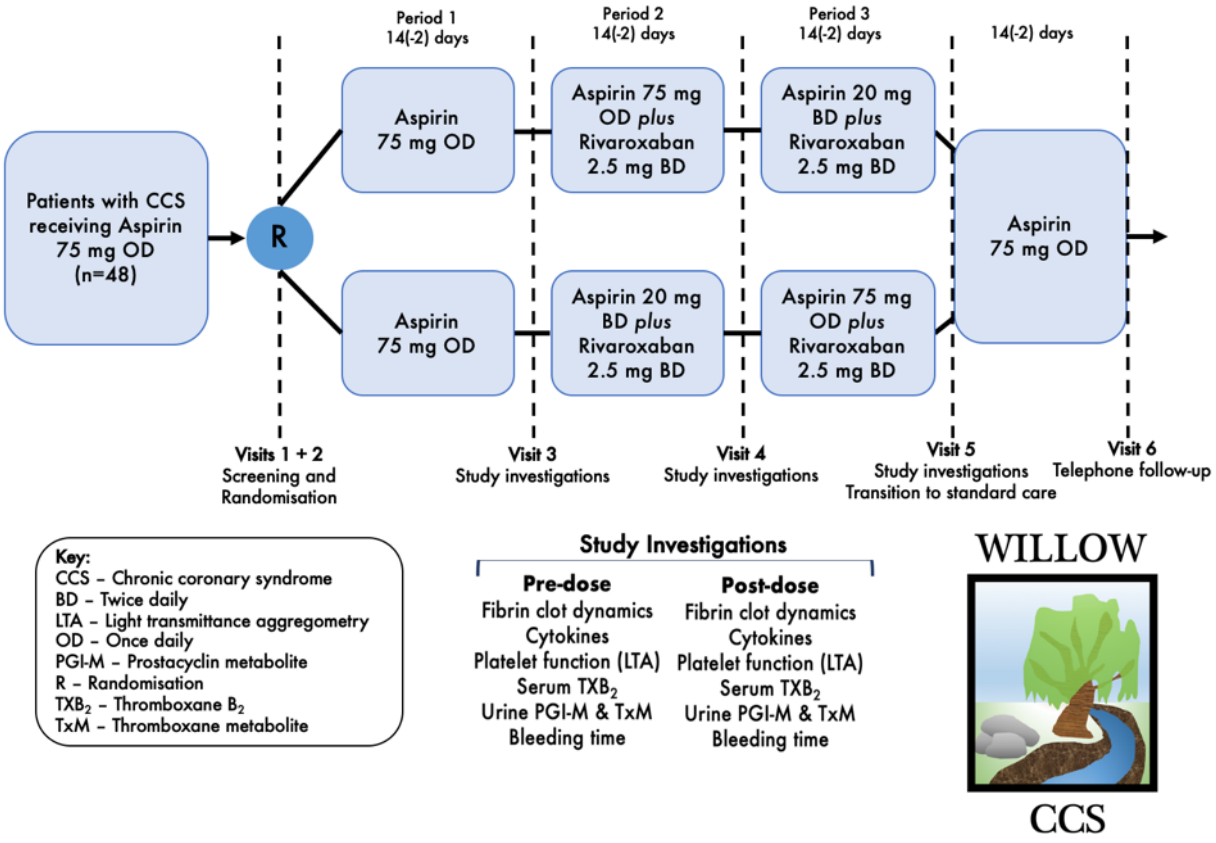

Figure 10.1 Design of the WILLOW CCS study.

IV. Concluding remarks

The work in this thesis has explored the pharmacodynamic effects of a novel approach to aspirin dosing during treatment with DAPT, coming at a time when minimising bleeding risk, inflammation and overintensity of treatment are recognised more than ever as important goals in the management of patients with IHD. These studies show that a nuanced approach to designing an aspirin regimen can lead to pharmacodynamic advantages in this respect and form a strong case for larger scale studies to examine effects on clinical outcomes.

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Appendix

Table A.1 Maximum aggregation responses before and after endotoxin injection when participants did not receive ticagrelor. Values are mean ± SD. P value for each treatment/agonist pairing generated by one-way ANOVA with timepoint as factor.

Time after endotoxin injection (hours) ⇒		-1	0	3	
Treatment regimen	Agonist	Maximum aggregation (%)			p
No aspirin	ADP 20 μmol/L	74.6 +/- 4.78	77.1 +/- 6.93	76.8 +/- 4.61	0.38
	Collagen 4 μg/mL	77.9 +/- 5.17	78.7 +/- 7.58	79.6 +/- 4.97	0.77
	Collagen 16 μg/mL	78.1 +/- 7.15	81.8 +/- 5.80	81.1 +/- 5.19	0.25
	AA 1 mmol/L	62 +/- 32.2	54.6 +/- 37.3	58.2 +/- 39.8	0.66
Aspirin 20 mg BD	ADP 20 μmol/L	64.0 +/- 4.67	69.6 +/- 9.42	68.3 +/- 6.21	0.062
	Collagen 4 μg/mL	70.7 +/- 7.98	66.5 +/- 15.9	72.3 +/- 13.8	0.23
	Collagen 16 μg/mL	74.0 +/- 8.54	74.9 +/- 12.7	79.2 +/- 8.11	0.097
	AA 1 mmol/L	1.63 +/- 1.43	1.09 +/- 1.37	1.72 +/- 1.34	0.23
Aspirin 75 mg OD	ADP 20 μmol/L	72.1 +/- 9.29	71.3 +/- 8.16	74.5 +/- 9.04	0.36
	Collagen 4 μg/mL	71.4 +/- 11.9	52.7 +/- 28.5	60.7 +/- 18.9	**0.037**
	Collagen 16 μg/mL	79.0 +/- 8.02	70.2 +/- 13.6	76.7 +/- 10.2	0.18
	AA 1 mmol/L	1.80 +/- 1.31	1.10 +/- 0.73	1.30 +/- 0.94	0.15
Aspirin 300 mg OD	ADP 20 μmol/L	69.3 +/- 6.77	69.6 +/- 7.67	71.5 +/- 8.11	0.78
	Collagen 4 μg/mL	57.6 +/- 13.9	43.4 +/- 22.7	41.0 +/- 26.2	**0.014**
	Collagen 16 μg/mL	72.3 +/- 8.65	61.0 +/- 14.9	63.4 +/- 18.7	**0.044**
	AA 1 mmol/L	1.63 +/- 1.85	1.54 +/- 2.01	2.30 +/- 2.86	0.36

Table A.2 Maximum aggregation responses before and after endotoxin injection when

participants received ticagrelor. Values are mean ± SD. P value for each treatment/agonist pairing generated by one-way ANOVA with timepoint as factor. [T]=Plus single 180 mg dose of ticagrelor 60 minutes prior to endotoxin,

Time after endotoxin injection (hours) ⇒		-1	0	3	
Treatment regimen	Agonist	Maximum aggregation (%)			p
No aspirin[T]	ADP 20 μmol/L	76.9 +/- 5.10	32.6 +/- 12.2	21.9 +/- 6.22	**<0.0001**
	Collagen 4 μg/mL	76.1 +/- 7.31	75.0 +/- 4.43	72.8 +/- 6.07	0.44
	Collagen 16 μg/mL	80.4 +/- 7.74	77.4 +/- 5.46	78.1 +/- 6.29	0.44
	AA 1 mmol/L	59.3 +/- 30.4	43.7 +/- 35.6	19.7 +/- 25.8	**0.0011**
Aspirin 20 mg BD[T]	ADP 20 μmol/L	69.5 +/- 5.91	33.2 +/- 18.2	24.0 +/- 12.7	**<0.0001**
	Collagen 4 μg/mL	68.5 +/- 18.9	37.5 +/- 28.9	39.0 +/- 27.3	**0.0004**
	Collagen 16 μg/mL	77.0 +/- 9.93	54.5 +/- 28.3	52.4 +/- 23.3	**0.0009**
	AA 1 mmol/L	1.33 +/- 0.88	0.91 +/- 0.79	1.50 +/- 0.79	0.27
Aspirin 75 mg OD[T]	ADP 20 μmol/L	66.8 +/- 4.08	41.2 +/- 16.4	26.6 +/- 7.57	**0.0003**
	Collagen 4 μg/mL	69.7 +/- 9.14	30.2 +/- 18.4	26.5 +/- 17.9	**<0.0001**
	Collagen 16 μg/mL	72.7 +/- 5.09	46.8 +/- 14.6	45.3 +/- 17.5	**0.0010**
	AA 1 mmol/L	2.50 +/- 1.77	1.37 +/- 0.74	2.12 +/- 0.83	0.26
Aspirin 300 mg OD[T]	ADP 20 μmol/L	65.9 +/- 5.40	32.7 +/- 15.3	26.9 +/- 9.89	**<0.0001**
	Collagen 4 μg/mL	65.5 +/- 8.34	22.0 +/- 13.2	18.3 +/- 8.05	**<0.0001**
	Collagen 16 μg/mL	68.6 +/- 5.91	36.2 +/- 17.6	34.0 +/- 16.4	**<0.0001**
	AA 1 mmol/L	1.80 +/- 1.03	1.22 +/- 0.83	2.00 +/- 1.76	0.060

Table A.3 Pairwise comparisons made following detection of a significant difference between timepoints for a regimen/agonist pairing by one-way ANOVA. Pairwise p values <0.05 are shown in bold. [T] = received a loading dose of ticagrelor 1 hour before endotoxin injection

Max aggregation			Timepoints compared (hours after endotoxin injection)					
Regimen			0 vs -1		3 vs -1		3 vs 0	
		p(ANOVA)	Mean difference (+/- SD)	p	Mean difference (+/- SD)	p	Mean difference (+/- SD)	p
Aspirin 75 mg OD	Collagen 4 µg/mL	0.037	-18.7 +/- 22.4	**0.027**	-10.7 +/- 9.8	**0.0071**	8.0 +/- 21.1	0.26
Aspirin 300 mg OD	Collagen 4 µg/mL	0.014	-14.2 +/- 14.8	**0.0098**	-17.3 +/- 19.5	**0.021**	-1.8 +/- 17.5	0.75
	Collagen 16 µg/mL	0.044	-11.3 +/- 14.6	**0.029**	-9.8 +/- 15.7	0.08	2.1 +/- 11.5	0.58
No aspirin[T]	ADP 20 µmol/L	<0.0001	-44.3 +/- 13.9	**<0.0001**	-55.8 +/- 7.1	**<0.0001**	-11 +/- 14.0	**0.035**
	AA 1 mmol/L	0.0011	-15.6 +/- 23.3	0.05	-38.5 +/- 30.7	**0.0033**	-21.9 +/- 27.1	**0.031**
Aspirin 20 mg BD[T]	ADP 20 µmol/L	<0.0001	-36.3 +/- 18.6	**<0.0001**	-45.4 +/- 12.3	**<0.0001**	-9.2 +/- 12.9	**0.032**
	Collagen 4 µg/mL	0.0004	-30.9 +/- 24.4	**0.0011**	-29.5 +/- 23.4	**0.0011**	1.4 +/- 11.2	0.67
	Collagen 16 µg/mL	0.0009	-22.6 +/- 22.3	**0.0049**	-24.7 +/- 17.9	**0.0006**	-2.1 +/- 11.2	0.53
Aspirin 75 mg OD[T]	ADP 20 µmol/L	0.0003	-25.6 +/- 16.0	**0.0027**	-40.3 +/- 8.8	**<0.0001**	-14.6 +/- 19.2	0.069
	Collagen 4 µg/mL	<0.0001	-39.5 +/- 16.1	**0.0002**	-43.3 +/- 16.5	**0.0001**	-3.8 +/- 12.1	0.41
	Collagen 16 µg/mL	0.001	-25.9 +/- 16.0	**0.0026**	-27.4 +/- 19	**0.0047**	-1.5 +/- 17.6	0.82
Aspirin 300 mg OD[T]	ADP 20 µmol/L	<0.0001	-33.6 +/- 11.8	**<0.0001**	-39.0 +/- 7.7	**<0.0001**	-4.6 +/- 13.0	0.32
	Collagen 4 µg/mL	<0.0001	-43.3 +/- 8.5	**<0.0001**	-47.2 +/- 9.2	**<0.0001**	-3.1 +/- 10.8	0.41
	Collagen 16 µg/mL	<0.0001	-32.8 +/- 18.6	**0.0007**	-34.6 +/- 19.8	**0.0004**	-0.6 +/- 14.5	0.91

Table A.4 Results of one-way ANOVA of maximum aggregation responses before and after endotoxin injection. P value for each treatment/agonist pairing generated for each timepoint by one-way ANOVA with treatment as factor.

		Timepoint (relative to endotoxin injection)		
Comparison	**Agonist**	**-1 hour***	**0 hours**	**+3 hours**
No aspirin vs. aspirin 20 mg BD vs. 75 mg OD vs. 300 mg OD	ADP 20 μmol/L	**<0.0001**	0.14	0.058
	Collagen 4 μg/mL	**0.0004**	**0.0017**	**0.0001**
	Collagen 16 μg/mL	**0.0084**	**0.0042**	**0.0065**
	AA 1 mmol/L	**<0.0001**	**<0.0001**	**<0.0001**
No aspirin plus ticagrelor vs. aspirin 20 mg BD plus ticagrelor vs. 75 mg OD plus ticagrelor vs. 300 mg OD plus ticagrelor	ADP 20 μmol/L	-	0.6	0.64
	Collagen 4 μg/mL	-	**<0.0001**	**<0.0001**
	Collagen 16 μg/mL	-	**0.0002**	**<0.0001**
	AA 1 mmol/L	-	**<0.0001**	**0.0068**
Aspirin 20 mg BD vs. aspirin 75 mg OD vs. aspirin 300 mg OD	ADP 20 μmol/L	0.38	0.88	0.22
	Collagen 4 μg/mL	**0.043**	0.074	**0.005**
	Collagen 16 μg/mL	0.059	0.075	**0.023**
	AA 1 mmol/L	0.35	0.72	0.5
Aspirin 20 mg BD plus ticagrelor vs. 75 mg OD plus ticagrelor vs. 300 mg OD plus ticagrelor	ADP 20 μmol/L	-	0.51	0.79
	Collagen 4 μg/mL	-	0.3	0.071
	Collagen 16 μg/mL	-	0.2	0.11
	AA 1 mmol/L	-	0.43	0.47

*pooled data from paired regimens

Table A.5 Pairwise comparisons between aspirin regimens for timepoint/agonist pairs identified to exhibit significant heterogeneity from ANOVA. Data shown are mean (95% CI).

Timepoint	Agonist	p (ANOVA)	aspirin 75 vs 20		300 vs 20		300 vs 75	
			Mean difference	p	Mean difference	p	Mean difference	p
-1 hour	Collagen 4 µg/mL	0.043	1.1 (-7.1 to 9.3)	0.79	-8.2 (-16.3 to -0.03)	**0.049**	-9.3 (-16.7 to -1.9)	**0.015**
+ 3 hours	Collagen 4 µg/mL	0.005	-11.2 (-29.9 to 7.4)	0.21	-26.3 (-51.7 to -0.9)	**0.044**	-18.1 (-31.7 to -4.5)	**0.016**
+ 3 hours	Collagen 16 µg/mL	0.023	-2.9 (-12.8 to 7.0)	0.52	-11.2 (-28.1 to 5.6)	0.16	-11.63 (-25.3 to 2.1)	0.084

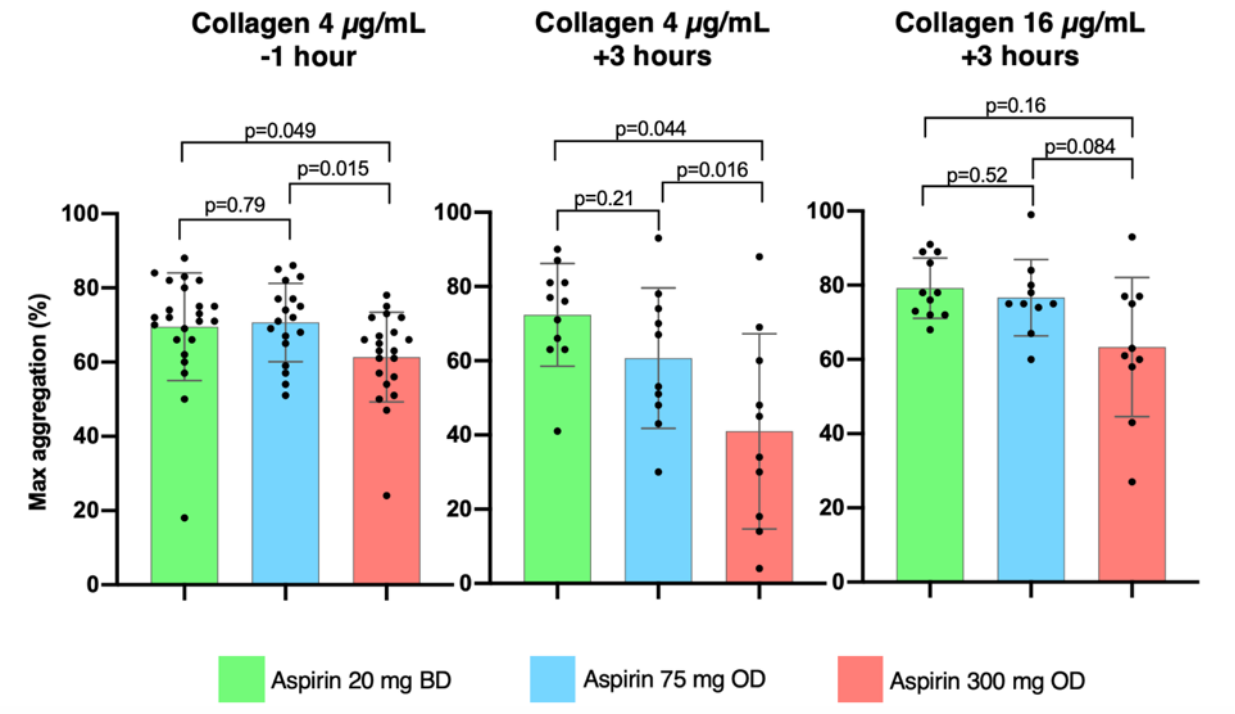

Figure A.1 Maximum aggregation responses compared between aspirin regimens for those timepoint/agonist combinations identified as exhibiting significantly heterogeneity during one-way ANOVA, showing results of pairwise comparisons (p values) using t-tests. Bars represent mean ± SD.

Table A.6 Final aggregation responses before and after endotoxin injection when participants did not receive ticagrelor. Values are mean ± SD. P value for each treatment/agonist pairing generated by one-way ANOVA with timepoint as factor.

Time after endotoxin injection (hours) ⇒		-1	0	3	
Treatment regimen	Agonist	Final aggregation (%)			p
No aspirin	ADP 20 μmol/L	71.8 +/- 4.96	74.8 +/- 7.11	74.6 +/- 4.76	0.26
	Collagen 4 μg/mL	75.7 +/- 5.12	76.6 +/- 7.38	77.6 +/- 4.97	0.32
	Collagen 16 μg/mL	75.8 +/- 6.66	79.8 +/- 5.86	78.7 +/- 5.47	0.23
	AA 1 mmol/L	60.5 +/- 32.0	53.0 +/- 37.1	56.5 +/- 39.5	0.37
Aspirin 20 mg BD	ADP 20 μmol/L	57.0 +/- 9.44	62.8 +/- 12.9	60 +/- 13.3	0.13
	Collagen 4 μg/mL	68.1 +/- 8.13	63.0 +/- 17.4	68.5 +/- 15.3	0.26
	Collagen 16 μg/mL	71.1 +/- 8.26	72.9 +/- 12.7	76.5 +/- 7.18	0.13
	AA 1 mmol/L	0.63 +/- 1.43	0.36 +/- 1.20	0.36 +/- 1.20	0.29
Aspirin 75 mg OD	ADP 20 μmol/L	65.8 +/- 13.7	64.7 +/- 13.8	68.0 +/- 12.6	0.52
	Collagen 4 μg/mL	67.3 +/- 14.7	48.6 +/- 28.8	55.0 +/- 22.0	0.029
	Collagen 16 μg/mL	76.7 +/- 7.81	67.7 +/- 13.4	73.8 +/- 10.7	0.18
	AA 1 mmol/L	0.30 +/- 0.94	0.00 +/- 0.00	0.00 +/- 0.00	0.34
Aspirin 300 mg OD	ADP 20 μmol/L	64.1 +/- 9.72	61.9 +/- 13.4	64.9 +/- 10.9	0.70
	Collagen 4 μg/mL	52.4 +/- 16.8	39.7 +/- 22.7	37.6 +/- 26.3	0.031
	Collagen 16 μg/mL	70.0 +/- 8.98	57.5 +/- 15.9	60.6 +/- 19.1	0.030
	AA 1 mmol/L	0.90 +/- 2.02	0.90 +/- 2.21	1.40 +/- 3.27	0.59

Table A.7 Final aggregation responses before and after endotoxin injection when participants did not receive ticagrelor. Values are mean ± SD. P value for each treatment/agonist pairing generated by one-way ANOVA with timepoint as factor. [T]= received loading dose of ticagrelor at -1 hour timepoint.

Time after endotoxin injection (hours) ⇒		-1	0	3	
Treatment regimen	Agonist	Final aggregation (%)			p
No aspirin[T]	ADP 20 μmol/L	74.7 +/- 5.29	13.0 +/- 18.3	3.30 +/- 4.27	<0.0001
	Collagen 4 μg/mL	74.2 +/- 7.19	72.6 +/- 4.13	69.0 +/- 6.53	0.15
	Collagen 16 μg/mL	77.1 +/- 6.59	74.8 +/- 5.54	75.4 +/- 6.09	0.52
	AA 1 mmol/L	57.9 +/- 29.6	42.0 +/- 34.9	13.6 +/- 24.4	0.0005
Aspirin 20 mg BD[T]	ADP 20 μmol/L	62.2 +/- 11.1	12.5 +/- 13.4	7.25 +/- 9.27	<0.0001
	Collagen 4 μg/mL	65.5 +/- 19.4	32.5 +/- 27.0	34.6 +/- 26.6	0.0001
	Collagen 16 μg/mL	74.7 +/- 10.6	51.6 +/- 27.6	49.0 +/- 22.7	0.0004
	AA 1 mmol/L	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	>0.99
Aspirin 75 mg OD[T]	ADP 20 μmol/L	61.3 +/- 6.80	19.6 +/- 20.3	3.62 +/- 4.03	<0.0001
	Collagen 4 μg/mL	66.6 +/- 10.6	24.6 +/- 14.4	22.3 +/- 14.7	<0.0001
	Collagen 16 μg/mL	70.2 +/- 4.86	42.5 +/- 14.4	41.1 +/- 16.2	0.0005
	AA 1 mmol/L	1.12 +/- 2.10	0.00 +/- 0.00	0.37 +/- 1.06	0.30
Aspirin 300 mg OD[T]	ADP 20 μmol/L	59.3 +/- 9.47	12.6 +/- 16.7	6.40 +/- 8.03	<0.0001
	Collagen 4 μg/mL	62.8 +/- 8.81	18.3 +/- 10.1	16.3 +/- 6.61	<0.0001
	Collagen 16 μg/mL	66.2 +/- 5.86	32.6 +/- 16.5	30.9 +/- 15.5	<0.0001
	AA 1 mmol/L	0.00 +/- 0.00	0.00 +/- 0.00	1.00 +/- 2.10	0.17

Table A.8 Platelet P-selectin expression after in vitro stimulation with ADP (30 μmol/L) at each timepoint for each regimen. Data shown are mean ± SD. [T]= received a loading dose of ticagrelor 1 hour prior to endotoxin injection.

		Platelet P-selectin expression	
Regimen	**Timepoint (hours)**	**% Population**	**MFI**
No aspirin	-1	45.8 +/- 10.2	697 +/- 363
	+6	29.8 +/- 17.7	479 +/- 184
Aspirin 20 mg BD	-1	43.8 +/- 15.9	628 +/- 233
	+6	29.2 +/- 19.8	457 +/- 210
Aspirin 75 mg OD	-1	50.5 +/- 19.2	831 +/- 409
	+6	30.8 +/- 12.2	476 +/- 165
Aspirin 300 mg OD	-1	40.9 +/- 13.4	652 +/- 300
	+6	23.4 +/- 13.9	420 +/- 170
No aspirin[T]	-1	48.5 +/- 18.6	688 +/- 265
	+6	6.56 +/- 6.73	253 +/- 48
Aspirin 20 mg BD[T]	-1	37 +/- 16.6	599 +/- 285
	+6	5.38 +/- 7.05	249 +/- 56.2
Aspirin 75 mg OD[T]	-1	39.9 +/- 14	551 +/- 188
	+6	6.01 +/- 9.35	252 +/- 63.2
Aspirin 300 mg OD[T]	-1	43.9 +/- 17.2	626 +/- 224
	+6	9.23 +/- 16.4	229 +/- 31.2

Table A.9 Forearm bleeding time 1 hour before and 6 hours after endotoxin injection.

[T]= received loading dose of ticagrelor 1 hour before endotoxin.

		Bleeding time (secs)
		Mean +/- SD
Baseline		
	All participants undergoing ≥1 endotoxin challenge	247.8 +/- 70.4
1 hour pre-endotoxin (immediately before last dose of study medication)		
Treatment group	No aspirin	263.1 +/- 55.7
	Aspirin 20 mg BD	280.5 +/- 62.5
	Aspirin 75 mg OD	343.5 +/- 108.2
	Aspirin 300 mg OD	331.0 +/- 101.0
3 hours post-endotoxin/4 hours post-last dose of study medication		
Treatment group	No aspirin	270.0 +/- 149.7
	Aspirin 20 mg BD	284.5 +/- 109.6
	Aspirin 75 mg OD	302.1 +/- 65.0
	Aspirin 300 mg OD	282.2 +/- 75.7
	No aspirin[T]	476.3 +/- 161.6
	Aspirin 20 mg BD[T]	419.5 +/- 162.2
	Aspirin 75 mg OD[T]	521.4 +/- 164.9
	Aspirin 300 mg OD[T]	565.6 +/- 159.0

[T]Plus single 180 mg dose of ticagrelor 60 minutes prior to endotoxin