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## **Anti-inflammatory effects of anti-platelet drugs Implications for atherosclerosis**

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**Anti-inflammatory effects of anti-platelet drugs:  
implications for atherosclerosis**

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*This thesis is submitted in partial fulfilment of the requirements for the  
degree of Doctor of Philosophy from the King's College London*

## Abstract

Cardiovascular disease secondary to atherosclerosis is the leading cause of death worldwide. The pathophysiology of atherosclerosis is multifactorial and complex. This thesis offers insight into the regulation of several potential biomarkers of cardiovascular disease and explores the effects of anti-platelet therapy in modulating these pathways.

CD14<sup>high</sup>CD16<sup>+</sup> monocytes are elevated in patients with atherosclerosis and may be predictive of future cardiovascular events. The initial study in this PhD project assessed the effects of anti-platelet drugs on the phenotype of circulating monocytes in 60 healthy volunteers in the presence of mild, systemic inflammation induced by the influenza immunisation. A significant rise in circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes developed following immunisation and anti-platelet therapy was subsequently shown to exert an immunomodulatory action by counteracting this response.

Netrin-1 is a laminin-like protein that is implicated in cardiovascular disease, including coronary artery disease. A series of experiments were performed to investigate the biomolecular mechanisms that regulate the synthesis of vascular netrin-1 in humans. Results showed that netrin-1 levels are directly modulated by changes in the production of vasoactive cyclooxygenase-derived molecules, such as prostaglandin E<sub>2</sub>, from the vascular endothelium.

Oxidised low-density lipoprotein was the final biomarker that was investigated. Results indicated that CD16<sup>+</sup> monocytes may regulate the clearance of oxidised lipoproteins and their systemic accumulation, possibly through the internalisation of circulating oxLDL / IgG immunocomplexes mediated by Fc  $\gamma$  receptors, including CD16.

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## List of publications derived from this thesis

### Articles:

- Layne K, Goodman T, Ferro A, Passacquale G. The effect of aspirin on circulating netrin-1 levels in humans is dependent on the inflammatory status of the vascular endothelium. *Oncotarget*. 2017 Sep 23;8(49):86548-86555. [PMID: 29156815](#)
- Layne K, Ferro A. Anti-platelet drugs in acute coronary syndrome. *European Cardiology Review*. 2017 Jul;12(1):33–7.
- Layne K and Ferro A. Traditional Chinese medicines in the management of cardiovascular diseases: a comprehensive systematic review. *British Journal of Clinical Pharmacology*. 2017 Jan;83(1):20-32., [PMID: 27195823](#)
- Layne K and Ferro A. Continuation of dual anti-platelet therapy after angioplasty beyond 12 months – effects on in-stent thrombosis. *www.cardio-debate.com*. 2016.
- Layne K, Di Giosia P, Ferro A, Passacquale G. Anti-platelet drugs attenuate the expansion of circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes under pro-inflammatory conditions. *Cardiovascular Research*. 2016 Jul 1;111(1):26-33. [PMID: 27118470](#)
- Layne K, Ferro A, Passacquale G. Netrin-1 as a novel therapeutic target in cardiovascular disease: to activate or inhibit? *Cardiovascular Research*. 2015 Sep 1;107(4):410-9. [PMID: 26209250](#)

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- Layne K, Goodman T, Ferro A, Passacquale G. Netrin-1 levels are reduced in healthy subjects in response to treatment with aspirin. *Clinical Therapeutics*. 2015 Aug 1; 37(8):e32–e33

- Layne K, Goodman T, Ferro A, Passacuale G. PP.07.13 Netrin-1 levels. Journal of Hypertension. 2015 Jun; 33 Suppl 1: e193
- Layne K, Di Giosia P, Ferro A, Passacuale G. Anti-inflammatory effects of anti-platelet drugs: implications for atherosclerosis. Journal of Hypertension. 2015 Jun;33 Suppl 1:e126. [PMID: 26102729](#)

Book chapters:

- Ferro A, Layne K. Platelet dynamics and role in the pathogenesis of atherosclerotic vascular ischemic conditions. Chapter from 'Cardiovascular thrombus: from pathology and clinical presentations to imaging, pharmacotherapy and interventions', Elsevier, to be published 2018.

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## Abbreviations

µg	Microgram
µl	Microlitre
AA	Arachidonic acid
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Allophycocyanin
ApoE	Apolipoprotein
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
CCL5	Chemokine (C-C motif) ligand 5
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
COX	Cyclo-oxygenase
CRP	C-reactive protein
Cy5	Complex-cyanine 5
DAPT	Dual anti-platelet therapy
DCC	Deleted in colorectal cancer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMR-1	EGF-like module containing, mucin-like, hormone receptor-like 1
eNOS	Endothelial nitric oxide synthase
F(ab') <sub>2</sub>	Fragment antibody binding domain
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HbA1c	Glycated haemoglobin
HDL	High-density lipoprotein
HIF-1	Hypoxia-inducible factor 1
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
Hs-CRP	High-sensitivity C-reactive protein
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular Adhesion Molecule 1
ICC	Intra-class correlation coefficient
IGF	Insulin-like growth factor
IgG	Immunoglobulin type G
IgM	Immunoglobulin type M
IL-10	Interleukin-10
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
IMT	Intima-media thickness
IQR	Interquartile range

ITGAM	Integrin alpha M
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LVEF	Left ventricular ejection fraction
M	Molar
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MFI	Median fluorescence intensity
Mg	Milligram
MI	Myocardial infarction
ml	Millilitre
MPA	Monocyte-platelet aggregates
MPO	Myeloperoxidase
NADH	Nicotinamide adenine dinucleotide + hydrogen
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Health and Care Excellence
nMFI	Normalised median fluorescence intensity
NSAID	Non-steroidal anti-inflammatory drug
NSTEMI	Non-ST-segment elevation myocardial infarction
oxLDL	Oxidised low-density lipoprotein
PAGE	Polyacrylamide gel electrophoresis
PAR1	Proteinase-activated receptor-1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PE	R-phycoerythrin
PER-CP	Peridinin-chlorophyll-protein
PG	Prostaglandin
PMN	Polymorphonuclear cell
PSGL-1	P-selectin glycoprotein ligand-1
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate



## **CHAPTER ONE:**

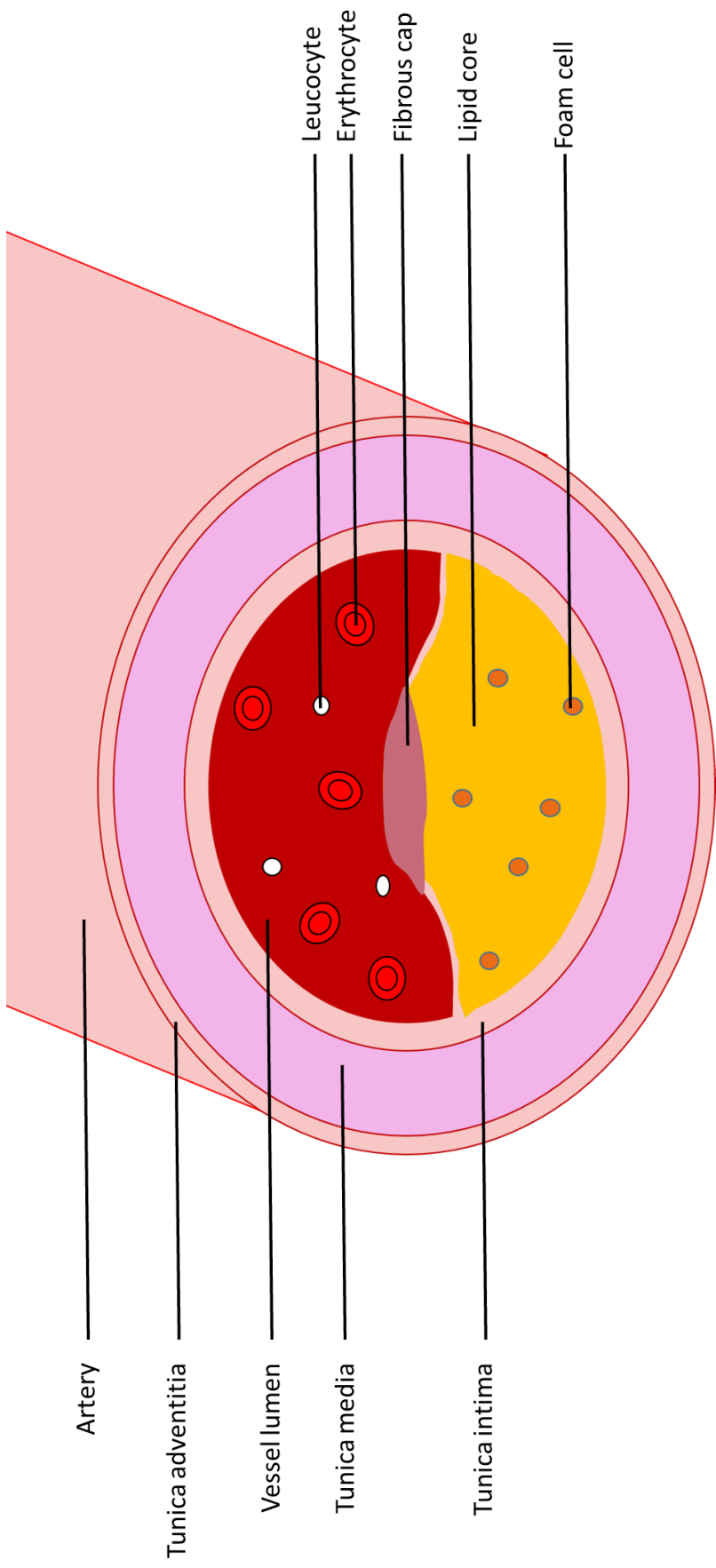
## **INTRODUCTION**

## **1.1. The role of inflammation in atherosclerosis**

Cardiovascular disease secondary to atherosclerosis is now the leading cause of death worldwide <sup>1</sup>. The pathophysiology of atherosclerosis is multifactorial and complex. This thesis will explore the inflammatory pathways that contribute to atherogenesis and assess the anti-atherogenic effects of anti-platelet drugs in patients with silent atherosclerosis.

### **1.1.1 Atherogenesis**

Atherosclerotic plaques develop over many years and frequently go unnoticed until a patient develops symptoms of ischaemia. The lesions typically develop in areas where there is a substantial change in blood flow velocity. The first stage of atherosclerosis occurs when serum lipoproteins accumulate within the arterial intima creating a state of chronic inflammation. The intimal cells become increasingly hypoxic as the arterial wall thickness increases. Monocytes are recruited to the subendothelial layer where the initial lesion, known as a fatty streak, develops. The monocytes differentiate into macrophages, which phagocytose the modified lipoproteins, in particular oxidised low-density lipoprotein (oxLDL) and form foam cells. Foam cells accumulate over time and aggregate into a necrotic, atheromatous core within the lesion. The plaque reduces the diameter of the vessel lumen, impairing arterial flow. Hypoxic conditions develop within the plaque and subsequent neovascularisation develops, increasing the likelihood of plaque haemorrhage. As the lesion increases in size, the endothelial shear stress to which it is subjected will rise accordingly. If the plaque ruptures, the lipid-rich contents of the atheromatous core are released and the subendothelial layer is exposed. Both plaque-bound and circulating blood-borne tissue factor bind to, and activate, factor VII, initiating the clotting cascade and thus platelet activation and aggregation. Platelets adhere to the ruptured lesion, enhancing the production of platelet agonists, thrombin and thromboxane A<sub>2</sub> (TXA<sub>2</sub>). Further platelet recruitment occurs and thrombus formation develops along with vasoconstriction of the already compromised vessels. Significant vessel occlusion will result in organ ischaemia and, ultimately, infarction, precipitating events such as stroke and myocardial infarction (MI) <sup>2,3</sup>.



Artery

Tunica adventitia

Vessel lumen

Tunica media

Tunica intima

Leucocyte

Erythrocyte

Fibrous cap

Lipid core

Foam cell

**Figure 1.1: Atherosclerotic plaque**

As atherosclerosis develops, the vessel lumen narrows, leading to reduced blood flow. The plaque forms a fibrous cap, which may rupture and expose the underlying lipid contents, precipitating platelet activation and, ultimately, thrombogenesis.

### **1.1.2 Inflammatory pathways associated with atherosclerosis**

The 'Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin' (JUPITER) study identified that elevated levels of high-sensitivity C-reactive protein (hs-CRP), a hepatically synthesised biomarker of inflammation produced in response to macrophage-derived interleukin-6 (IL-6), were associated with cardiovascular events, including MI and stroke in patients with no previously known cardiac disease <sup>45</sup>. Hs-CRP was found to be a stronger predictor of cardiovascular disease than the traditionally used low-density lipoprotein (LDL) levels, demonstrating the importance of underlying inflammation in the development of cardiovascular disease. Hs-CRP, however, is a non-specific acute phase response protein, and rises in most pathologies where an inflammatory response is generated, including malignancy, auto-immune disease, late-stage pregnancy and bacterial infection. Hs-CRP has yet to be causally related to coronary heart disease and a reduction in baseline levels has not been proven to improve prognosis <sup>67</sup>.

Patients with chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease have an increased risk of developing cardiovascular disease in later life, suggesting a possible link between inflammatory processes and atherogenesis <sup>8</sup>. Pro-inflammatory biomarkers have been shown to have significant prognostic value in MI <sup>9</sup>, and a recent clinical trial has also identified potential benefits arising from administration of an anti-inflammatory therapy (colchicine) in the management of acute ST-segment elevation MI (STEMI) <sup>10</sup>. This thesis will focus on three novel inflammatory biomarkers: intermediate CD14<sup>high</sup>CD16<sup>+</sup> monocytes, netrin-1, and oxLDL, addressing their role in the development of cardiovascular disease and whether anti-platelet therapy can modify their expression.

### **1.1.3 Oxidised low-density lipoprotein in atherosclerosis**

Clinical evidence has shown that dysregulation of the innate and adaptive immune systems occurs during atherogenesis, which is typically accompanied by accumulation of pro-immunogenic factors, such as oxLDL, that are able to activate innate cell effectors (primarily

myeloid cells), subsequently leading to an adaptive immunological response with production of circulating autoantibodies (Immunoglobulin (Ig) M and IgG anti-oxLDL antibodies). Classical risk factors for cardiovascular disease are poorly correlated with the levels of these immunological mediators and thus the question arises as to whether pharmacological targeting of oxLDL and its autoantibodies, or reducing monocyte/macrophage activity, may represent a viable strategy to improve treatment in patients with cardiovascular disease.

To date, LDL has been among the preferred therapeutic targets, and drugs such as 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) that reduce circulating oxLDL levels are prescribed abundantly in both primary and secondary prevention of cardiovascular disease. New immunomodulatory agents continue to be developed in order to reduce levels of circulating oxLDL, by either reducing synthesis of oxLDL, such as can occur with certain phospholipase A<sub>2</sub> inhibitors<sup>11 12</sup>, or increasing clearance of the molecules via passive immunisation strategies to shift the synthesis of anti-oxLDL autoantibodies away from IgM production and in favour of IgG that is thought to be protective<sup>13 14</sup>. The effectiveness of these therapeutic strategies remains to be defined and whilst several lipoprotein-associated phospholipase A<sub>2</sub> inhibitors have entered Phase III clinical trials, they have yet to demonstrate a significant reduction in cardiovascular events when used alongside conventional pharmacotherapy<sup>15 16</sup>. Immunisation agents are still at the pre-clinical stage of development and further definition of their therapeutic potential is required<sup>17</sup>. Characterisation of the modulatory effect of conventional therapy on oxLDL-induced immunological dysfunction may promote further advance in this field.

Previous work by our group has demonstrated inflammation can lead to a change in the phenotype of circulating monocytes in terms of their expression of the CD16 molecule<sup>18</sup>. This latter acts as a Fc  $\gamma$  receptor for IgG antibodies<sup>19</sup> and as such may mediate the phagocytosis of immune complexes consisting of IgG-opsonised oxLDL, thus contributing to their clearance and, in turn, regulating accumulation of pro-immunogenic LDL<sup>20</sup>. Evidence has also emerged that

myeloid CD16 directly binds to oxLDL via the specific recognition of malondialdehyde (MDA) epitopes contained in modified lipoproteins by the Fc  $\gamma$  receptor <sup>21</sup>.

Expansion of the CD16<sup>+</sup> monocyte pool occurs in the peripheral blood of patients with cardiovascular disease <sup>22 23</sup>; whether this occurrence has pro-atherogenic implications or is rather a protective mechanism potentially implicated in the detoxification of the blood from oxLDL is unknown.

#### **1.1.4 Immunisation as a model of inflammation**

Over recent years, immunisation has become a well-established model to study the response to mild stimulation of the inflammatory system <sup>24 25</sup>, and has been shown to generate a similar acute phase protein response to that of chronic systemic inflammatory conditions, such as rheumatoid arthritis <sup>26</sup>. *Salmonella typhi* immunisation, in particular, has been used to investigate cardiovascular physiology, where the widespread inflammation induced by the immunisation causes profound dysfunction of the arterial endothelium in healthy volunteers, as evidenced by impaired vasodilatation in response to both physical and pharmacological dilator stimuli <sup>27 28</sup>. These generalised inflammatory changes post-immunisation may be modified by systemic pre-treatment with aspirin, although locally administered aspirin is unable to reverse established immunisation-induced endothelial dysfunction <sup>29</sup>.

In 2004, Posthouwer *et al* showed that influenza immunisation can serve as an *in vivo* model to investigate a generalised pro-inflammatory state. Using healthy subjects, they demonstrated that following influenza immunisation, there is a consistent elevation in the inflammatory biomarker CRP <sup>30</sup>. The same model was subsequently used to identify that acute inflammation leads to higher circulating levels of activated platelets and to formation of monocyte-platelet aggregates (MPA), which in turn promotes expansion of the CD14<sup>high</sup>CD16<sup>+</sup> monocytic subset <sup>31</sup>. A subsequent study to assess endothelial function, as measured by flow-mediated vasodilatation and soluble intracellular adhesion molecule-1 (ICAM-1) levels, in patients with human immunodeficiency virus infection undergoing either influenza A or sham immunisation

identified that the active immunisation cohort developed a leucocytosis, indicative of systemic inflammation, as well as impaired endothelial function <sup>32</sup>.

Acute infections, particularly with the influenza virus, are strongly associated with cardiovascular events <sup>33</sup>. Despite the transient pro-inflammatory response generated post-immunisation, influenza immunisation does not appear to increase the frequency of cardiovascular events in the short-term, and has consistently been shown to protect against MI in the medium to long-term <sup>34 35</sup>. Patients with cardiovascular disease in both the UK and the USA are advised to undergo influenza immunisation on an annual basis <sup>36 37</sup>.

It is unclear whether anti-platelet therapy is able to modulate the effectiveness of the immune response following vaccination. Current guidelines advise against the use of paracetamol or ibuprofen in children post-immunisation as it is generally accepted that the use of antipyretics may result in a blunted immune response <sup>38 39</sup>. Indeed, COX-2 function is a major determinant of antibody synthesis and both aspirin and ibuprofen have been shown to reduce IgM and IgG synthesis in human peripheral blood mononuclear cells <sup>40</sup>. It would therefore stand to reason that aspirin use may reduce the effectiveness of immunisation against influenza and other infections, although a randomised controlled trial assessing the development of antibodies against influenza subtypes A and B post-vaccination in elderly participants taking either aspirin or a placebo showed that aspirin actually augmented the immune response, with higher levels of specific antibodies directed against influenza being generated <sup>41</sup>. To date, there has been minimal research investigating the effect of anti-platelet drugs on immune responses to vaccination.

## **1.2 Platelets**

Platelets, otherwise known as thrombocytes, are approximately 2.5 µm anuclear, disc-shaped cytoplasmic fragments whose primary physiological function is to contribute to haemostasis <sup>42</sup>, although they play further roles in inflammatory processes. Platelets are derived from megakaryocyte precursor cells, which are present within the bone marrow. Thrombopoietin, a

glycoprotein growth factor, is the primary regulator of megakaryocyte production and development, alongside numerous cytokines and hormones. As megakaryocytes mature, they enlarge to allow high concentrations of ribosomes to accumulate, from which platelet-specific proteins will eventually be formed<sup>43</sup>. Although this process has not been fully elucidated, *in vitro* data suggest that pseudopodial elongations, termed proplatelets, emerge from the mature megakaryocyte, and platelets are budded off from the ends of these processes<sup>44</sup>. Each megakaryocyte can release thousands of platelets, which enter the systemic circulation or are reserved within the spleen<sup>43</sup>. Circulating platelets have a life span of 8-10 days, and are typically present at a concentration of 150-400 x10<sup>9</sup> per litre of blood<sup>45</sup>.

The platelet structure is maintained by a complex actin cytoskeleton formation, while the cell surface is covered by invaginations of the plasma membrane forming a network of tubes termed the open canalicular system<sup>46</sup>. This allows substances to be transported from the surface to within the platelet, whilst additionally providing a mechanism of secreting storage granules<sup>47</sup>. The open canalicular system, along with the surface membranes of platelet storage granules, allows a 2-4 fold increase in platelet surface area<sup>48</sup>.

There are three main classes of storage granules within platelets. The most abundant, and most thoroughly characterised, are the alpha granules, which contain hundreds of soluble proteins, including:

- 1) Haemostatic proteins, such as fibrinogen and von Willebrand factor.
- 2) Coagulation factors, including Factors V, XI, and XIII.
- 3) Cellular adhesion molecules, including P-selectin and CD63.
- 4) Cytokines, including platelet-factor 4 and chemokine (C-C motif) ligand 5 (CCL5).
- 5) Growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF)<sup>48</sup>.

Additionally, there are delta (also known as dense) granules, which contain platelet activation factors, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin,



histamine, and ionised calcium <sup>49</sup>. Lysosomal granules contain hydrolytic enzymes, which are released upon platelet activation <sup>50</sup>.

### **1.2.1 Platelet activation and function**

The classically described function of platelets is to prevent haemorrhage. In the presence of an intact, healthy endothelium, circulating platelets remain in a resting state. Platelets move into an activated state in response to several factors that signal vascular trauma, whereby they adhere to damaged endothelium and locally mediate both thrombotic and inflammatory events via the secretion of cytokines and interaction with leucocytes. This results in chemotaxis and the development of an inflammatory milieu within the arterial wall.

When endothelial damage has occurred, the extracellular matrix is exposed and collagens are released. Collagens stimulate a change in the conformation of platelets, which change from a discoid shape to a spherical one, and develop dendritic pseudopodia <sup>51</sup>. The collagens bind to von Willebrand factor, which interacts with glycoprotein Ib/V/IX, a transmembrane protein complex on the surface of platelets that mediates the tethering of activated platelets to the site of vascular injury <sup>52-55</sup>. The collagen receptor, glycoprotein VI, is also expressed on the platelet surface and binds to collagen to induce the release of secondary platelet agonists, including TXA<sub>2</sub>, ADP, thrombin, and adrenaline, which serve to stimulate further autocrine and paracrine platelet activation <sup>54</sup>.

Platelet agonists act on G-protein coupled receptors, including those linked to the G<sub>q</sub>, G<sub>13</sub>, and G<sub>i</sub> proteins, to activate the platelet integrin, glycoprotein IIb/IIIa <sup>56</sup>. This receptor mediates platelet adhesion via binding to fibrinogen and forming bridges between platelets. A haemostatic platelet plug subsequently develops, which increases in size as further platelet activation is propagated.

### **1.2.1.1 Thrombin**

Thrombin forms a key part of the initiation of the coagulation cascade, and is produced upon cleavage of prothrombin by activated factor Xa. Thrombin acts as a serine protease to convert fibrinogen to fibrin, which subsequently polymerises into multimeric strands to create a mesh that, along with platelets, forms the haemostatic plug<sup>57</sup>. Thrombin additionally converts factor XIII to XIIIa, which induces the formation of covalent cross-links between the fibrin strands, thus further stabilising and strengthening the clot<sup>58</sup>.

Thrombin binds to protease-activated receptors on endothelial cells, activating the cells and thus promoting platelet adhesion and cytokine release, and triggering endothelial cell production of platelet-activating factor<sup>59</sup>. Thrombin can additionally modify vascular tone by direct and indirect effects on vascular smooth muscle cells and post-capillary vessels<sup>60-62</sup>, partially via the promotion of Ca<sup>2+</sup> release from intracellular stores and Ca<sup>2+</sup> influx across the plasma membrane<sup>63</sup>. This is of particular clinical relevance in the context of subarachnoid haemorrhage, where thrombin accumulates at the site of the bleed and amplifies cerebral vasospasm; the contractile response to thrombin is then typically augmented by the upregulation of protease-activated receptor-1 (PAR1)<sup>64</sup>.

### **1.2.1.2 Adenosine diphosphate**

ADP is a platelet agonist that interacts with platelet purinergic P2 receptors, including P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2X<sub>1</sub><sup>65</sup>. Upon binding to ADP, platelets undergo a conformational change, developing a spiculated, spherical shape, enabling the release of granular contents (containing additional ADP) and thus further potentiating platelet aggregation<sup>66 67</sup>. As with thrombin, ADP enhances intracellular stored Ca<sup>2+</sup> release through inositol phosphate production and rapid calcium influx via the plasma membrane<sup>65</sup>. The P2Y<sub>1</sub> G<sub>q</sub>-protein coupled receptor mediates the platelet structural changes, mobilises calcium, and stimulates the phospholipase C and inositol phosphate signalling pathways. P2Y<sub>12</sub> is a G<sub>i</sub>-protein coupled receptor that blocks adenylyl cyclase signalling and reduces intracellular cyclic adenosine monophosphate (cAMP) levels. The

fall in cAMP inhibits phosphorylation of vasodilator-stimulated phosphoprotein, which subsequently induces activation of the GP IIb/IIIa receptor and platelet aggregation <sup>68</sup>. P2X<sub>1</sub> functions as a ligand-gated ion channel, allowing rapid calcium influx upon binding of ADP <sup>67</sup>. Patients with abnormal storage and release of ADP from dense granules typically present with prolonged bleeding times due to impaired platelet aggregation <sup>69</sup>.

### **1.2.1.3 Thromboxane A<sub>2</sub>**

Binding of ADP to P2Y<sub>12</sub> stimulates phospholipase A<sub>2</sub>-induced release of arachidonic acid (AA) from phospholipid. AA is converted to prostaglandin (PG) G<sub>2</sub> by cyclo-oxygenase-1 (COX-1), which is then reduced by a peroxidase to PGH<sub>2</sub> and subsequently converted to TXA<sub>2</sub> via TXA<sub>2</sub> synthase <sup>65 66</sup>. The thromboxane/PGH<sub>2</sub> receptor is present on platelets, as well as monocytes, erythrocytes and endothelial cells. TXA<sub>2</sub> binds to the thromboxane/PGH<sub>2</sub> receptor, which has two main isoforms: the G<sub>q</sub>-coupled TP $\alpha$ , and the G<sub>i</sub>-coupled TP $\beta$  receptors. These two receptors differ in their C-terminal cytoplasmic domains <sup>70</sup>, with the TP $\alpha$  isoform primarily being expressed on platelets <sup>71</sup>.

TXA<sub>2</sub> binding to the TP $\alpha$  receptor leads to activation of both G<sub>s</sub> and G<sub>i</sub> proteins, with consequent activation of both phospholipase C and adenylyl cyclase, whilst its binding to TP $\beta$  results in G<sub>i</sub> activation and consequent inhibition of adenylyl cyclase; the net result in platelets being their activation leading to the abovementioned conformational changes and aggregation <sup>72</sup>. A series of experiments using transfected cell lines, showed that TXA<sub>2</sub> binding to the TP $\alpha$  receptor stimulated adenylyl cyclase and thus increased cAMP formation, whilst opposite effects were seen with the TP $\beta$  receptor <sup>73</sup>.

Aside from platelet activation, TXA<sub>2</sub> plays roles in atherogenesis, vascular remodelling, and immunogenicity <sup>74 75</sup>, and is a potent vasoconstrictor <sup>76</sup>. TXA<sub>2</sub> increases the expression of endothelial adhesion molecules, such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), and plays complex roles in endothelial cell migration <sup>75</sup>. Mutations in the TP receptors result in a varied clinical picture, ranging from impaired haemostasis when reduced receptor activity

is present to predisposition to thrombosis and infarction<sup>77 78</sup>. Due to the short half-life of TXA<sub>2</sub>, its metabolite, 11-dehydro TXB<sub>2</sub> is commonly used when measured in urine as an indirect measure of TXA<sub>2</sub><sup>79</sup>.

### **1.2.2 Anti-platelet agents**

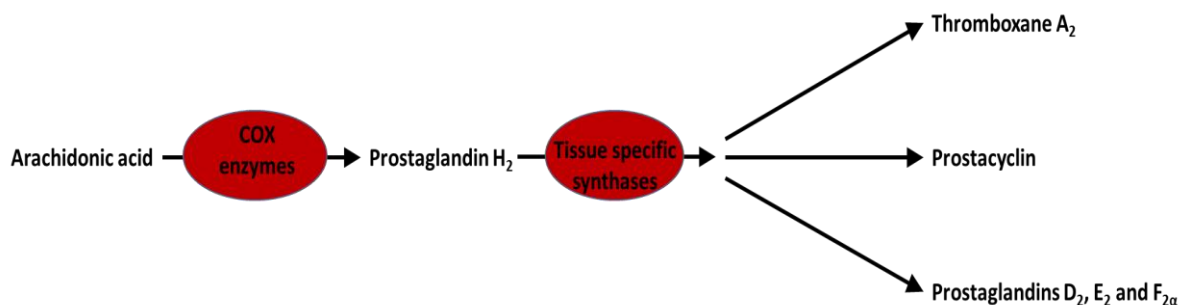
Anti-platelet agents act via a variety of mechanisms to inhibit platelet aggregation. They are the cornerstone of treatment in cardiovascular disease and are used prophylactically in patients with significant atherosclerosis who are at risk of thrombotic events. Anti-platelet agents, particularly aspirin, have been used for decades and the drug class continues to evolve as novel agents with increasingly efficacious anti-platelet actions are identified. The main risk associated with all forms of anti-platelet therapy is bleeding, and physicians need to carefully weigh the possible adverse effects against the benefits of prescribing these drugs to patients with cardiovascular disease. Aspirin, which has been recognised to have anti-thrombotic effects since the 1960s, continues to be prescribed almost ubiquitously for patients with acute coronary syndrome (ACS), and P2Y<sub>12</sub> antagonists are now often added in; such dual anti-platelet therapy (DAPT) confers greater anti-thrombotic efficacy but at the risk of increased bleeding. Over recent years, it has become apparent that these drugs may also exert powerful anti-inflammatory effects that provide additional benefit in the management of ACS.

Anti-platelet therapy may improve outcomes not only through anti-thrombotic properties but also via their anti-inflammatory effects, although their relative contribution in this context remains a subject of debate. What is clear, however, is that increased anti-thrombotic efficacy improves outcomes post-ACS, but carries the price of increased bleeding risk, so that at some point diminishing returns accrue from ever more efficacious anti-platelet therapy. The challenge for the future is to better predict the benefit / risk ratio in individual patients, so that the intensity of anti-platelet therapy can be optimised on a personalised basis. The following sections of this thesis will discuss anti-platelet agents that are currently used in clinical practice.

### 1.2.2.1 Cyclo-oxygenase enzymes and eicosanoids

The COX enzyme converts AA to PGs. There are 2 human isoforms – COX-1, which is expressed in most tissues and COX-2, which is typically present in areas of active inflammation<sup>80</sup>. As mentioned above, the COX enzyme converts AA into PGG<sub>2</sub>, which is subsequently reduced to PGH<sub>2</sub><sup>81</sup>.

Eicosanoids are a large group of signalling molecules derived from AA, including PGs, TXs, and leukotrienes; of these, prostacyclin (also known as PGI<sub>2</sub>) and TXA<sub>2</sub>, are important in maintaining platelet homeostasis. Prostacyclin inhibits platelet activation while TXA<sub>2</sub> increases platelet activation. PGE<sub>2</sub> is among the most abundantly expressed eicosanoids and plays complex and often contrasting roles in the modulation of inflammation, from initiation of pyrexia to resolution of inflammatory processes<sup>82 83</sup>.



**Figure 1.2: Cyclo-oxygenase dependent pathways**

The figure shows the conversion of arachidonic acid to prostaglandins, prostacyclin, and thromboxane A<sub>2</sub> via the cyclo-oxygenase enzyme.

### 1.2.2.1.1 Aspirin

Aspirin, otherwise known as acetylsalicylic acid, irreversibly inhibits both COX enzymes through acetylation of a critical serine residue, although its effect on COX-1 is at least ten fold greater than that on COX-2<sup>80</sup>. By predominantly inhibiting COX-1, TXA<sub>2</sub>-induced platelet activation and aggregation are blocked<sup>84</sup>. Aspirin achieves maximum platelet inhibition within 2 hours following a loading dose and inhibits aggregation in circulating platelets for at least 5 days<sup>85 86</sup>. There has been some debate over the past decade as to whether aspirin resistance is a clinically significant issue, requiring patients deemed to be 'poor responders' to switch to an alternative anti-platelet agent<sup>87 88</sup>. It has since been shown that whilst platelet function assays may produce variable results, and thus suggest that aspirin resistance is present, platelet COX-1 activity, as reflected by TXA<sub>2</sub> levels, is uniformly and persistently suppressed by low-dose aspirin<sup>89</sup> and it is now generally accepted that true pharmacological resistance to aspirin is rare<sup>90</sup>.

The analgesic and topical antiseptic properties of salicylates have been recognised since at least 3000 BC, with the Ancient Egyptians and Sumerians documenting the medicinal benefits of willow and other salicylate-rich trees and plants, such as meadowsweet<sup>91</sup>. Hippocrates prescribed willow tea to labouring women to relieve pain in the fifth century BC<sup>92</sup> and the Roman encyclopaedist, Aulus Celsus, documented the anti-inflammatory effects of willow leaf in his *De Medicina* treatise in the first century AD<sup>93</sup>.

Acetylsalicylic acid was initially synthesised by Charles Frédéric Gerhardt in 1853<sup>94</sup> and subsequently marketed as a commercially available drug in 1899 by the Bayer pharmaceutical company. The drug was named 'aspirin', with the 'a' representing the acetyl group that was added to salicylic acid to create the drug, the 'spir' deriving from the plant, *Spiraea ulmaria*, from which the salicylates were isolated, and 'in' being a common suffix for drugs at the time<sup>95</sup>. The drug achieved prominence in the Spanish influenza pandemic of 1918 and remained unchallenged in its position as the leading analgesic agent until the 1950s, when paracetamol was brought to the market.

Alongside its analgesic and antipyretic effects, aspirin has been recognised as an inhibitor of platelet function for around 50 years <sup>96</sup> and, to date, remains the most commonly prescribed drug worldwide <sup>97</sup>. The pharmacologists, John Vane and Priscilla Piper, established that administration of aspirin impaired the production of a compound that they termed 'rabbit aorta contracting substance' <sup>98 99</sup>. Vane later identified this compound as a PG <sup>100 101</sup>, finally elucidating aspirin's mechanism of action, for which he was awarded the 'Nobel Prize in Physiology and Medicine' in 1982.

#### **1.2.2.2 Adenosine diphosphate receptor antagonists**

Clopidogrel, prasugrel and ticagrelor are all ADP antagonists, a class of therapeutic agents that bind selectively to the P2Y<sub>12</sub> receptor to inhibit platelet function <sup>102</sup>. The thienopyridine, ticlopidine was the first drug in this class but is seldom prescribed now, following reports of serious adverse reactions, in particular neutropaenia <sup>103</sup> and thrombotic thrombocytopenic purpura <sup>104</sup>.

##### **1.2.2.2.1 Clopidogrel**

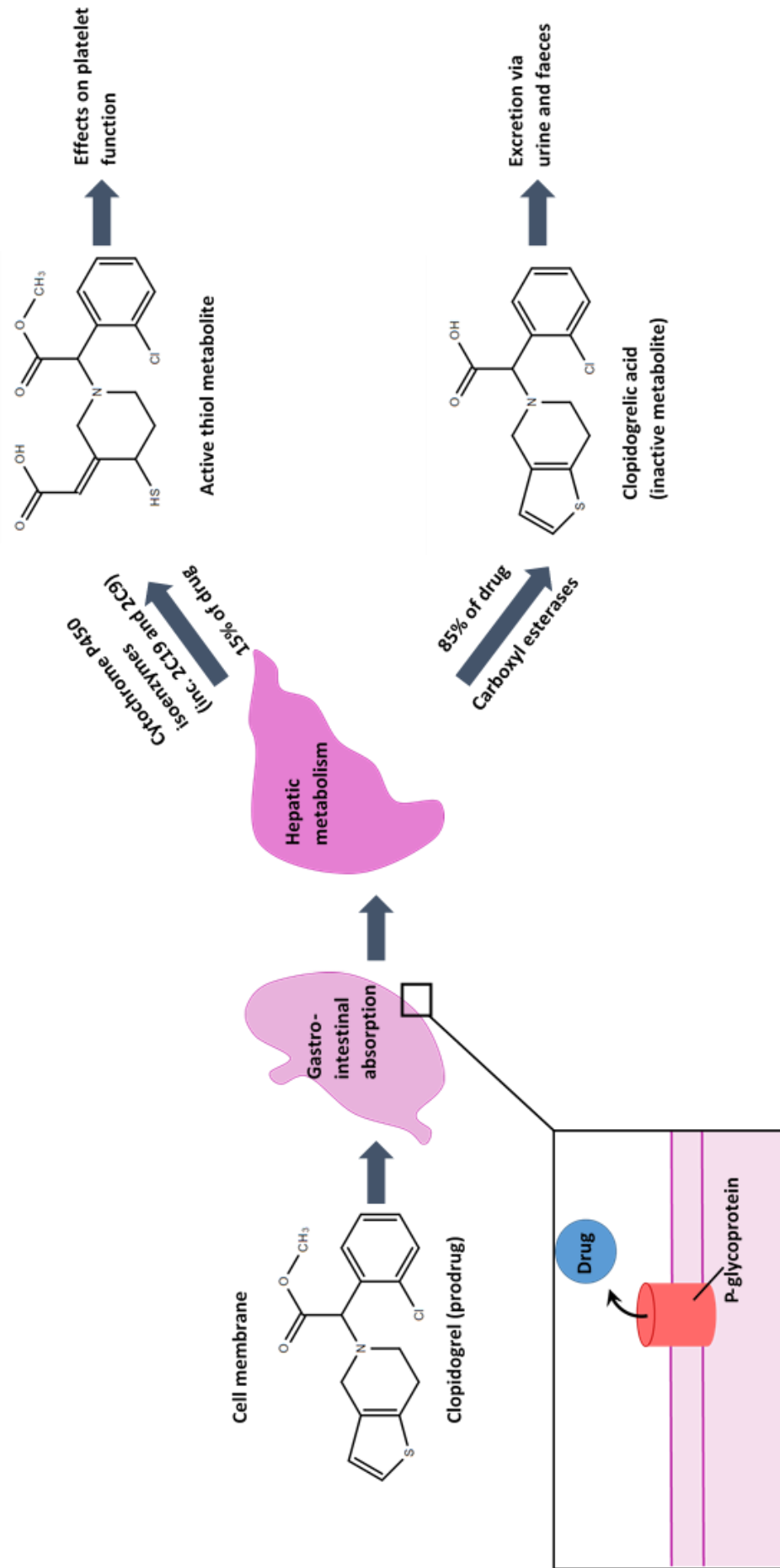
The second generation thienopyridine prodrug, clopidogrel, is currently the most commonly prescribed ADP receptor antagonist. It is administered orally and up to 85 % of the absorbed drug undergoes hepatic metabolism by carboxyl esterases to form an inactive carboxylic acid derivative, clopidogrellic acid, whilst the remaining 15 % is metabolised into the active thiol product by cytochrome P450 isoenzymes <sup>102 105</sup>; see Figure 1.3. Although clopidogrel has a relatively short half-life of 6 hours <sup>106</sup>, the thiol metabolite covalently binds to the P2Y<sub>12</sub> receptor, inducing an irreversible conformational change in the receptor and thus impairing thrombotic function for the remaining lifespan of the affected platelet. Genetic polymorphisms in cytochrome P450 enzymes, particularly CYP2C19 and CYP2C9, may result in impaired generation of the active thiol metabolite in patients taking clopidogrel, resulting in lack of efficacy <sup>107</sup>.

Variable responses to clopidogrel may additionally result from mutations in the ABCB1 gene that encodes the P-glycoprotein involved in clopidogrel absorption <sup>107</sup>.

The standard dosing regime is a 300 mg loading dose and 75 mg daily maintenance dose. Following this, steady state ADP inhibition is typically achieved within 3-7 days, with a 40 – 60 % reduction in ADP-induced platelet aggregation from baseline <sup>106</sup>. Randomised controlled trials have demonstrated that clopidogrel is more effective than aspirin in preventing cardiovascular events in patients with vascular disease <sup>108</sup>, further reduces mortality in patients with MI when used alongside aspirin <sup>109</sup>, and improves outcomes in patients undergoing PCI when used in combination with aspirin <sup>110 111</sup>. The 'Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events' (CAPRIE) study showed that clopidogrel administration was associated with similar adverse effects to those observed with aspirin, including gastrointestinal discomfort and increased bleeding, but the overall safety profile of clopidogrel 75 mg daily was considered to be least as good as that of aspirin 325 mg daily <sup>108</sup>. However, the inter-patient unpredictability in clopidogrel responsiveness (with some patients not responding at all) due to the abovementioned factors led to the development of newer P2Y<sub>12</sub> antagonists.



Figure 1.3: Clopidogrel metabolism

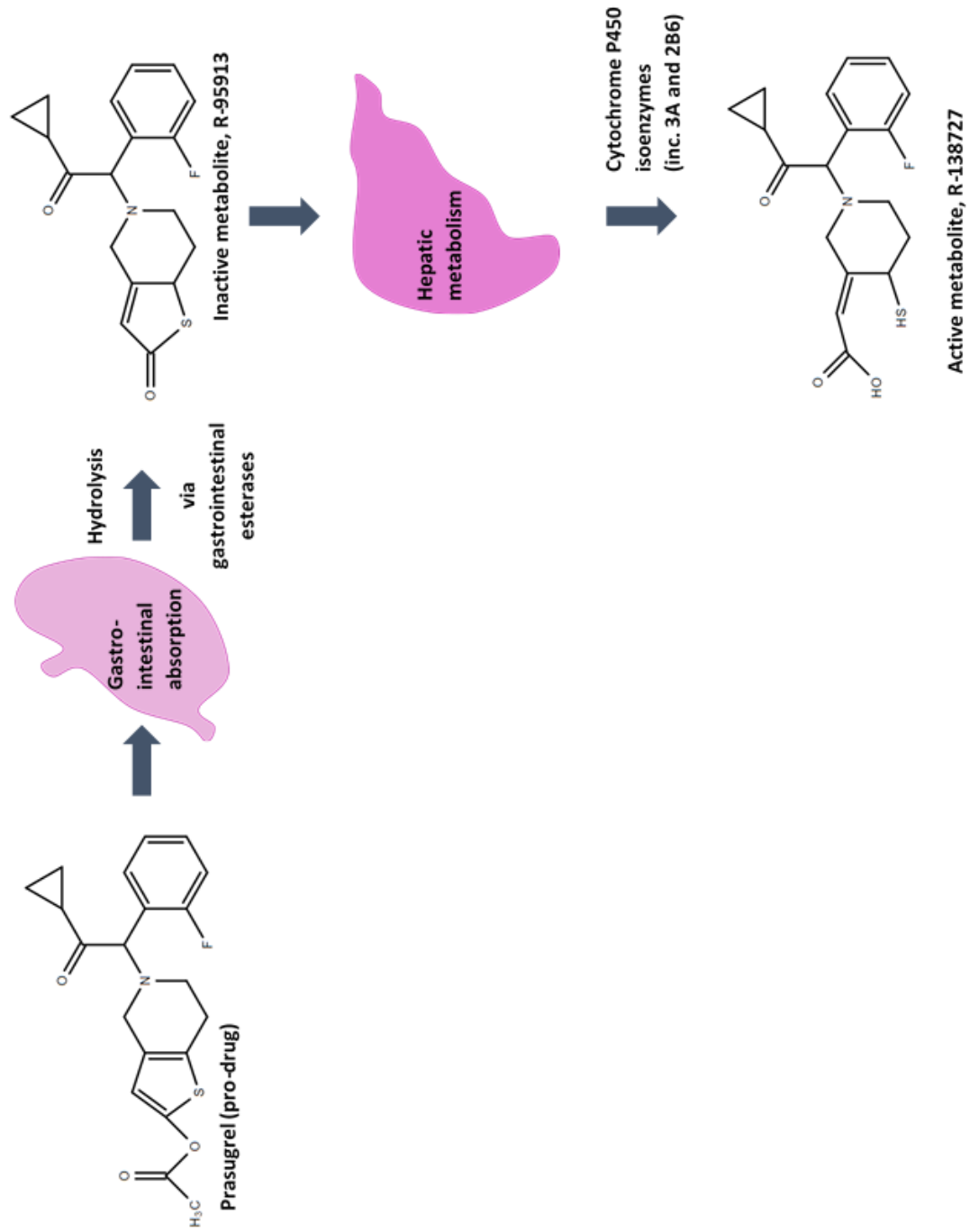


#### 1.2.2.2.2 Prasugrel

Prasugrel is an oral thienopyridine prodrug that is hydrolysed by esterases to the metabolite, R-95913. This inactive metabolite is then activated by cytochrome P450 enzymes, forming the active metabolite R-138727; Figure 1.4. As with clopidogrel, the active metabolite subsequently binds irreversibly via a covalent bond to the platelet P2Y<sub>12</sub> receptor and thus inhibits platelet function <sup>112</sup>.

A loading dose of 60 mg is given, followed by 5 – 10 mg daily maintenance dosing <sup>113</sup>. Peak plasma concentration is reached within 30 minutes and the drug has a half-life of 7 hours <sup>112</sup>. Phase I and II studies have demonstrated that prasugrel has a faster onset of action than clopidogrel, as well as being more efficacious and more predictable in its anti-platelet action <sup>114-116</sup>. The TRITON-TIMI 38 phase III study found that, in patients with ACS undergoing PCI, prasugrel was more effective than clopidogrel in reducing further ischaemic events although it conveyed a higher risk of major bleeding <sup>117</sup>. Further analysis of a subgroup of patients with STEMI undergoing PCI found that prasugrel was more effective than clopidogrel in preventing additional cardiovascular events without any increased risk of minor or major bleeding <sup>118</sup>. Among patients with ACS without ST-elevation who did not undergo PCI, prasugrel was not found to be superior to clopidogrel in preventing ischaemic events <sup>119</sup>.

Figure 1.4: Prasugrel metabolism



### 1.2.2.2.3 Ticagrelor

Unlike clopidogrel and prasugrel, ticagrelor is a cyclo-pentyltriazolo-pyrimidine ADP antagonist that has distinct pharmacokinetic and pharmacodynamic properties. Ticagrelor binds directly to the P2Y<sub>12</sub> receptor and alters its conformation, resulting in reversible inhibition. The drug does not require metabolic activation and thus exhibits a comparatively rapid onset and offset of effect, necessitating comparatively frequent dosing to achieve steady ADP inhibition<sup>120</sup>. Plasma levels of ticagrelor peak at 1.5 – 3 hours post-ingestion and reach steady state after 2 – 3 days<sup>121</sup>. Although metabolic activation is not required for initiation of its anti-platelet effects, the drug does have an active metabolite, AR-C124910XX, which is produced following the interaction of the parent drug with cytochrome P450<sup>120 121</sup>.

Ticagrelor is administered as a loading dose of 180 mg, followed by maintenance dosing of either 60 or 90 mg twice daily. The 'PLATElet inhibition and patient Outcomes' (PLATO) study showed that ticagrelor was superior to clopidogrel in reducing mortality and further cardiovascular events in patients presenting with ACS, regardless of the presence or absence of CYP2C19 and CYP2C9 polymorphisms<sup>122</sup>. The DISPERSE-2 trial showed that there was no increase in major bleeding events in patients with non-ST segment ACS taking ticagrelor compared with clopidogrel, however there were significantly more minor bleeding events<sup>123</sup>. Ticagrelor achieves higher levels of platelet inhibition than clopidogrel<sup>124</sup>, likely due to a combination of factors, including the aforementioned genetic variations in absorption and metabolism of clopidogrel.

Dyspnoea is a well-documented adverse effect of ticagrelor use, although to date the mechanism of this physiological response is unclear, particularly as this is not a prominent feature of other drugs in this class. Adenosine activates pulmonary vagal C nerve fibres, inducing dyspnoea<sup>125</sup>. It has been postulated that ADP receptor inhibition results in higher levels of extracellular adenosine, due to drug-induced inhibition of a sodium-independent equilibrative nucleoside transporter and subsequent reduced adenosine clearance<sup>126 127</sup>. Although other ADP-receptor inhibitors, such as clopidogrel, bind irreversibly to P2Y<sub>12</sub> receptors, they have a

comparatively short half-life and thus when they inhibit receptors on cells other than platelets, such as neurons, their effects are transient as the presence of a cell nucleus allows synthesis of new receptors<sup>126</sup>. Additionally, twice daily dosing of ticagrelor may result in consistently higher plasma drug concentrations compared with other ADP-receptor antagonists that are dosed once daily<sup>126</sup>.

In view of the greater platelet inhibition and consequent improved outcomes that are observed with prasugrel and ticagrelor compared with clopidogrel, many cardiology centres now recommend that the latter is not used as a first line P2Y<sub>12</sub> inhibitor in the management of acute STEMI<sup>128</sup>.

#### **1.2.2.2.4 Cangrelor**

Cangrelor is a novel P2Y<sub>12</sub> inhibitor that, like ticagrelor, binds directly to the receptor and induces reversible blockade. The drug is a non-thienopyridine adenosine triphosphate analogue that is administered intravenously and has shown promising results in clinical trials to date<sup>129</sup>. The drug has a rapid onset and offset of action, reaches steady state within a few minutes, and achieves greater than 90 % inhibition of platelet activation resulting from the P2Y<sub>12</sub> pathway<sup>130</sup>.

In a series of randomised-controlled studies, when compared with current standard therapy, however, no significant differences in mortality or further MI were observed when patients were treated with either clopidogrel or cangrelor before<sup>131</sup> or during PCI<sup>132</sup>. A double-blind placebo-controlled trial involving 11,145 patients subsequently found that cangrelor significantly reduced the rate of ischemic events during PCI, with no increase in severe bleeding, compared with clopidogrel<sup>133</sup>. At present, cangrelor has been approved by US and European regulatory agencies for use in patients undergoing PCI, although it has not yet been recommended by the National Institute for Health and Care Excellence (NICE) for use in the UK due to a relative lack of clear data<sup>134 135</sup>

### 1.2.2.3 Glycoprotein IIb/IIIa inhibitors

The platelet integrin complex glycoprotein IIb/IIIa represents the final common pathway of platelet activation<sup>56 136</sup>. This molecule mediates platelet binding to fibrinogen thereby forming bridges between platelets. A haemostatic platelet plug subsequently develops, which increases in size as further platelet activation is propagated<sup>56</sup>. Glycoprotein IIb/IIIa receptor inhibitors block this pathway and thus reduce thrombogenesis. Of the drugs in this class, abciximab (a monoclonal antibody fragment), tirofiban (a small, non-peptide molecule), and eptifibatid (a cyclic heptapeptide derived from rattlesnake venom) are used in clinical practice<sup>136 137</sup>. Abciximab additionally binds to integrin receptors on leucocytes and endothelial cells, thus reducing the adhesion of platelets to these cells<sup>137</sup>.

Glycoprotein IIb/IIIa inhibitors have played varying roles as anti-platelet agents over the past 20 years<sup>136</sup>. Data from several large-scale meta-analyses looking at glycoprotein IIb/IIIa inhibitor clinical trials in the medical management of non-ST-elevation ACS indicate a significant reduction in further MI and overall mortality in patients treated with these agents<sup>138 139</sup>, and although they were formerly used as key therapies in the management of acute MI for several years, glycoprotein IIb/IIIa inhibitors have been gradually phased out in favour of the P2Y<sub>12</sub> inhibitors. A similar trend has followed with regard to the use of glycoprotein IIb/IIIa inhibitors as prophylactic anti-thrombotic agents in patients undergoing PCI, where their use has been declining in favour of novel anti-thrombotic / anticoagulant drugs. The TRITON-TIMI 38 study found that prasugrel significantly reduced the risk of cardiovascular events in patients with ACS after PCI regardless of whether or not a GP IIb/IIIa inhibitor was used concurrently<sup>140</sup>. The 'Intracoronary Stenting and Antithrombotic Regimen—Rapid Early Action for Coronary Treatment' (ISAR – REACT) trial enrolled 2159 patients with coronary artery disease who underwent elective PCI following pre-treatment with clopidogrel 600 mg and either abciximab or placebo; there was no observable clinical benefit in those receiving abciximab over the 30 days post-procedure<sup>141</sup>. The ISAR – REACT 2 study subsequently assessed 2022 patients with UA or non-STEMI (NSTEMI) undergoing PCI who were pre-treated with 600 mg clopidogrel and

either abciximab or placebo, and found that abciximab significantly reduced the incidence of adverse events, although only in those patients with elevated troponin levels at presentation<sup>142</sup>. In the UK, current NICE guidance recommends glycoprotein IIb/IIIa inhibitors as an adjunct to PCI for all patients with diabetes undergoing elective PCI, and for those patients undergoing complex procedures, but not for the routine management of ACS or PCI<sup>143</sup>.

#### **1.2.2.4 Dipyridamole**

Dipyridamole is a drug that alters platelet function via two main mechanisms. Firstly, it blocks the reuptake of adenosine into platelets, endothelial cells and erythrocytes, leading to increased extracellular concentrations of adenosine<sup>144</sup>. This stimulates adenylyl cyclase activity, thus increasing cAMP levels. The rise in cAMP results in enhanced phosphorylation of vasodilator-stimulated phosphoprotein and subsequent reduced GP IIb/IIIa receptor activity and platelet aggregation<sup>145</sup>. Secondly, dipyridamole inhibits the platelet cAMP-phosphodiesterases that inactivate cAMP, further augmenting the reduction in platelet aggregation<sup>144</sup>.

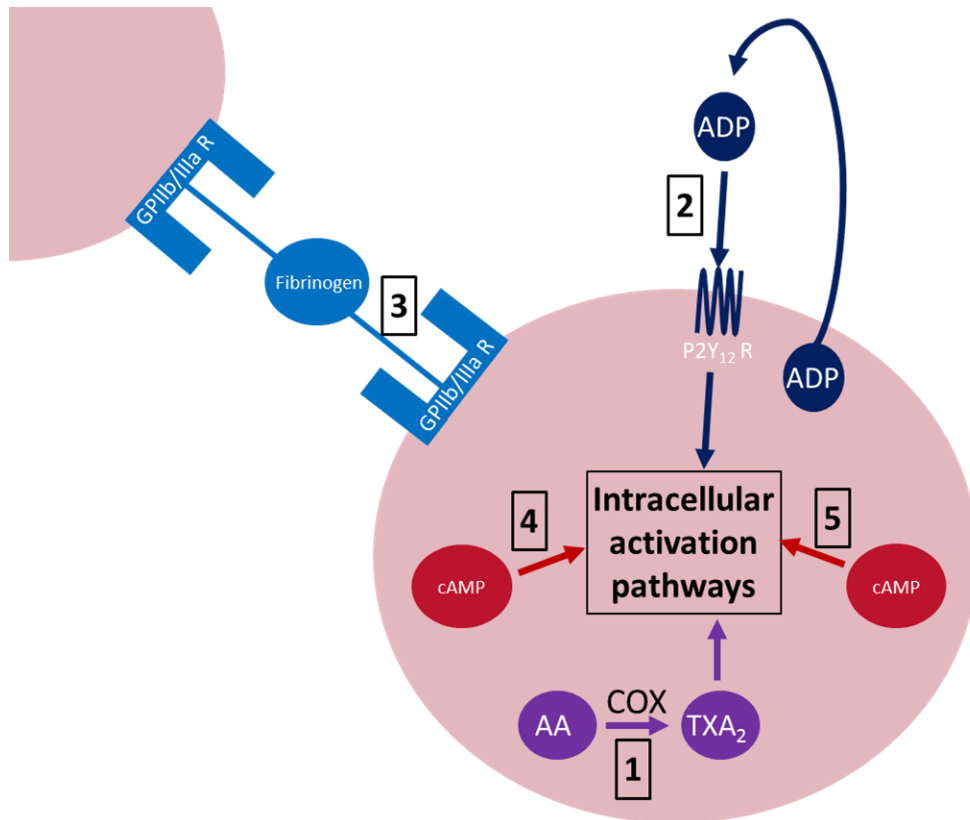
Until relatively recently, dipyridamole, used in combination with aspirin, formed the mainstay of secondary prevention of ischaemic stroke. Data from the 'Prevention Regimen For Effectively avoiding Second Stroke' (PROFESS) study published in 2008 found that there were no significant differences in outcome in treating patients with either aspirin plus extended-release dipyridamole or clopidogrel, although there were more bleeding events with the dipyridamole regime (4.1 % vs 3.36 %; hazard ratio 1.15)<sup>146</sup>, and thus the general preference for monotherapy over combination therapy has led to a change in guidelines, favouring clopidogrel<sup>147</sup>. Dipyridamole continues to be used in the secondary prevention of ischaemic stroke in patients who do not tolerate ADP-receptor inhibitors, and is frequently prescribed alongside the coumarin anticoagulant, warfarin, in the prevention of postoperative thromboembolic events in patients with mechanical heart valve replacements<sup>148 149</sup>.

### **1.2.2.5 Cilostazol**

Cilostazol is a quinolinone inhibitor of the phosphodiesterase 3 isoenzyme that, as mentioned above, breaks down cAMP, and the drug therefore reduces platelet aggregation. Cilostazol is currently licensed for the treatment of intermittent claudication pain in patients with peripheral arterial disease, although the main benefits from the drug in this context are derived from the vasodilating, rather than anti-thrombotic, effects of elevated cAMP levels <sup>150</sup>. Cilostazol is currently not recommended for use in patients with peripheral arterial disease in the UK, due to its apparent inferiority compared to other vasodilating agents <sup>151</sup>.

Cilostazol may potentially play a role in the future management of transient ischaemic attacks and ischaemic strokes. Recent meta-analysis data identified that the drug significantly reduces the recurrence of stroke compared to aspirin, with fewer occurrences of intracranial haemorrhage than with aspirin, clopidogrel, and DAPT as long-term therapy, although the majority of patients involved in these studies were from East Asia and it remains to be seen whether comparable results are obtained in Western populations <sup>152 153</sup>.





**Figure 1.5: Sites of action for anti-platelet agents**

This figure shows the pathways that are interrupted by anti-platelet drugs to impair platelet activation and aggregation. 1) Aspirin acts to inhibit the activity of the cyclo-oxygenase enzyme and thus attenuates the production of PGs and thromboxane; 2) The adenosine diphosphate (ADP) receptor antagonists bind to the P2Y<sub>12</sub> receptor to prevent ADP-induced platelet activation; 3) Glycoprotein IIb/IIIa inhibitors impair platelet adhesion by preventing the formation of fibrinogen bridges between platelets; 4) Dipyridamole blocks the re-uptake of adenosine into platelets and inhibits the platelet cyclic adenosine monophosphate (cAMP)-phosphodiesterases, both of which lead to an increase in cAMP levels, thus reducing platelet aggregation; 5) Cilostazol inhibits the phosphodiesterase 3 isoenzyme that breaks down cAMP.

### **1.2.3 Anti-platelet drugs in primary prevention**

Although guidelines vary throughout the world, it is generally accepted that anti-platelet therapy should not be given for primary prevention of cardiovascular disease in patients who have no comorbidities. Most physicians use a system, such as the Framingham risk score or the QRISK2 score, to calculate a patient's individual risk of suffering a cardiovascular event based on a series of established risk factors before deciding whether the benefit of commencing anti-platelet therapy outweighs the risks.

Despite the fact that it is not licensed for this indication, aspirin is usually prescribed as the first-line anti-platelet therapy in primary prevention in cases where anti-platelet therapy is thought to be of benefit<sup>154</sup>. The British Hypertension Society recommends that aspirin only be used in primary prevention in hypertensive patients who are aged over 50 years with 10 year cardiovascular risk of at least 20 %<sup>155</sup>. In patients with type 2 diabetes mellitus, the current NICE recommendations are to consider aspirin therapy in patients who are aged over 50 years, or who otherwise have significant cardiovascular risk factors<sup>26</sup>. The European Society of Cardiology, however, does not recommend the use of anti-platelet agents in primary prevention<sup>156</sup>, based on the findings of a meta-analysis published shortly after the aforementioned NICE guidelines, that reported no benefit from aspirin use in preventing major cardiovascular events in diabetic patients<sup>157</sup>.

### **1.2.4 The role of anti-platelet drugs in the management of myocardial infarction**

ACS is a term used to encompass unstable angina (UA) and MI with or without electrocardiographic (ECG) evidence of ST-segment elevation. Anti-platelet therapy has formed the backbone of ACS management for decades and current guidelines recommend that all patients routinely receive a loading dose of aspirin, followed by maintenance therapy unless contraindicated<sup>158 159</sup>. There is a wide range of maintenance dosages of aspirin that are prescribed in this context, ranging from 75 to 325 mg daily in different countries<sup>160</sup>, although in the UK, a loading dose of 300 mg aspirin is given, followed by a daily maintenance dose of 75

mg. In patients who have suffered ACS, recommended secondary prevention therapy typically entails twelve months of DAPT, followed by lifelong aspirin, whilst patients with stable angina are given aspirin monotherapy<sup>36 37</sup>. Alongside aspirin therapy, patients with UA or NSTEMI who are considered to have a predicted 6-month mortality of greater than 1.5 % (which is the case for the vast majority of such patients) are usually treated with a loading dose of 300 mg clopidogrel<sup>161</sup>. Those with acute STEMIs will go on to receive coronary reperfusion therapy, usually with percutaneous coronary intervention (PCI) or, less commonly now, with fibrinolytic therapy, and also receive a second anti-platelet agent, namely clopidogrel, prasugrel or ticagrelor depending on local guidelines.

### **1.2.5 The role of anti-platelet drugs in the management of stroke**

NICE guidelines recommend that patients who present with signs of a transient ischaemic attack and are considered by the ABCD2 scoring system to have a high risk of stroke are immediately offered either 300 mg aspirin or 300 mg clopidogrel (although this would be an off-label use of the latter)<sup>28</sup>, based on data suggesting that anti-platelet therapy conveys an 80 % reduction in the risk of early recurrent stroke<sup>162</sup>.

In the UK, patients who have a confirmed acute ischaemic stroke are typically managed in a highly specialist unit where thrombolytic therapy may be given depending on the clinical presentation. Otherwise, patients are immediately given 300 mg aspirin to continue for 14 days, followed by once daily 75 mg clopidogrel<sup>31</sup>.

### **1.2.6 Dual anti-platelet therapy in coronary artery disease**

DAPT is routinely commenced in patients who have undergone PCI, involving both drug-eluting and (more rarely these days) bare-metal stents, with the aim of reducing the risk of subsequent in-stent thrombosis. Although the introduction of drug-eluting stents has markedly decreased the occurrence of re-stenosis, in-stent thrombosis remains a significant complication. The presence of a foreign body within the coronary artery induces platelet adhesion and activation,

and potentially thrombus formation, until a layer of endothelial cells has covered the surface of the stent.

Current guidelines recommend that DAPT should be continued for between 6 and 12 months post stent-insertion <sup>163 164</sup>. Meta-analysis data have generally supported the use of short term DAPT (<12 months) regimes, in view of the reduced bleeding rates without an apparent increase in ischaemic complications <sup>165-167</sup>.

It is likely that these recommendations will soon be reviewed, following the recent publication of results from the 'Dual Antiplatelet Therapy (DAPT) Study', a large-scale randomised controlled trial, showing that the continuation of DAPT beyond 12 months post-PCI with drug-eluting stent placement yielded a reduction in the occurrence of in-stent thrombosis, although there was an increased bleeding risk <sup>168</sup>. This data has since facilitated the development of a prediction score to enable clinicians to identify patients who are likely to receive greater benefit from continuing DAPT after 12 months, based on bleeding and ischaemic risk factors, although this has yet to be fully validated <sup>169</sup>.

Although a clear consensus on the optimal duration of DAPT post-PCI insertion may take some time to reach, a pragmatic approach at present is for clinicians to continue adhering to the national guidelines, whilst considering the bleeding and ischaemic risks of their individual patient and adjusting the recommended DAPT duration accordingly.

**Table 1.1: Characteristics of anti-platelet drugs**

<b>Drug</b>	<b>Site of action</b>	<b>Time to peak action</b>	<b>Half-life</b>	<b>Typical dose administered</b>	<b>Route / dosing interval</b>
<b>Aspirin</b>	Inhibition of COX enzyme	1-2 hours	15-20 mins	In Europe loading dose 300 mg, maintenance dose 75 mg	Oral Once daily
<b>Clopidogrel</b>	Thienopyridine prodrug irreversibly binds to the P2Y <sub>12</sub> receptor	45 mins	6 hours	Loading dose 300 – 600 mg, maintenance dose 75 mg	Oral Once daily
<b>Prasugrel</b>	Thienopyridine prodrug, irreversibly binds to the P2Y <sub>12</sub> receptor	30 mins	7 hours	Loading dose 60 mg, maintenance dose 10 mg	Oral Once daily
<b>Ticagrelor</b>	Cyclo-pentyltriazolo-pyrimidine drug, reversibly binds to P2Y <sub>12</sub> receptor	1.5 hours	7 hours	Loading dose 180 mg, maintenance dose 90 mg	Oral Twice daily
<b>Cangrelor</b>	Adenosine triphosphate analogue, reversibly binds to P2Y <sub>12</sub> receptor	2 mins	3-6 mins	Bolus 30 mcg/kg injection then 4 mcg/kg/min infusion	Intravenous One-off treatment
<b>Abciximab</b>	Glycoprotein IIb/IIIa receptor inhibitor	30 mins	30 mins	Bolus 0.25 mg/kg injection then 0.125 mcg/kg/min infusion	Intravenous One-off treatment
<b>Dipyridamole</b>	Inhibitor of platelet cAMP-phosphodiesterase	2-2.5 hours	10-12 hours	75 – 200 mg immediate release  200 mg modified release	Oral 3-4 times daily  Oral Twice daily
<b>Cilostazol</b>	Inhibitor of phosphodiesterase 3 isoenzyme	2-2.5 hours	11-13 hours	100 mg	Twice daily

### **1.3 Monocytes**

Monocytes are mononuclear white blood cells that play a pivotal role in the human adaptive immune response. Monocytes comprise approximately 1-8 % of circulating leucocytes and have a half-life of around 1-3 days, after which they move into tissues and differentiate into macrophages or dendritic cells<sup>170</sup>. Monocytes migrate to sites of local inflammation where their roles include phagocytosis and pro- or anti-inflammatory cytokine production.

#### **1.3.1 The role of monocytes in atherosclerosis**

Monocytes play a pivotal role in the development of atherosclerosis via formation and deposition of lipid-laden foam cells within the arterial tunica intima, which contribute to plaque instability<sup>171</sup>. Monocytes are further implicated in the development of atherosclerosis and plaque rupture due to their involvement in plaque neovascularisation. As plaque size increases, impaired oxygen diffusion develops and the proximal cells of the tunica intima become hypoxic<sup>172</sup>. Cellular oxygen levels become depleted and hypoxia-inducible factor 1 (HIF-1) production is upregulated, which induces expression of angiogenic signalling molecules, including vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), which serve to enhance the development of adventitial vasa vasorum and intraplaque microvessels<sup>172</sup>. Monocytes strongly express receptors to VCAM-1, which is produced by intraplaque microvessels<sup>173-175</sup>. The chronic inflammation present in atherosclerosis triggers increased expression of endothelial markers of activation, including VCAM-1, ICAM-1 (also known as CD54) and P-selectin (CD62P). There is additional upregulation of monocyte chemoattractant protein-1 (MCP-1), one of the primary chemokines involved in regulating migration and infiltration of monocytes, and its monocytic receptor, C-C chemokine receptor type 2 (CCR2)<sup>176 177</sup>.

Evidence suggests that circulating monocytes may also serve as endothelial progenitor cells, further amplifying angiogenesis<sup>173 178</sup>. The newly-formed micro-vessels are fragile and prone to haemorrhaging. As erythrocytes extravasate from bleeding neo-vessels, their lipid-rich cellular contents contribute to further evolution of the atheromatous lesion, while the free haemoglobin

potentiates reactive oxygen species (ROS) generation and pro-inflammatory cytokine generation<sup>179</sup>. Intraplaque haemorrhage is one of the principal determinants of plaque rupture<sup>180</sup>.

### **1.3.2 Monocyte phenotypes and their differential contribution to atherosclerosis**

Until recently, human monocytes were divided into two major subsets based on their surface expression of CD14 and CD16: the CD14<sup>+</sup>CD16<sup>-</sup> and the CD14<sup>+</sup>CD16<sup>+</sup> groups. Around 85-90 % of monocytes comprise the CD14<sup>+</sup>CD16<sup>-</sup> or 'classical' group, which are involved in phagocytosis and pro-inflammatory cytokine production<sup>177 181</sup>. The CD14<sup>+</sup>CD16<sup>+</sup> subgroup has since been subdivided into the CD14<sup>high</sup>CD16<sup>+</sup> or 'intermediate' group and the CD14<sup>low</sup>CD16<sup>+</sup> or 'non-classical' monocyte group. The CD14<sup>high</sup>CD16<sup>+</sup> group express the monocyte chemoattractant protein-1 receptor, also known as CCR2, which enhances vascular monocyte recruitment and their subsequent transendothelial migration<sup>177</sup>. CD14<sup>high</sup>CD16<sup>+</sup> monocytes are highly pro-inflammatory<sup>182</sup> while the CD14<sup>low</sup>CD16<sup>+</sup> group express genes involved in cytoskeletal rearrangement and demonstrate high motility and patrolling behaviour<sup>183</sup>. See Table 1.2 for further details regarding monocyte subsets. There are additional, smaller, poorly-characterised subsets of monocytes, including the CD56<sup>+</sup> group, which may be associated with certain autoimmune diseases<sup>184</sup>.

**Table 1.2: Monocyte subset characteristics**

Monocyte subsets:	Classical	Intermediate	Non-classical
<b>CD14 expression</b>	++	++	+
<b>CD16 expression</b>	-	+	+
<b>CCR2 expression</b>	+	+	-
<b>Population %</b>	84.8 <sup>183</sup>	5.4 <sup>183</sup>	9.2 <sup>183</sup>
<b>Primary functions</b>	Phagocytosis <sup>177</sup> Tissue repair <sup>183</sup> Immune response <sup>183</sup> <sup>185</sup>	Angiogenesis <sup>185</sup> Phagocytosis <sup>183</sup>	Cytoskeleton rearrangement <sup>183</sup>
<b>Cytokine response</b>	Response to cell-surface Toll-like receptors <sup>186</sup>	Response to cell-surface Toll-like receptors <sup>186</sup>	Response to viruses via TLR7-TLR 8-MyD88-MEK pathway <sup>186</sup>
<b>Inflammatory effects</b>	Weakly pro-inflammatory (IL-6 <sup>187</sup> , IL-1 $\beta$ <sup>186</sup> , TNF- $\alpha$ correlation)	1. Highly pro-inflammatory (IL-6 <sup>187</sup> , IL-1 $\beta$ <sup>186</sup> , TNF- $\alpha$ correlation)  2. Anti-inflammatory (IL-10 correlation <sup>187</sup> )	Weakly pro-inflammatory (TNF- $\alpha$ correlation <sup>183</sup> )



Prior to the subdivision of the CD16<sup>+</sup> monocyte group, it was well-recognised that the CD16<sup>+</sup> subset was a major producer of serum tumour necrosis factor alpha (TNF- $\alpha$ ) in response to inflammatory challenges <sup>182</sup>. It was subsequently found that CD16<sup>+</sup> monocytes were expressed at relatively low levels in healthy individuals but showed amplified expression in the presence of coronary atherosclerosis, suggestive of a possible role for CD16<sup>+</sup> monocytes in atherogenesis <sup>188</sup>. Recently, a correlation between intima-media thickness (IMT) and CD14<sup>high</sup>CD16<sup>+</sup> cell counts has been observed <sup>189</sup>.

The association between an increased CD14<sup>high</sup>CD16<sup>+</sup> phenotype and the presence of atherosclerosis may be partially explained by the presence of the CCR2 receptor on the surface of this particular monocyte subset, which regulates the migration and infiltration of monocytes <sup>190</sup>. Our group has previously shown that in the context of acute inflammation the circulating CD14<sup>high</sup>CD16<sup>+</sup> numbers expand, and that this cell subset exhibits increased adhesiveness to the vascular endothelium <sup>31</sup>.

A multitude of clinical studies have highlighted the association between raised levels of CD16<sup>+</sup> monocytes and coronary disease. CD16<sup>+</sup> monocyte counts were elevated in patients with UA compared to matched control subjects with stable CAD. Among the UA patients, those with intermediate-high risk of MI had significantly higher counts of the CD14<sup>high</sup>CD16<sup>+</sup> subset <sup>23</sup>. Tapp *et al* found a correlation between CD14<sup>high</sup>CD16<sup>+</sup> counts and peak troponin-T (TnT) levels post-STEMI, as well as a correlation between CD14<sup>high</sup>CD16<sup>+</sup> counts and left ventricular ejection fraction (LVEF) post-STEMI <sup>187</sup>. A study by Rogacev *et al* in 2012 using 951 patients referred for elective coronary angiography showed that a higher CD14<sup>high</sup>CD16<sup>+</sup> count was predictive of cardiovascular events, including MI, ischaemic stroke and death from all cardiovascular causes <sup>22</sup>.

Conversely, Jaipersad *et al* showed that the CD14<sup>high</sup>CD16<sup>-</sup>, rather than CD14<sup>high</sup>CD16<sup>+</sup> or CD14<sup>low</sup>CD16<sup>+</sup>, subset was predictive of carotid and systemic atherosclerosis severity, and intraplaque neovascularisation <sup>173</sup>. These differences may be partially explained by the heterogeneity of subject groups recruited to the studies, as well as differences in monocyte

gating strategies during flow cytometry. Certainly, the majority of studies suggest that the CD14<sup>high</sup>CD16<sup>+</sup> subset is highly pro-inflammatory compared with its CD14<sup>high</sup>CD16<sup>-</sup> and CD14<sup>low</sup>CD16<sup>+</sup> counterparts, and that this subset is strongly associated with the presence of cardiovascular disease. Whether CD14<sup>high</sup>CD16<sup>+</sup> levels represent a more specific biomarker of cardiovascular risk than hs-CRP remains to be determined.

### **1.3.3 Monocyte-platelet interactions**

Platelets move from a resting state to an activated state in response to endothelial-derived activating factors, such as ADP and thrombin. Activated platelets adhere to damaged endothelium and locally mediate both inflammatory and thrombotic events via the secretion of cytokines and interactions with leucocytes, which results in chemotaxis and development of an inflammatory milieu within the arterial wall.

Activated platelets form complexes with leucocytes, particularly monocytes, as P-selectin, a platelet surface adhesion molecule expressed on platelet plasmalemma upon activation, binds to its ligand, P-selectin glycoprotein ligand-1 (PSGL-1)<sup>191,192</sup>, which is constitutively expressed by circulating monocytes. These MPA are measurable in the peripheral blood and serve as an easily quantifiable marker of platelet activation and appear to predict cardiovascular events, such as MI<sup>193</sup> and ischaemic stroke<sup>194</sup>. Functionally, MPA formed at the site of a vascular injury recruit circulating monocytes and facilitate their adhesion to the endothelium, where, following adhesion and migration into the subintima, they differentiate to macrophages and contribute further to atherogenesis<sup>195</sup>. Platelet activation and subsequent MPA formation are increased in the presence of high shear stress within blood vessels, such as occurs around the site of atherosclerotic lesions<sup>196</sup>, and circulating MPA levels in humans correlate with coronary plaque size<sup>197</sup>. P-selectin levels are independently associated with carotid atherosclerotic lesions in humans<sup>198</sup>.

The aforementioned model of acute inflammation, influenza immunisation, has been used to demonstrate that acute inflammation leads to higher circulating levels of activated platelets and

MPA formation, which promotes expansion of the CD14<sup>high</sup>CD16<sup>+</sup> subset <sup>31</sup>. This evidence supports the hypothesis that the increased degree of circulating MPA formation reported in patients with cardiovascular risk factors or established atherosclerotic disease, could represent a key event in the expansion of circulating CD16<sup>+</sup> monocytes, which also occurs in the presence of cardiovascular disease. Targeting platelet activation could therefore counteract the development of the more pro-atherogenic CD14<sup>high</sup>CD16<sup>+</sup> phenotype with a subsequent beneficial effect on atherosclerosis progression. Consistent with this hypothesis, previous experiments conducted in apolipoprotein knockout (ApoE<sup>-/-</sup>) mice has demonstrated that platelet inhibition, as achieved by either aspirin or clopidogrel administration, counteracts the blood monocytes that accompanies disease progression in this animal model of atherosclerosis, thus reducing inflammation <sup>31</sup>.

Although they have no effect on circulating lipid levels, anti-platelet drugs act to disrupt pathways in which platelets contribute to atherogenesis, including MPA-driven endothelial recruitment of monocytes and thrombus formation in areas of endothelial damage <sup>199 200</sup>. The pharmacological efficacy of the multiple classes of anti-platelet agents in counteracting anti-atherogenic mechanisms is variable. Administration of aspirin does not affect circulating levels of MPA, whereas clopidogrel appears to impair MPA formation <sup>201 202</sup>. Their distinct mechanisms of action may have differing effects on the intracellular pathways that finally lead to P-selectin expression on activated platelets <sup>203</sup>.

#### **1.4. Netrin-1**

Netrins are a class of laminin-like proteins, which were first identified as axonal guidance cues during embryonic development <sup>204</sup>. The netrin family was named from the Sanskrit word 'netr' which means 'one who guides' <sup>205</sup>. Netrin-1 is a secreted protein that mediates axonal chemoattractant activity via binding to the deleted in colorectal cancer (DCC) and neogenin receptors, and chemorepulsion via the uncoordinated-5 (UNC5) receptors <sup>206-208</sup>.

The role of netrin-1 in cardiovascular disease and states of acute inflammation is an emerging area of research. The identification of DCC and UNC5 receptors on cell types other than neurons, has supported the hypothesis that netrin-1 could have additional functions outside the central nervous system. Over the past decade it has become apparent that netrin-1 has involvement in multiple physiological responses, ranging from atherosclerosis to inflammation, as well as potentially functioning as a marker of renal function (Figure 1.6), making it an attractive potential therapeutic target. In recent years, netrin-1 has been identified as a key modulator of atherosclerosis, although its precise role in this disease – protective or deleterious – has been the subject of much debate.

#### **1.4.1 Netrin-1 in atherosclerosis**

van Gils *et al* have shown that human macrophage foam cells from human coronary artery plaques express netrin-1 and UNC5B, the latter being responsible for the inhibitory effect of netrin-1 on macrophage migration that ultimately results in netrin-1 dependent myeloid cell retention within plaques <sup>209</sup>. In LDLR<sup>-/-</sup> mice fed a Western diet, deletion of netrin-1 in haematopoietic cells reduced atheroma size and complexity and promoted macrophage migration from plaques <sup>209</sup>. This same group also showed that netrin-1 and UNC5B expression in macrophages is upregulated in hypoxic conditions and protects macrophages from apoptosis <sup>210</sup>. As netrin-1 appears to stimulate progression of atherosclerosis by retaining macrophages within atheromatous lesions, thus amplifying the cycle of chronic inflammation, identifying a method of interrupting this pathway could prove to be of therapeutic benefit.

This detrimental effect of netrin-1 on atherosclerosis progression contradicts the evidence provided by Khan *et al*, in which LDLR<sup>-/-</sup> mice underwent intravenous viral delivery of human netrin-1 copy DNA (cDNA) and, when compared with untreated control mice, were found to have lower levels of nitrotyrosine (a marker of ROS), as well as of CD68, integrin alpha M (ITGAM), and EGF-like module-containing mucin-like hormone receptor-like 1 (EMR-1), all of which are markers of macrophage and monocyte activity <sup>211</sup>. The netrin-1 treated mice

demonstrated a reduction in plaque formation, presumably through the prevention of monocytes migrating into atherosclerotic plaques <sup>211 212</sup>. Focal application of netrin-1 in this situation highlights another potential pathway in atherogenesis where modification of netrin-1 expression may retard plaque formation. Of note, a reduction in the endothelial expression of netrin-1 under pro-atherogenic conditions has been reported by van Gils *et al*, suggesting that suppression of netrin-1 within the vasculature in response to pro-atherogenic factors could impede plaque development <sup>209</sup>.

In 2012, Delloye-Bourgeois *et al* identified that certain cancer cells produce a truncated intranuclear form of netrin-1, as opposed to the well-characterised, full-length, secreted netrin-1 <sup>213</sup>. The majority of studies prior to this had not differentiated between these isoforms, and it has since been postulated that measurement of different isoforms of netrin-1 may explain some of the conflicting data surrounding its role in atherosclerosis <sup>203</sup>.

#### **1.4.2 The relationship between netrin-1 and inflammation**

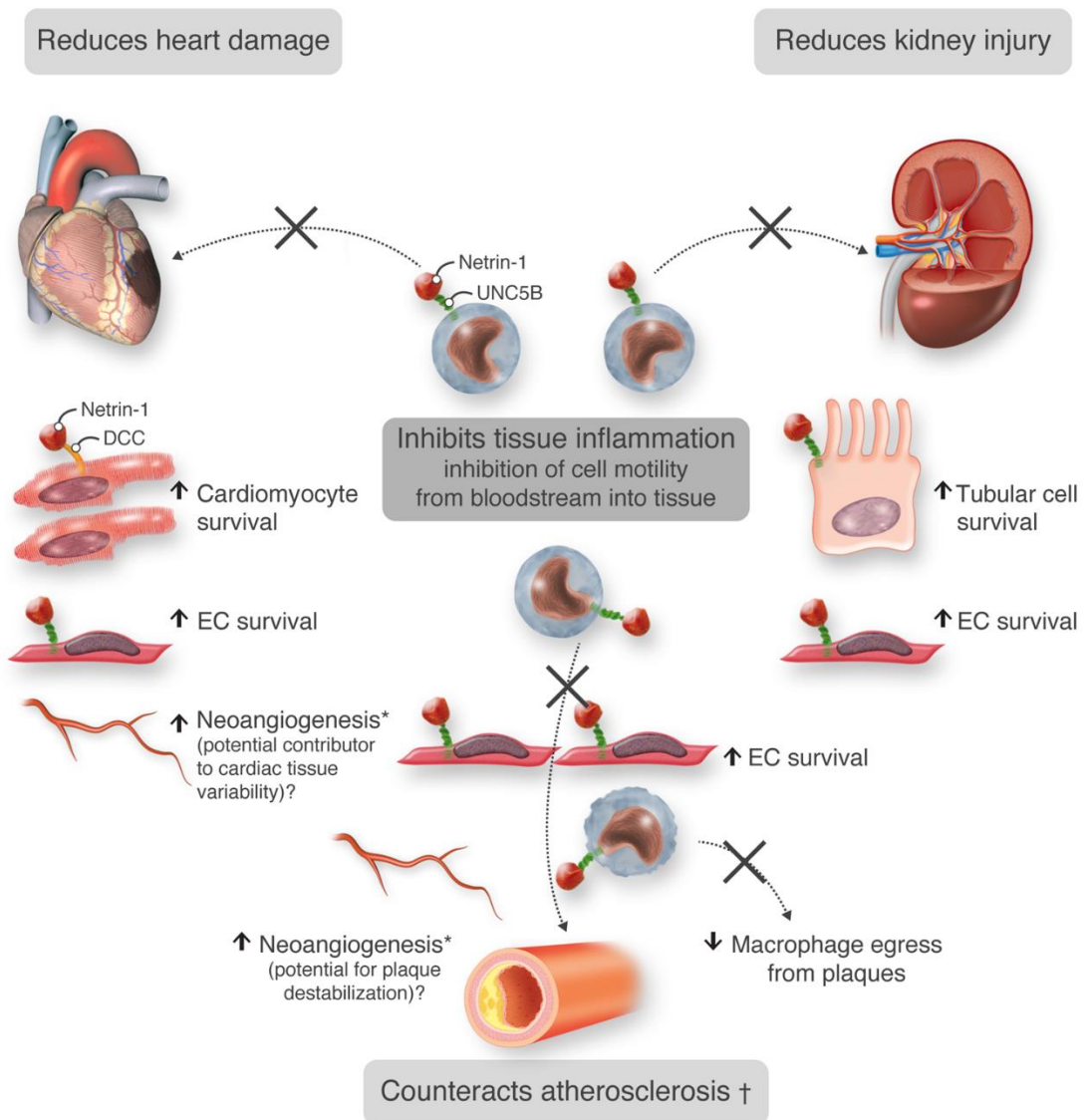
Murine models of myocardial ischaemia-reperfusion injury have shown that elevated netrin-1 expression has a cardioprotective effect, partly achieved by reducing the infiltration of neutrophils and recruitment of macrophages that serve to further amplify the pro-apoptotic inflammatory response <sup>214 215</sup>. Similar results have been shown in models of renal ischaemia-reperfusion injury <sup>216</sup> as well as other pro-inflammatory states, including acute lung injury, peritonitis and sepsis <sup>217 218</sup>.

Both *in vitro* and *in vivo* studies have repeatedly demonstrated that netrin-1 and UNC5B modulate leucocyte migration in pro-inflammatory states <sup>218</sup>. UNC5B is strongly expressed on leucocytes and increased netrin-1 expression attenuates leucocyte migration and leucocyte-driven inflammatory responses <sup>218-220</sup>. These findings have generated interest in a potential role for netrin-1 in the modification of inflammatory processes.

Administration of netrin-1 suppresses COX-2 expression via regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B). There is a subsequent reduction in COX-2

metabolites that mediate neutrophil infiltration, interferon- $\gamma$ -induced macrophage-activation and TXA<sub>2</sub><sup>221</sup>. Netrin-1 additionally suppresses the production of T<sub>H</sub>1 helper cell cytokines, which generate interferon- $\gamma$ <sup>219</sup>.

The degree to which suppression of the inflammatory response is desirable, and whether this could have detrimental effects in the context of infection, remain unclear.



\* Effect on neoangiogenesis within plaques and ischaemic myocardium needs to be explored

† Beneficial effect on atherosclerosis onset and progression requires further investigation since it can vary depending on disease stage

**Figure 1.6: Biological roles of netrin-1**

The diverse actions of netrin-1 in cardiovascular and renal disease. Netrin-1 directly modulates survival and migration of different cell types, including cardiomyocytes, leucocytes, and endothelial and tubular renal cells, through engagement of cell-specific receptors, as indicated. These effects result in netrin-1-dependent cardioprotection and reduction of kidney damage in response to ischaemia. Netrin-1 also confers anti-atherogenic protection, by repelling monocyte arterial infiltration. However, netrin-1-induced inhibition of resident macrophage egress from atherosclerotic plaques could be detrimental. The effect of netrin-1 on neoangiogenesis may be beneficial in the context of cardiac ischaemia but detrimental for atherosclerotic plaque destabilization. The anti-inflammatory action of netrin-1 is

mediated by direct inhibition of leucocyte motility, as well as by a protective effect on target organs that negatively feeds back on inflammatory cell trafficking within the tissue. UNC5B, unco-ordinated-5 receptor B; DCC, deleted in colorectal cancer receptor; EC, endothelial cells. Figure taken from Layne *et al.*, 2015 <sup>222</sup>.

#### **1.4.3 Anti-platelet therapy and netrin-1**

As mentioned above, netrin-1 modulates macrophage migration into and out of plaque sites <sup>209</sup> and thus represents a potential target for modifying progression of atherosclerosis. In 2015, Passacuale *et al* published a series of experiments where the relationship between endothelial netrin-1 expression and anti-platelet therapy was explored using both *in vitro* and *in vivo* models. They found that aspirin, but not clopidogrel, reduced vascular endothelial permeability and increased netrin-1 production in the ApoE<sup>-/-</sup> mouse model of atherosclerosis, which in turn led to reduced monocyte infiltration of atherosclerotic plaques <sup>203</sup>. These findings suggest that anti-platelet therapy with aspirin may have the potential to modify circulating netrin-1 levels and thus modulate atherosclerosis generation. This, together with the previously mentioned beneficial effect of platelet inhibition on blood monocytosis observed in ApoE<sup>-/-</sup> mice, points to the existence of a mutual interaction between platelet activity, endothelial chemorepulsion against monocyte infiltration, and monocyte phenotype that can be positively modulated by anti-platelet strategies.

#### **1.5 Aims**

The aims of this PhD were as follows:

- Chapter Two: To assess the effects of anti-platelet drugs on the phenotype of circulating monocytes in healthy volunteers in the presence of mild, systemic inflammation
- Chapter Three: To investigate the biomolecular mechanisms that regulate the synthesis of serum netrin-1 in humans



- Chapter Four: To explore the role of CD16 in the transduction of the signalling pathways triggered by oxLDL

**CHAPTER TWO:**

**THE EFFECT OF ANTI-PLATELET THERAPY ON**

**CIRCULATING CD16<sup>+</sup> MONOCYTES**

**IN HEALTHY SUBJECTS**

## **2.1 Introduction**

Circulating human monocytes consist of a heterogeneous cell population generally classified into three main subtypes: the 'classical' CD14<sup>high</sup>CD16<sup>-</sup>, 'intermediate' CD14<sup>high</sup>CD16<sup>+</sup>, and 'non-classical' CD14<sup>low</sup>CD16<sup>+</sup> cells<sup>223</sup>. The prevalence of these subpopulations in the peripheral blood changes under pro-inflammatory conditions, with an expansion of the 'intermediate' subset typically occurring in the context of atherosclerosis-related inflammation<sup>188</sup>. The functional implications of such changes in terms of disease progression remain elusive, although a positive correlation between the level of CD14<sup>high</sup>CD16<sup>+</sup> monocytes and the presence of subclinical atherosclerosis has been demonstrated<sup>189</sup>. More importantly, intermediate monocytes have proved to be an independent predictive factor for future cardiovascular events<sup>22</sup>, and a strong correlation between their blood level and coronary plaque vulnerability has been detected<sup>224-227</sup>. This indicates that CD14<sup>high</sup>CD16<sup>+</sup> cells may represent a novel biomarker of cardiovascular risk and a potential target for preventative therapeutic strategies.

In this respect, previous work undertaken by our group suggests that anti-platelet drugs may have a modulatory action on the phenotype of circulating monocytes, and additionally that platelet activation is a key determinant in the acquisition of a CD16<sup>+</sup> profile by human monocytes<sup>31</sup>. Furthermore, using ApoE<sup>-/-</sup> mice, our group has demonstrated the efficacy of platelet inhibition *in vivo*, as achieved by either aspirin or clopidogrel administration, in counteracting the blood monocytosis and expansion of circulating Ly6C<sup>low</sup> cells (the murine counterpart of CD16<sup>+</sup> monocytes) that accompany disease progression in this well-established animal model of atherosclerosis<sup>203</sup>. In the current clinical study, I have assessed the effect of anti-platelet drugs currently used in cardiovascular prophylaxis on the phenotype of circulating monocytes in healthy human subjects in the context of a pro-inflammatory stimulus. I have used influenza immunisation as an experimental model of acute inflammation, in line with our group's previous report showing an increase in the pool of circulating CD14<sup>high</sup>CD16<sup>+</sup> cells in response to this vaccine<sup>31</sup>, and also in keeping with other researchers who have previously used immunisations,

particularly the seasonal influenza and *Salmonella typhi* vaccines, in clinical studies to generate mild, systemic inflammation and investigate its relevance to cardiovascular pathophysiology <sup>27</sup>

28 30

## **2.2 Hypothesis and aim**

Levels of circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes increase in atherosclerotic patients and are predictive of future cardiovascular events. Platelet activation has been identified as a crucial determinant in the acquisition of a CD16<sup>+</sup> phenotype by classical CD14<sup>high</sup>CD16<sup>-</sup> cells. I aimed to test the hypothesis that anti-platelet therapy modulates the phenotype of circulating monocytes in the context of systemic inflammation.

## **2.3 Methods**

### **2.3.1 Participant recruitment**

Seventy five healthy subjects were studied, before and 48 hours after receiving seasonal influenza immunisation. Subjects were recruited from workers employed by Guy's and St Thomas' NHS Foundation Trust (GSTT) who attended the Occupational Health Department requesting influenza immunisation. None of the subjects were taking regular medications, aside from the combined oral contraceptive pill, and had not taken anti-platelet medication in the preceding fortnight. Participants were randomly assigned to receive either anti-platelet therapy or no treatment, according to the following scheme:

- Group 1 (aspirin 300): aspirin 300 mg orally immediately following immunisation and another 300 mg dose 24 hours later; n = 15.
- Group 2 (aspirin 75): aspirin 75 mg orally immediately following immunisation and another 75 mg dose 24 hours later; n = 15.
- Group 3 (clopidogrel): clopidogrel 300 mg orally immediately following immunisation, with a further dose of clopidogrel 75 mg 24 hours later; n = 15.

- Group 4 (ticagrelor): ticagrelor 90 mg orally twice daily for 48 hours, the first dose being taken immediately following immunisation; n = 15.

- Group 5 (untreated): no anti-platelet treatment given following immunisation; n = 15.

I carried out 'directly observed tablet' taking with the first dose of each medication for each participant.

### **2.3.2 Influenza immunisation**

The 2014/15 National Health Service (NHS) trivalent seasonal influenza immunisation, an inactivated (split virion) vaccination, specifically targeting 'A/California/7/2009 (H1N1)pdm09-like virus', 'A/Texas/50/2012 (H3N2)-like virus', and 'B/Massachusetts/2/2012-like virus', manufactured by Sanofi Pasteur MSD Limited, was administered via intramuscular injection into the upper arm by a trained healthcare professional, as part of normal Occupational Health procedures.

### **2.3.3 Collection of blood samples**

Blood samples were collected immediately prior to immunisation and again 48 hours later. 16 ml whole blood was obtained from the antecubital vein using a 23-gauge butterfly needle and collected directly into three vacutainer tubes.

The first vacutainer tube contained a serum gel separator, and blood was left to clot within this tube for 10 minutes at room temperature. The blood was centrifuged for 15 minutes, 1500 x g, at room temperature to obtain serum that was stored at -80 °C for subsequent analysis.

The other two vacutainer tubes contained ethylenediaminetetraacetic acid (EDTA). One tube was processed by the ViaPath laboratory at GSTT, London, to obtain cell counts using a Beckmann DX 7 analyser, while the other was used for subsequent flow cytometry analysis and preparation of plasma aliquots for future use. Plasma was prepared by centrifuging the blood for 10 minutes, 1500 x g, at 4 °C and was subsequently stored at -80 °C for subsequent analysis.

#### **2.3.4 Immunostaining**

100 µl whole blood was removed from the EDTA vacutainer tube and incubated with 5 µl of both R-phycoerythrin (PE)-mouse anti-human CD14 (BD Bioscience) and fluorescein isothiocyanate (FITC)-conjugated anti-human CD16 (3g8 clone, which reacts with both CD16a and CD16b isoforms of CD16; BD Bioscience) for 20 minutes at 4 °C. 1 ml BD FACS lysing solution (BD Bioscience) was used to lyse the erythrocytes. The samples were incubated for 10 minutes, whilst protected from light, and then centrifuged for 5 minutes, 1500 x g, at room temperature. Supernatant was discharged and the pellets were washed twice with 1 ml phosphate-buffered saline (PBS) / 0.2% bovine serum albumin (BSA) / 0.1% sodium azide. Samples were again centrifuged for 5 minutes, 1500 x g, at room temperature, then fixed with 250 µl 1% paraformaldehyde. Immunostaining took place within an hour of the samples being obtained. After processing, samples fixed in 1% paraformaldehyde were stored at 4 °C for up to 48 hours prior to flow cytometry analysis.

#### **2.3.5 Whole blood flow cytometry**

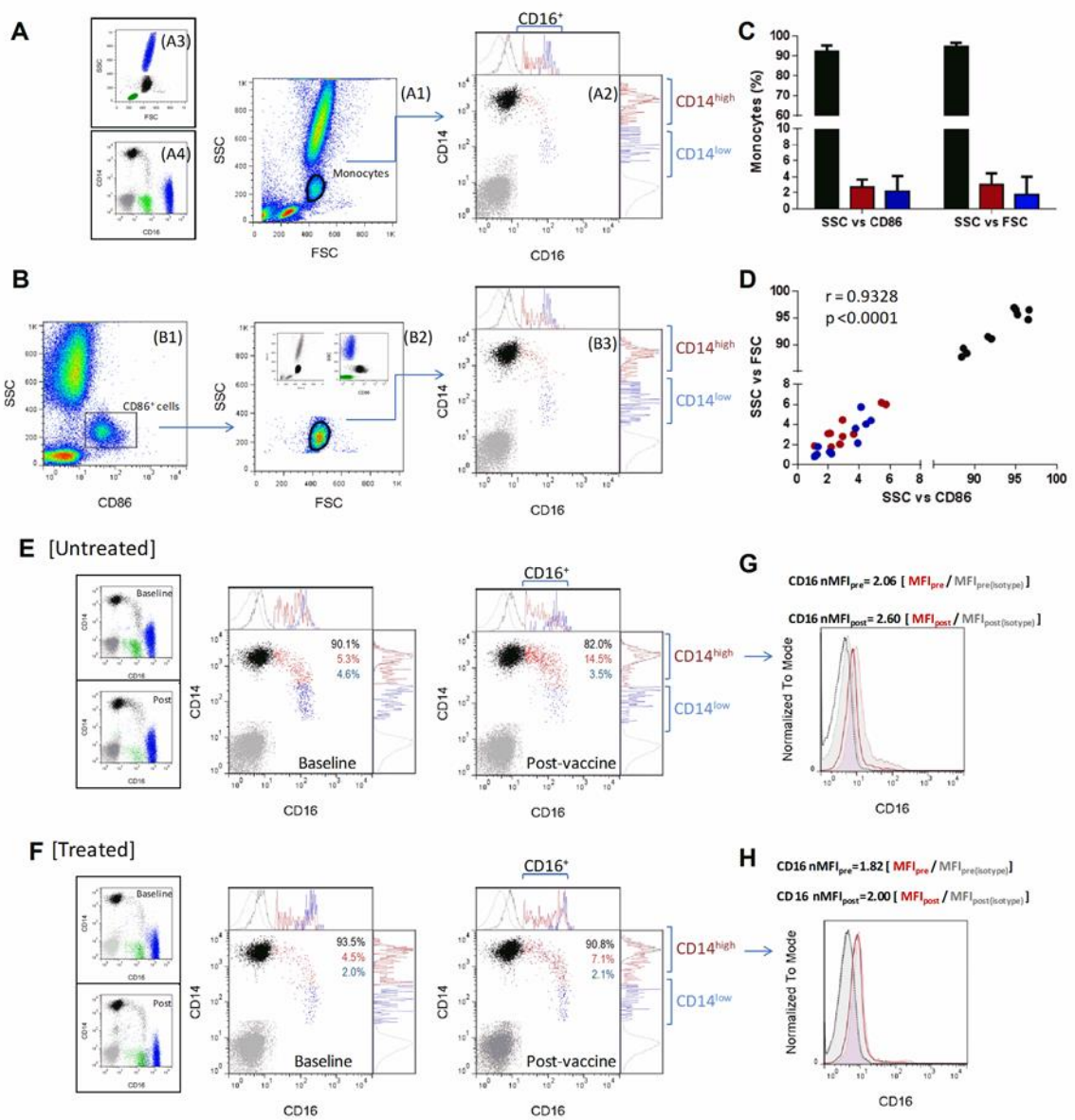
The immunostained samples were analysed using a BD FACSCalibur (BD Bioscience) flow cytometer to determine monocyte subset distribution. A total of 100,000 events were acquired and post-acquisition analysis was performed using FlowJo (version 10) software.

Monocytes were identified on a forward (FSC) – versus side scatter (SSC) plot and gated to analyse expression of CD14 and CD16 fluorescence, in order to distinguish the different subsets of monocytes: “classical” CD14<sup>high</sup>CD16<sup>-</sup>, “intermediate” CD14<sup>high</sup>CD16<sup>+</sup> and “non-classical” CD14<sup>low</sup>CD16<sup>+</sup> cells. The percentage of each monocyte subset with respect to total monocytes was calculated and absolute numbers were obtained based on the full blood count results. Every analysis was performed independently by two blinded researchers (Dr Gabriella Passacquale and I).

In a subgroup of 10 participants, Dr Gabriella Passacquale repeated measures of monocyte subset cell count on 2 consecutive measurements, 2 days apart and before immunisation administration, in order to assess variability in monocyte subset cell count in the absence of any intervention (immunisation either in the presence or absence of pharmacological treatment). Such an analysis showed inter-assay variation of <2 % for all the different monocytic subsets. Moreover, our gating strategy for monocyte characterisation (which was based on a FSC-versus SSC monocyte profile) was directly compared with a flow cytometry methodology that also included the pan-monocytic marker antibody allophycocyanin (APC)-mouse anti-human CD86, in the antibody panel <sup>228</sup>. In this case, cells with a typical monocytic FSC versus SSC profile and positive to CD86 staining were gated to analyse CD14 and CD16 expression.

Prevalence of the different monocyte subpopulations obtained with the two gating strategies were analysed on SPSS (version 23) to calculate the intraclass correlation coefficient (ICC) using an absolute agreement definition. The ICCs were 0.961 (95 % CI: 0.859-0.989;  $p < 0.0001$ ), 0.924 (95 % CI: 0.711-0.980;  $p < 0.0001$ ) and 0.915 (95 % CI: 0.695-0.977;  $p < 0.0001$ ) for the classical, intermediate and non-classical subset respectively; Figure 2.1.

In order to increase robustness of data, we also analysed the level of CD16 expression on monocytes using a normalised median fluorescence intensity (nMFI) strategy, in agreement with previously published methods used to monitor changes in cell immunophenotype in repeated measures <sup>229</sup>. A representative analysis of flow cytometry data is shown in Figure 2.1.



**Figure 2.1: Flow cytometry analysis**

As a validation study of our methodology, a comparison was carried out between the gating strategy for monocytes used in the current study (panel A, technique 1) and the use of a pan-monocytic marker, namely CD86, (panel B, technique 2).

In ‘Technique 1’, monocytes were identified based on their scatter properties using an autogating tool (as shown in the FSC vs SSC plot, A1). In ‘Technique 2’, they were initially identified as CD86<sup>+</sup> cells using



manual rectangular gating (B1) followed by a SSC vs FSC dot plot to further gate monocytes with a typical scatter property using an autogating tool (SSC vs CD86 plot, B2). Within the gated populations (A2, B3), CD14<sup>high</sup> and CD14<sup>low</sup> monocytes were identified, with “high” denoting an expression of CD14 approximately 100-fold higher and “low” as 10-fold higher than the isotype, as underlined in the corresponding histograms. Similarly, positivity for CD16 was evaluated based on the isotype control (in grey). The prevalence of each monocyte subset, obtained using a rectangular gating strategy, was calculated over the total monocytes. Data (median with IQR) are reported in the graph (C, classical CD14<sup>high</sup>CD16<sup>-</sup> in black, intermediate CD14<sup>high</sup>CD16<sup>+</sup> in red and non-classical CD14<sup>low</sup>CD16<sup>+</sup> in blue). A high level of concordance between the two strategies was noted, as evidenced by an intra-class correlation coefficient (ICC) of 0.961 (95 % CI: 0.859-0.989; p<0.0001), 0.924 (95 % CI: 0.711-0.980; p<0.0001) and 0.915 (95 % CI: 0.695-0.977; p<0.0001) for the classical, intermediate and non-classical subsets respectively. The correlation analysis (Spearman’s correlation analysis) between the two techniques is graphed in D. Both gating strategies enabled restriction of analysis to the CD14<sup>+</sup> population thus excluding potential interference from cell types such as neutrophils (blue, typically CD14<sup>-</sup>CD16<sup>high</sup>), and a subpopulation of lymphocytes (green, CD14<sup>-</sup>CD16<sup>low</sup>, likely NK cells) that are shown in the side panels (A3, A4) and overlaid with the analysed monocytes (black) and isotype control (grey).

Dot plots representative of untreated and treated participants are shown in panels E and F respectively. Since significant differences between groups were observed in the prevalence and cell number of CD14<sup>high</sup>CD16<sup>-</sup> (black) and CD14<sup>high</sup>CD16<sup>+</sup> (red) subsets, the level of CD16 expression was also specifically analysed within the CD14<sup>high</sup> population using a normalised median fluorescence intensity (nMFI) strategy. The histograms (G and H) show overlay of the baseline (empty histogram) and post-immunisation (filled histogram) intensity of fluorescence for CD16 of the stained samples (red). Also shown are the fluorescence levels of the antibody isotype controls (in grey, with dotted histogram and filled histogram for baseline and post-immunisation samples respectively). The median fluorescence intensity (MFI) calculated in CD14<sup>high</sup> monocytes at baseline (pre) and post-immunisation (post) were normalised by the MFI of the corresponding isotype control as indicated in the figure.

### **2.3.6 Serum clinical biochemistry**

All serum clinical biochemistry was measured by ViaPath laboratory at St Thomas' Hospital, London. Serum creatinine levels were obtained by using the creatininase enzymatic method on a Roche C8000 analyser. Liver enzyme and lipid levels were also measured using a Roche Cobas 8000 analyser. Hs-CRP was measured using a DadeBehring BN2 analyser. Glycosylated haemoglobin levels were measured from whole blood samples using a Menarini Hb9210 analyser. All of the aforementioned assays were performed by Viapath staff at St Thomas' Hospital.

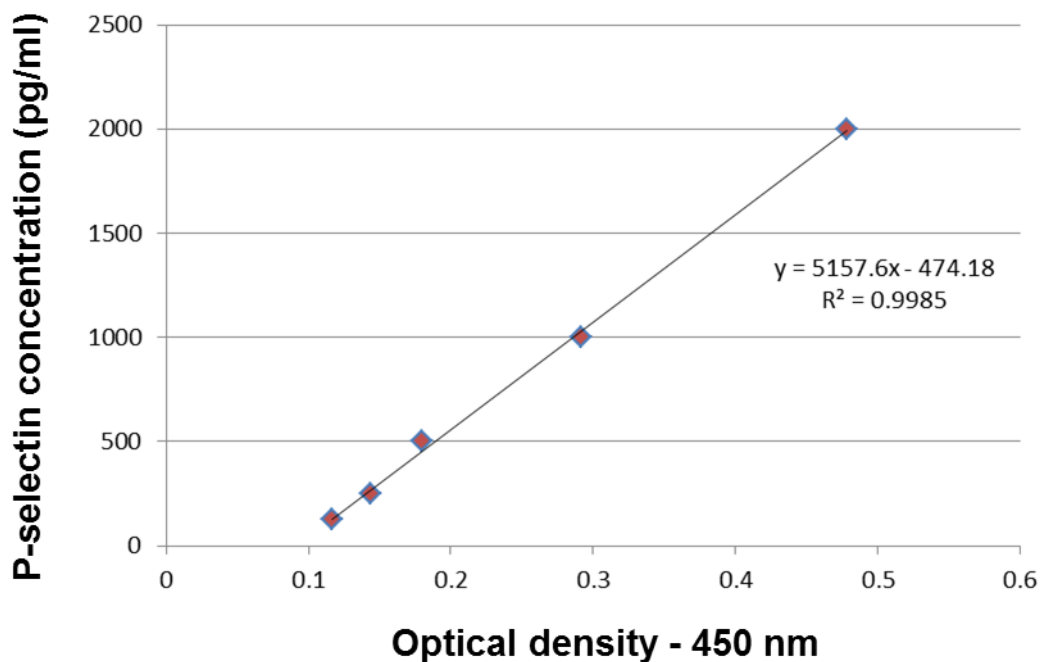
### **2.3.7 Enzyme-linked immunosorbent assays**

Enzyme-linked immunosorbent assays (ELISAs) were carried out to measure plasma levels of P-selectin using commercially available kits (BEK1189, Biospes), as per manufacturer instructions (inter-assay variability was found to be <5 %).

Briefly, 100 µl of either plasma samples or standards ranging from 0 – 2000 pg/ml were loaded in duplicate onto a 96-well plate coated with anti-P-selectin polyclonal antibody. The plate was incubated for 90 minutes at 37 °C. The liquid was then removed from the wells and 100 µl biotin-conjugated anti-human P-selectin antibody was added to the wells. The plate was incubated for 60 minutes at 37 °C and was then washed three times with wash buffer from the kit. 100 µl avidin-biotin-peroxidase complex was added to each well and the plate was incubated for 30 minutes at 37 °C. The plate was washed five times and 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well. The plate was incubated at 37 °C in the dark for 15-30 minutes, until various shades of blue colouration could be detected within the standard wells. 100 µl stop solution (typically sulphuric acid, at a concentration of around 0.16 M) was added to each well to prevent the reaction from continuing, and the colour was observed to change from blue to yellow. The plate was subsequently analysed using a microplate optical density reader at 450 nm wavelength. A standard curve was constructed by plotting the optical densities of the standards against the corresponding known P-selectin concentrations; Figure 2.2. Plasma P-

selectin concentrations in the different samples were read from the standard curve using the respective optical density readings.

ELISA kits were also used to measure serum levels of CD16a (SEB278Hu; Cloud-Clone Corp.), and CD16b (SEB271Hu; Cloud-Clone Corp.) using a similar technique to that stated above, with variations based on manufacturer instructions. ELISA kits to measure IL-1 $\beta$  (MBS263843; MyBioSource, USA), IL-6 (0Kaa00012\_96W; Aviva Systems Biology, USA), and TNF- $\alpha$  (GWV-SKR066; GenWay Biotech Inc, USA) were used but the cytokine levels in this healthy volunteer population were too low to be accurately detected, and thus a Luminex<sup>®</sup> ultrasensitive cytokine multiplex assay was performed instead (see below).



**Figure 2.2: A representative standard curve from the P-selectin enzyme-linked immunosorbent assay**

The optical density reading (at 450 nm) was plotted against the P-selectin concentration to obtain a standard curve, from which sample concentrations were extrapolated.

### **2.3.8 Luminex assay**

A magnetic Luminex® ultrasensitive cytokine multiplex assay (Life Technologies Corporation) was performed to measure levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in serum. Manufacturer instructions were followed.

Briefly, a custom-made antibody bead solution was vortexed for 30 seconds and then sonicated for 30 seconds to prevent the beads from clumping. 25  $\mu$ l of beads were added to each well in a 96 –well plate; the plate was protected from light to prevent photobleaching. The wells were washed twice with pre-prepared wash solution (using a handheld magnet underneath the plate to keep the beads in place). 50  $\mu$ l pre-prepared incubation buffer was added to all wells. Either 100  $\mu$ l of diluted standards, or 50  $\mu$ l of assay diluent followed by 50  $\mu$ l of sample (diluted to 1:100 with pre-prepared assay diluent) was added to each well and the plate was incubated at room temperature for 2 hours on an orbital shaker.

The liquid was removed and the plate was washed twice. 100  $\mu$ l biotinylated detector antibody was added to each well and the plate was incubated at room temperature for an hour on an orbital shaker. The wells were washed twice and 100  $\mu$ l streptavidin-R-phycoerythrin conjugate was added to each well. The plate was incubated at room temperature for 30 minutes on an orbital shaker. The wells were washed four times and the plate was subsequently read using a Luminex® Flex Map 3D Analyser.

### **2.3.9 Sample size calculation**

The sample size needed for the study was calculated in accordance with previously published data that showed a standard deviation for CD16<sup>+</sup> monocytes and total monocytes within a healthy population of 5 %, with a difference in means between pre and post-immunisation values of 7.5 %<sup>31</sup>. Using a minimum detectable difference in means between anti-platelet treatments and placebo at the end of the study of 4 %, at power 0.9 and significance level 0.05, this yielded a sample size of 15 per group.

### **2.3.10 Statistical analysis**

Statistical analyses were performed using SPSS (version 23) software. A Shapiro–Wilk normality test was run for all variables measured in the study. The effect of treatments on monocyte subtypes, inflammatory biomarkers (hs-CRP and cytokines), and platelet activation (soluble P-selectin), which were all found not to be normally distributed, were compared using a non-parametric analysis of co-variance (rank ANCOVA) with Bonferroni *post hoc* correction for multiple comparison analysis, using baseline values as a covariate. Baseline vs. post-immunisation comparison of the analysed variables was also analysed within each group by Wilcoxon matched-pairs signed-rank test. Associations between variables were assessed by a Spearman's correlation analysis. A *p* value <0.05 was taken as statistically significant. Data are expressed as the mean ± standard error of mean (SEM) or median and interquartile ranges (IQR) for parametric and non-parametric variables, respectively.

## **2.4 Results**

### **2.4.1 Anti-platelet drugs do not influence hs-CRP rise post-immunisation**

General characteristics of the study population stratified across groups are shown in Table 2.1. Influenza immunisation induced an acute inflammatory response as evidenced by an increase in hs-CRP post-immunisation in all study groups; Figure 2.3. None of the anti-platelet drugs modified the rise in hs-CRP in response to immunisation administration; Figure 2.3.

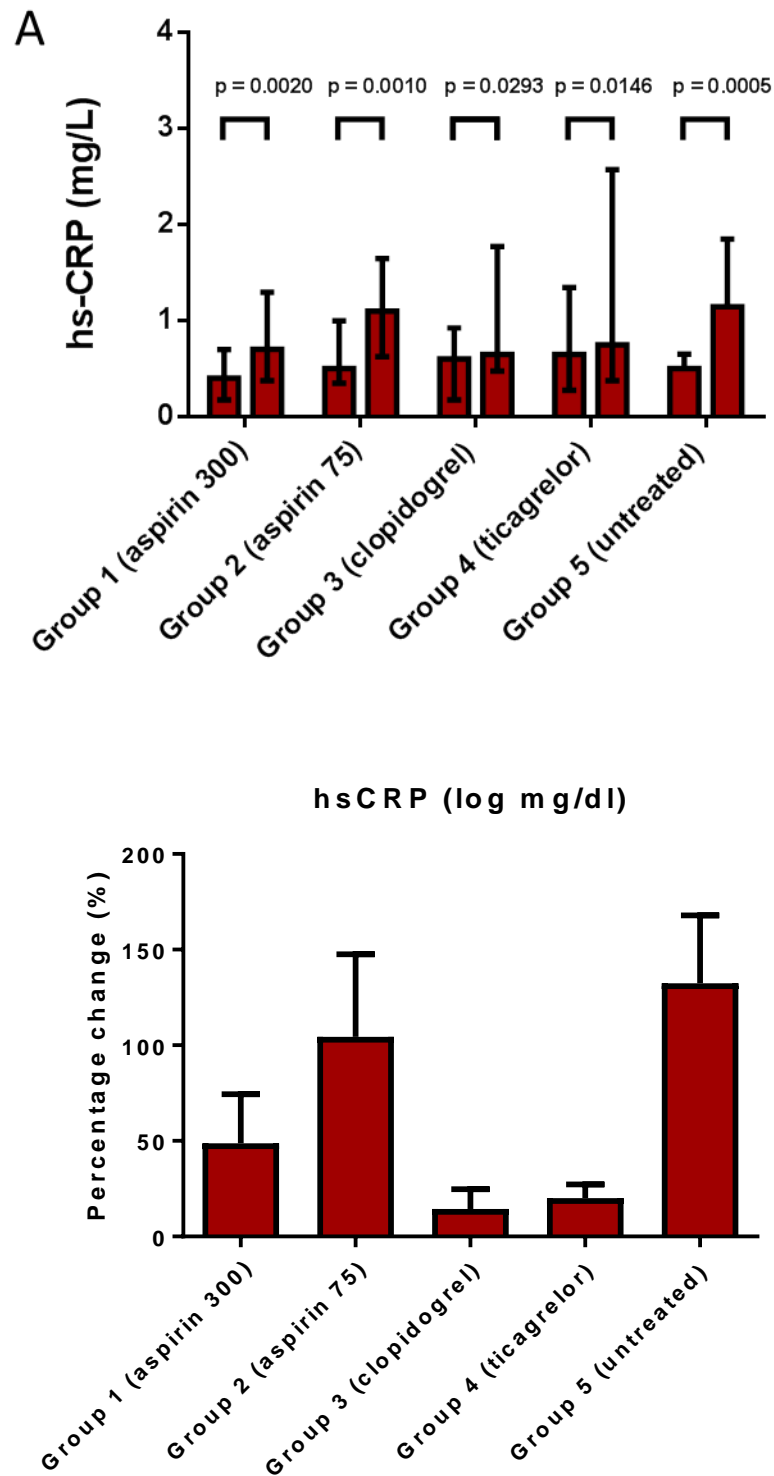
As above, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels were measured using a Luminex<sup>®</sup> assay. There were no significant differences between post-immunisation and baseline levels of any of these pro-inflammatory cytokines in any of the groups; Figure 2.4.

	Gender (n)	Age (years)	Systolic blood pressure (mmHg)	Current / ex-smokers (n)	BMI (kg/m <sup>2</sup> )	HDL chol (mmol/l)	LDL chol (mmol/l)	Triglycerides (mmol/l)	Leucocytes (x10 <sup>9</sup> /l)	Platelets (x10 <sup>9</sup> /l)	Monocytes (x10 <sup>9</sup> /l)	HbA1c (mmol/mol)	Creatinine (μmol/l)
Group 1 (aspirin 300) n = 15	4 male 11 female	32.00 (30.00 - 35.25)	117 ± 4	2	22.78 (20.81 - 26.00)	1.872 ± 0.08	2.74 (2.42 - 3.33)	1.08 (0.72 - 1.32)	5.99 ± 0.31	219.5 (193.4 - 240.8)	0.5 (0.4 - 0.5)	32.50 (30.75 - 36.00)	73.53 ± 2.72
Group 2 (aspirin 75) n = 15	3 male 12 female	33.50 (28.75 - 41.25)	121 ± 5	3	25.23 (21.87 - 28.47)	1.751 ± 0.10	2.87 (2.43 - 3.43)	1.00 (0.80 - 1.39)	6.01 ± 0.33	211.0 (184.0 - 240.0)	0.5 (0.4 - 0.5)	32.00 (29.75 - 34.00)	70.07 ± 3.27
Group 3 (clopidogrel) n = 15	5 male 10 female	36.00 (31.5 - 43.50)	123 ± 4	2	25.12 (20.61 - 28.42)	1.735 ± 0.09	2.49 (2.24 - 3.15)	1.07 (0.81 - 1.50)	6.13 ± 0.38	228.0 (204.0 - 251.0)	0.4 (0.3 - 0.5)	33.00 (31.00 - 34.00)	72.79 ± 3.17
Group 4 (ticagrelor) n = 15	4 male 11 female	34.50 (28.50 - 41.00)	124 ± 5	3	23.71 (20.04 - 29.00)	1.77 ± 0.06	2.76	0.99 (0.76 - 1.64)	6.08 ± 0.30	224 (188.2 - 242.3)	0.4 (0.3-0.5)	32.00 (30.25 - 36.25)	70.98 ± 3.33
Group 4 (untreated) n = 15	6 male 9 female	37.00 (31.00 - 43.50)	125 ± 6	1	23.63 (21.83 - 28.66)	1.793 ± 0.09	2.20 (1.77 - 3.03)	0.93 (0.69 - 2.04)	6.23 ± 0.27	219.5 (171.3 - 248.8)	0.5 (0.4 - 0.6)	34 (31.00 - 36.00)	73.10 ± 3.54

**Table 2.1: Baseline characteristics of the study population**

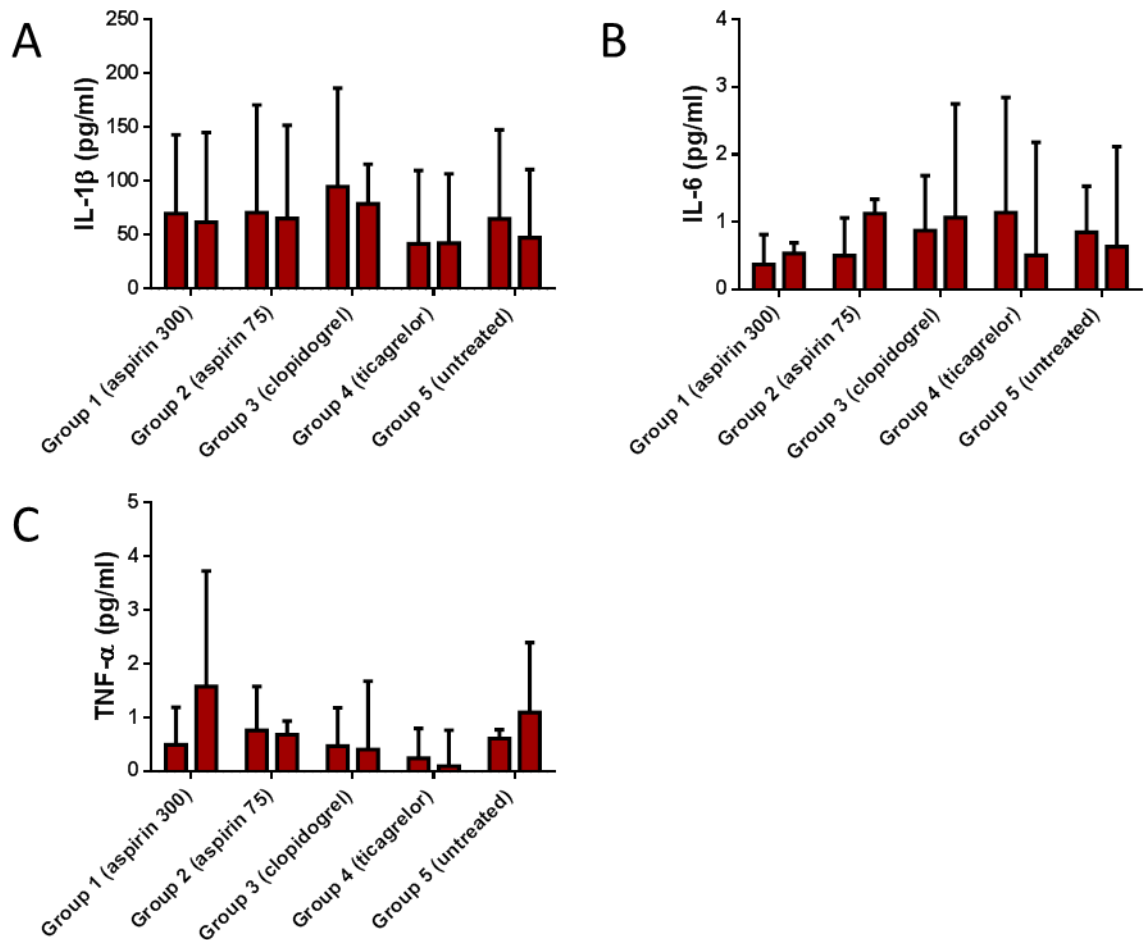
Biometric data and cardiovascular risk profiles for the subjects in all groups are shown. Values are expressed as the mean ± SEM or median (IQR). BMI: body mass index;

HDL: high density lipoprotein; LDL: low density lipoprotein.



**Figure 2.3: High-sensitivity C-reactive protein levels**

Panel A: absolute values of hs-CRP pre- and post-immunisation are shown for all groups. Panel B: the percentage change from baseline in hs-CRP is shown for all groups.



**Figure 2.4: Cytokine levels pre- and post- immunisation**

The absolute values for IL-1 $\beta$  (A), IL-6 (B), and TNF- $\alpha$  (C) at baseline and post-immunisation are shown.

Data are reported as median with IQR. Rank ANCOVA was used for between-group comparison and identified no significant differences.

	Hs-CRP (mg/L)		IL-1 $\beta$ (pg/ml)		IL-6 (pg/ml)		TNF- $\alpha$ (pg/ml)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Group 1 (aspirin 300)	0.40 (0.18-0.70)	0.70 (0.38-1.25)	70 (40-123)	62 (45-130)	0.37 (0.14-0.80)	0.53 (0.21-0.64)	0.50 (0.43-0.81)	1.58 (0.29-3.61)
Group 2 (aspirin 75)	0.51 (0.35-1.00)	1.05 (0.63-1.65)	71 (46-142)	66 (63-122)	0.50 (0.22-0.96)	1.12 (0.41-1.27)	0.77 (0.21-1.45)	0.69 (0.39-0.74)
Group 3 (clopidogrel)	0.58 (0.18-0.93)	0.65 (0.48-1.78)	95 (48-171)	79 (61-102)	0.87 (0.38-1.39)	1.06 (0.58-2.54)	0.47 (0.31-0.53)	0.41 (0.26-0.74)
Group 4 (ticagrelor)	0.63 (0.28-1.33)	0.73 (0.35-2.58)	42 (30-63)	43 (35-59)	1.14 (0.14-2.54)	0.50 (0.29-1.86)	0.25 (0.14-0.67)	0.10 (0.10-0.57)
Group 5 (untreated)	0.45 (0.38-0.65)	1.15 (0.77-1.87)	65 (39-115)	48 (30-95)	0.85 (0.43-1.43)	0.63 (0.47-1.59)	0.62 (0.54-0.72)	1.10 (0.57-1.82)

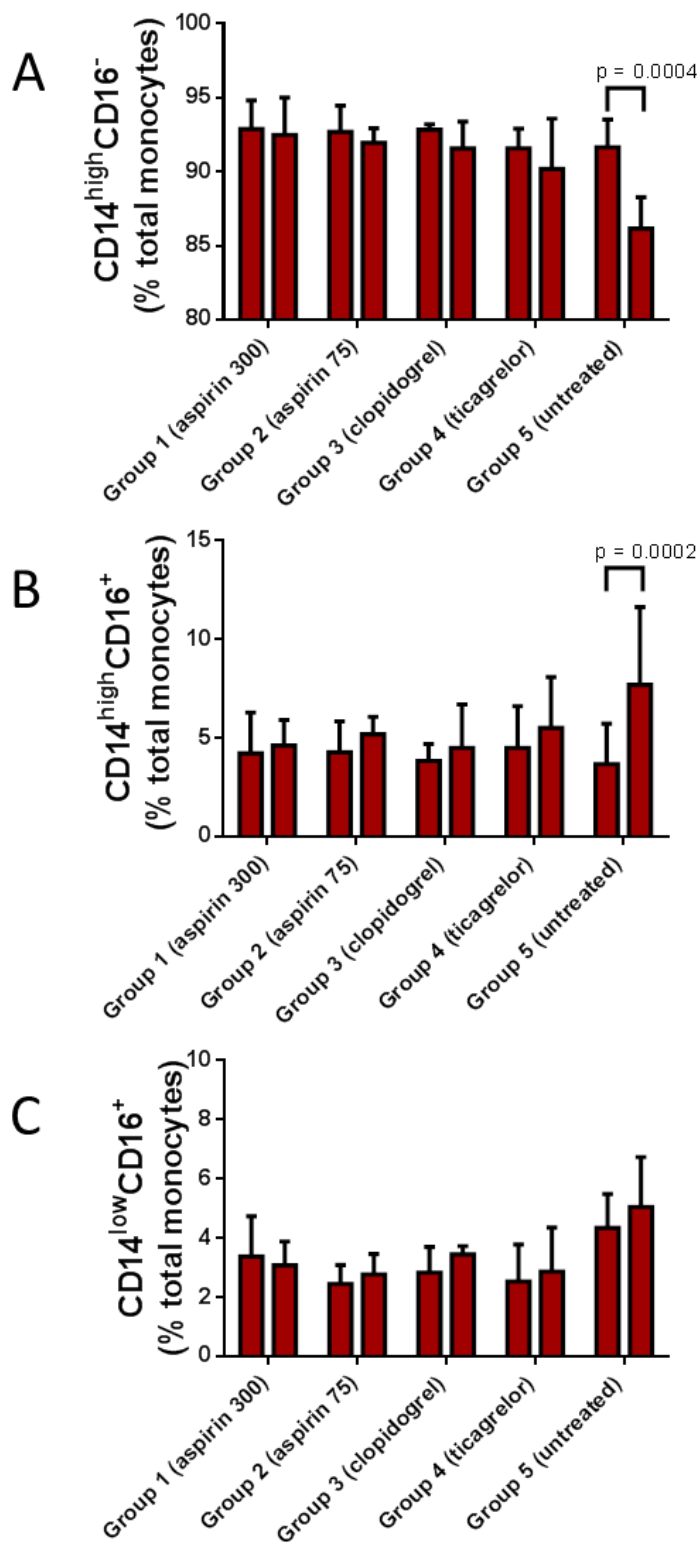
**Table 2.2: Pre- and post-immunisation levels of hs-CRP and cytokines**



#### **2.4.2 Expansion of intermediate CD14<sup>high</sup>CD16<sup>+</sup> monocytes in response to influenza immunisation is attenuated by all anti-platelet treatments**

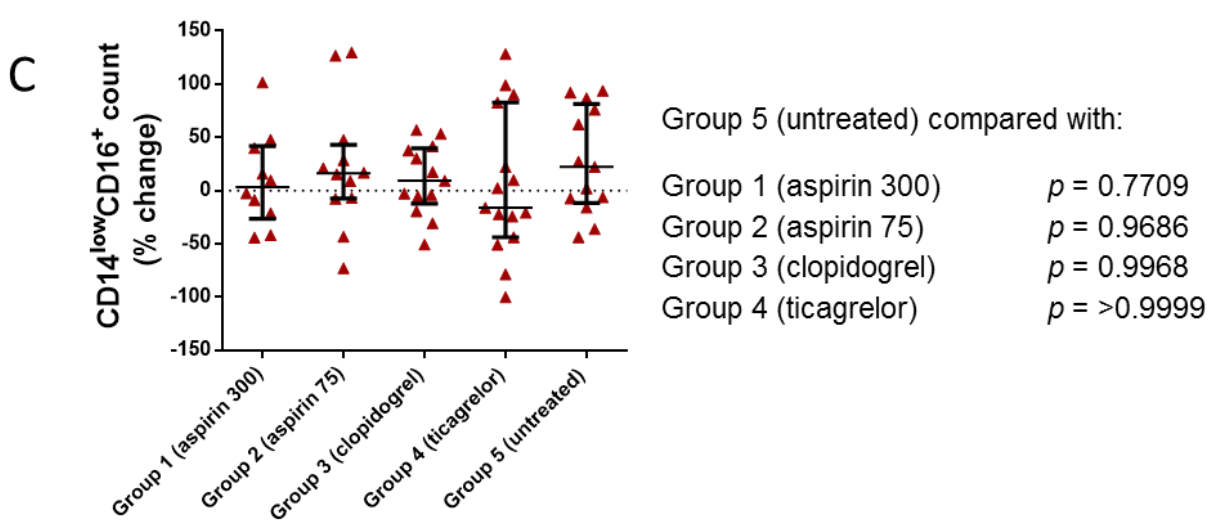
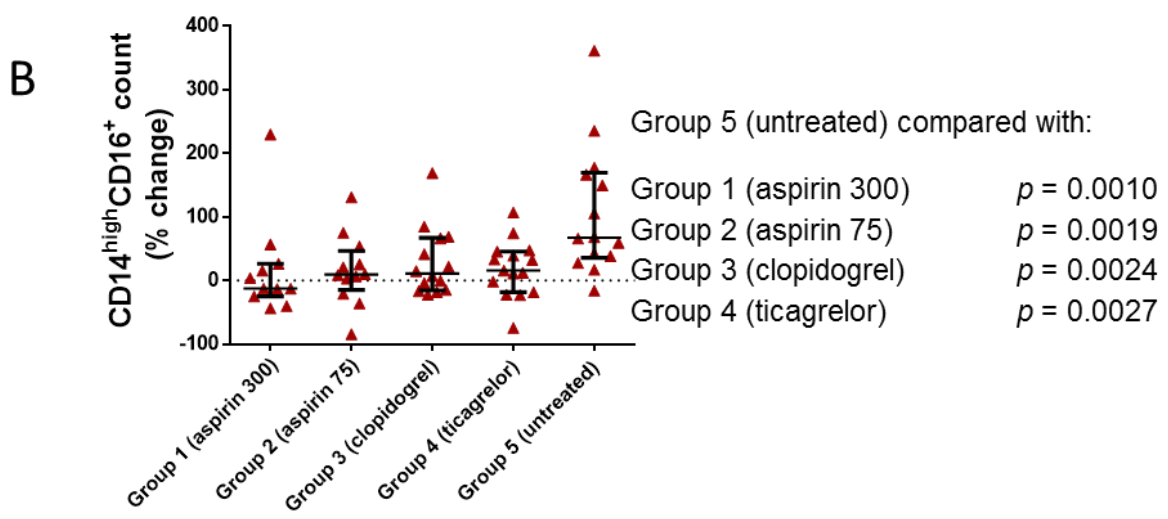
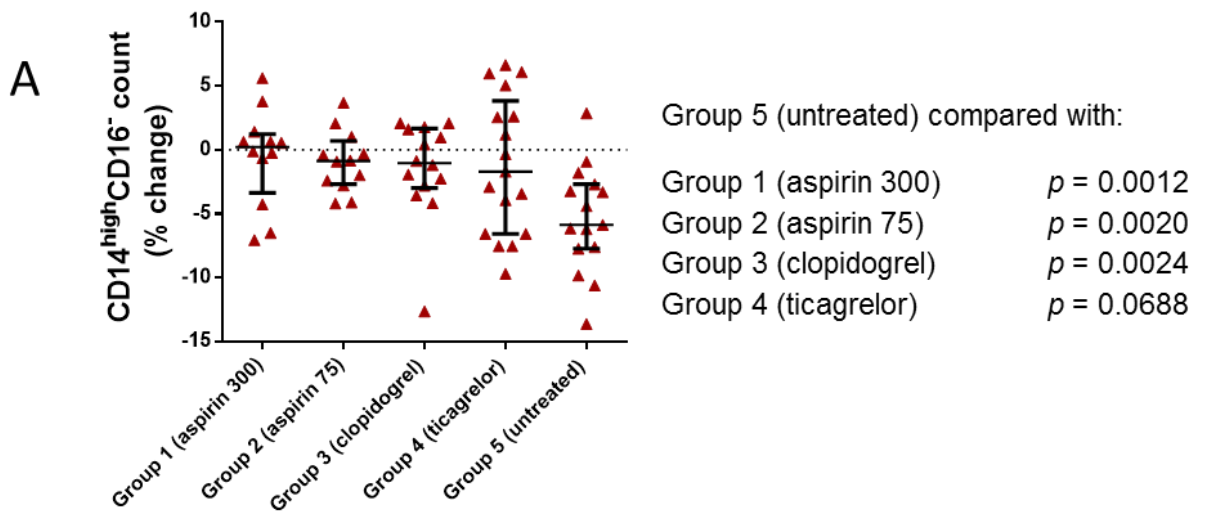
The baseline monocyte phenotype was similar between groups, with a typical preponderance of the 'classical' CD14<sup>high</sup>CD16<sup>-</sup> subtype (median of 92.7 % of total monocytes) over the 'intermediate' CD14<sup>high</sup>CD16<sup>+</sup> (4.2 %) and 'non-classical' CD14<sup>low</sup>CD16<sup>+</sup> (3.2 %) subpopulations in all participants (Figures 2.5-2.6, Table 2.2). Whilst the total monocyte cell count remained stable post-immunisation in the whole study population, differences emerged between groups as regards the distribution of the different subsets. Results from the participants not treated with any anti-platelet drugs showed a significant increase in both percentage (from a median of 3.7 to 7.8 %;  $p = 0.0002$ ) and absolute number (from a median of 18.07 to 33.40 cells/ $\mu$ L;  $p = 0.0002$ ) of the CD14<sup>high</sup>CD16<sup>+</sup> subtype; and a concomitant reduction in both the percentage (from a median of 91 to 86 %;  $p = 0.0004$ ) and cell count (from a median of 443.90 to 399.00 cells/ $\mu$ L;  $p = 0.002$ ) of classical CD14<sup>high</sup>CD16<sup>-</sup> monocytes was noted. No change in the non-classical CD14<sup>low</sup>CD16<sup>+</sup> population was observed (Figures 2.5-2.6, Table 2.2).

All of the tested anti-platelet regimes abrogated the above changes in monocyte phenotype induced by immunisation. Although no statistically significant differences were observed between the treated groups, there was a trend towards aspirin 300 mg exerting a stronger effect in limiting the expansion of CD14<sup>high</sup>CD16<sup>+</sup> count post-immunisation (median percentage change from baseline was -12.6 in Group 1 vs. +67.3 in untreated;  $p = 0.0010$ ). Attenuation in the CD14<sup>high</sup>CD16<sup>+</sup> increase post-immunisation was also observed in Group 2 (aspirin 75) (median percentage increase was 9.3;  $p = 0.0019$  vs. untreated) and, although less pronounced, in Group 3 (clopidogrel) (median percentage increase was 10.8;  $p = 0.0024$  vs. untreated) and Group 4 (ticagrelor) (median percentage increase was 16.0;  $p = 0.0027$  vs. untreated). Typical monocyte phenotype profiles are shown in Figure 2.7.



**Figure 2.5: Monocyte subset prevalence**

Graphs A, B and C show the prevalence of each monocyte subset (expressed as percentage over total monocytes) at baseline and post-immunisation in the different groups; A = CD14<sup>high</sup>CD16<sup>-</sup>, B= CD14<sup>high</sup>CD16<sup>+</sup>, and C = CD14<sup>low</sup>CD16<sup>+</sup>.

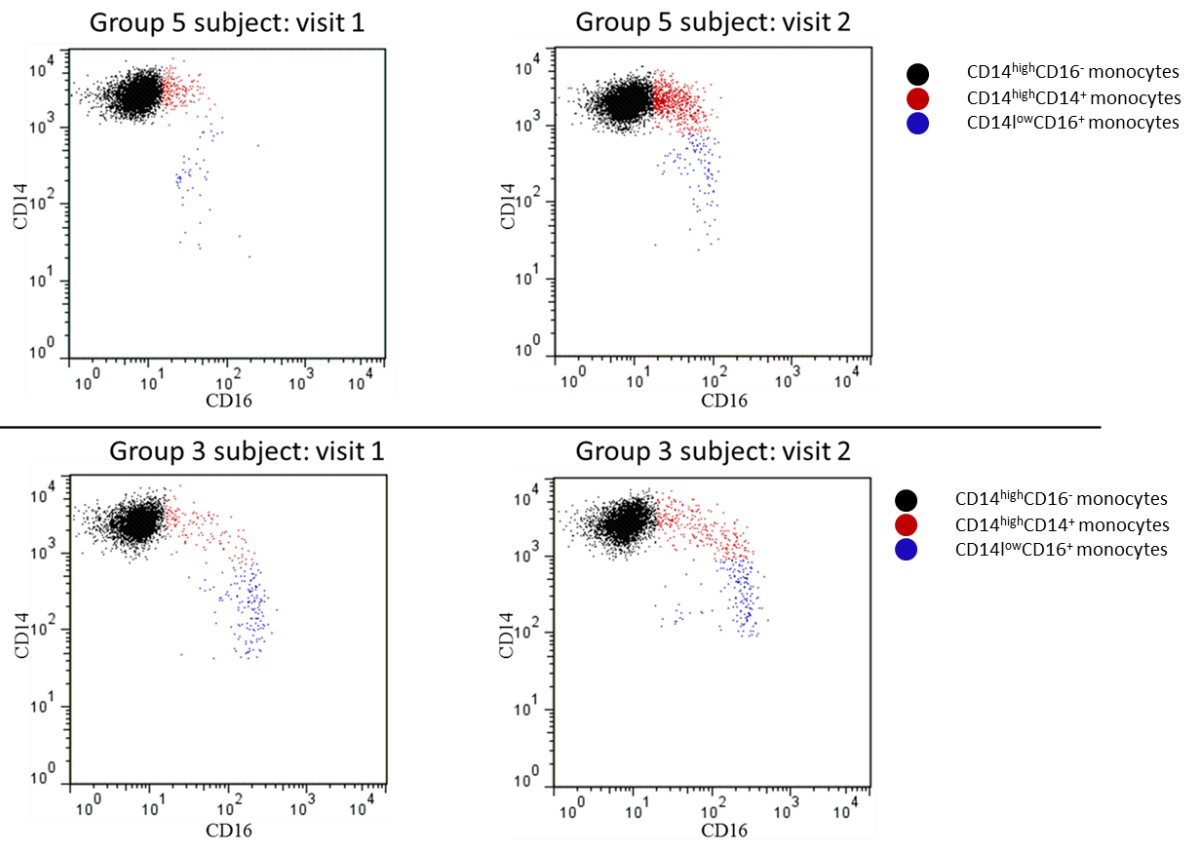


**Figure 2.6: Monocyte percentage change**

The percentage change from baseline of CD14<sup>high</sup>CD16<sup>-</sup>, CD14<sup>high</sup>CD16<sup>+</sup>, and CD14<sup>low</sup>CD16<sup>+</sup> cell count in each of the groups is reported in A, B, and C, respectively.

**Table 2.3: Monocyte subsets expressed in absolute cell numbers**

	<b>CD14<sup>high</sup>CD16<sup>-</sup> count (cells/<math>\mu</math>l)</b>	<b>CD14<sup>high</sup>CD16<sup>+</sup> count (cells/<math>\mu</math>l)</b>	<b>CD14<sup>low</sup>CD16<sup>+</sup> count (cells/<math>\mu</math>l)</b>
<b>Group 1 (aspirin 300)</b>			
<b>Baseline</b>	438.50 (312.70 - 470.80)	17.70 (11.77 - 26.50)	13.32 (8.70 - 18.84)
<b>Day 2 post-vaccination</b>	443.00 (313.30 - 466.90)	18.40 (15.85 - 29.60)	13.33 (11.46 - 17.90)
<b>p vs Group 5 (untreated)</b>	0.0009	0.0012	0.1720
<b>Group 2 (aspirin 75)</b>			
<b>Baseline</b>	462.50 (442.80 - 472.10)	22.70 (16.60 - 30.93)	11.83 (8.50 - 15.46)
<b>Day 2 post-vaccination</b>	451.20 (443.60 - 460.00)	28.28 (16.92 - 29.34)	12.45 (10.61 - 17.05)
<b>p vs Group 5 (untreated)</b>	0.0023	0.0021	0.0972
<b>Group 3 (clopidogrel)</b>			
<b>Baseline</b>	370.80 (250.00 - 465.80)	15.98 (12.59 - 19.42)	11.17 (8.46 - 17.73)
<b>Day 2 post-vaccination</b>	359.00 (245.30 - 456.50)	20.39 (12.35 - 27.05)	13.68 (8.03 - 18.98)
<b>p vs Group 5 (untreated)</b>	0.0020	0.0023	0.1990
<b>Group 4 (ticagrelor)</b>			
<b>Baseline</b>	422.40 (397.80 - 465.90)	17.90 (12.50 - 25.40)	12.40 (8.21 - 18.14)
<b>Day 2 post-vaccination</b>	413.50 (285.05 - 460.80)	19.24 (13.00 - 27.85)	14.75 (10.31 - 18.78)
<b>p vs Group 5 (untreated)</b>	0.0027	0.0035	0.3470
<b>Group 5 (untreated)</b>			
<b>Baseline</b>	443.90 (336.10 - 570.80)	18.07 (12.15 - 29.14)	15.41 (11.73 - 21.35)
<b>Day 2 post-vaccination</b>	399.00 (264.60 - 531.60)	33.40 (24.08 - 53.61)	22.19 (13.49 - 28.37)



**Figure 2.7: Typical monocyte phenotype profile**

Comparison of the typical changes in monocyte phenotype profile in a subject who did not receive anti-platelet therapy (top images) with one who did (bottom images), showing with an increase in CD14<sup>high</sup>CD16<sup>+</sup> monocytes and a reduction in CD14<sup>high</sup>CD16<sup>-</sup> monocytes post-immunisation in the untreated subject.

### **2.4.3 The rise in CD14<sup>high</sup>CD16<sup>+</sup> monocytes in response to immunisation is directly correlated with CD16 expression**

CD16 expression, specifically analysed within the CD14<sup>high</sup> population, was studied using the nMFI strategy. Group 5 (untreated) showed a significant rise in monocytic CD16 levels post-immunisation (median percentage change from baseline was +20.92;  $p = 0.002$  vs. baseline) that was abolished by all anti-platelet drugs, with aspirin 300 mg daily appearing to be the most efficacious of the treatments in this regard (median percentage increase from baseline in Group 1 was -2.1;  $p = 0.006$  vs. Group 5; in Groups 2, 3 and 4, it was +0.5, +2, and +1.5 respectively;  $p = 0.018, 0.022, \text{ and } 0.0021$  respectively vs. untreated). Likewise, a strong direct correlation was found between the increase from baseline of CD14<sup>high</sup>CD16<sup>+</sup> cell count and CD16 nMFI ( $r = 0.5$ ;  $p = 0.0005$ ) (Figure 2.8). While attenuating the increase in CD14<sup>high</sup>CD16<sup>+</sup> cells, the anti-platelet regimes counteracted the reduction in CD14<sup>high</sup>CD16<sup>-</sup> monocytes observed in untreated participants post-immunisation (Figure 2.9). The two monocyte subsets showed a strong inverse correlation with each other ( $r = -0.8$ ;  $p < 0.0001$ ).

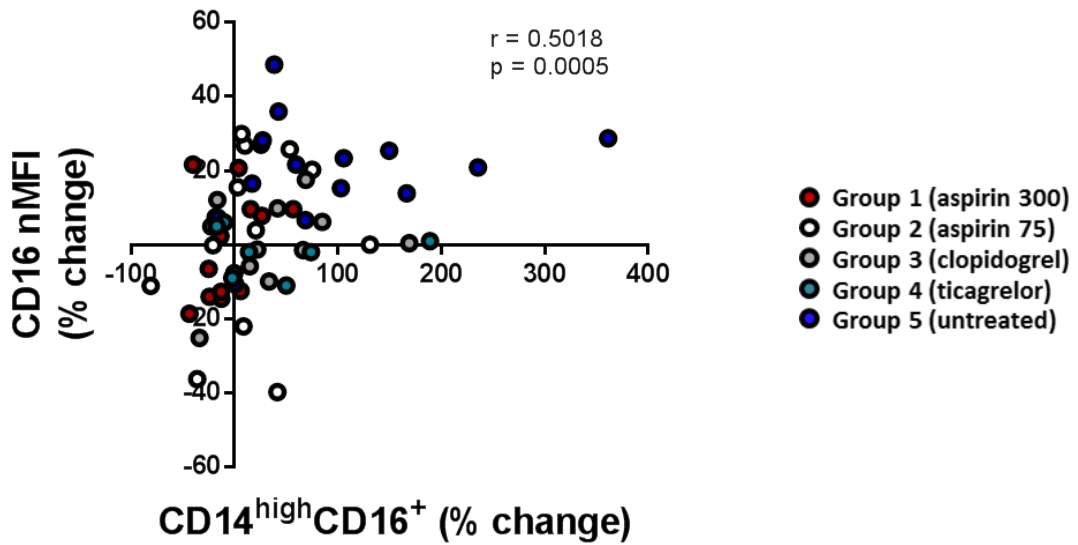


Figure 2.8: CD14<sup>high</sup>CD16<sup>+</sup> cell count and CD16 normalised median fluorescence intensity correlation

This graph shows the correlation between percentage change in CD14<sup>high</sup>CD16<sup>+</sup> cell count and CD16 nMFI.

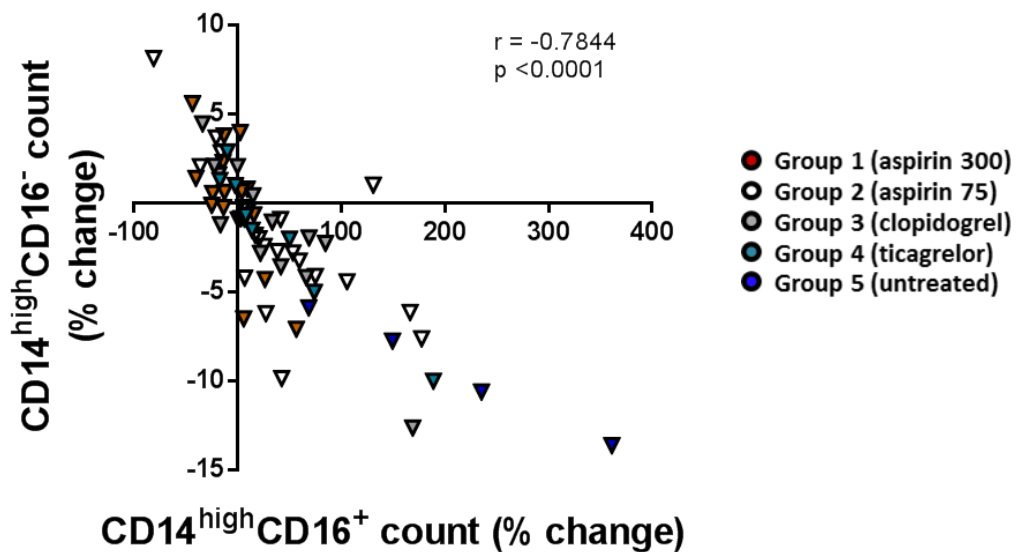


Figure 2.9: CD14<sup>high</sup>CD16<sup>+</sup> and CD14<sup>high</sup>CD16<sup>-</sup> cell count correlation

This graph shows the correlation between percentage change in CD14<sup>high</sup>CD16<sup>+</sup> and CD14<sup>high</sup>CD16<sup>-</sup> cell count.

#### **2.4.4 As monocytes acquire a CD14<sup>high</sup>CD16<sup>+</sup> phenotype, there is upregulation of polymorphonuclear leucocyte cell surface expression of CD16**

In addition to the observed upregulation of CD16 on circulating monocytes, in Group 5 (untreated) there was a rise in surface CD16 expression on PMNs in response to the influenza immunisation. PMN nMFI increased from  $203.50 \pm 17.93$  to  $332.50 \pm 54.90$  ( $p = 0.0262$ ). This response was counteracted by all anti-platelet drugs, which in fact reduced the level of CD16 expression on PMNs; see Table 2.3 and Figure 2.10 A.

When the percentage change in PMN surface CD16 in Group 5 (untreated) was compared to the four anti-platelet therapy groups using a multiple comparison analysis, it was found to be significantly higher than each of the other groups; see Figure 2.10 A.

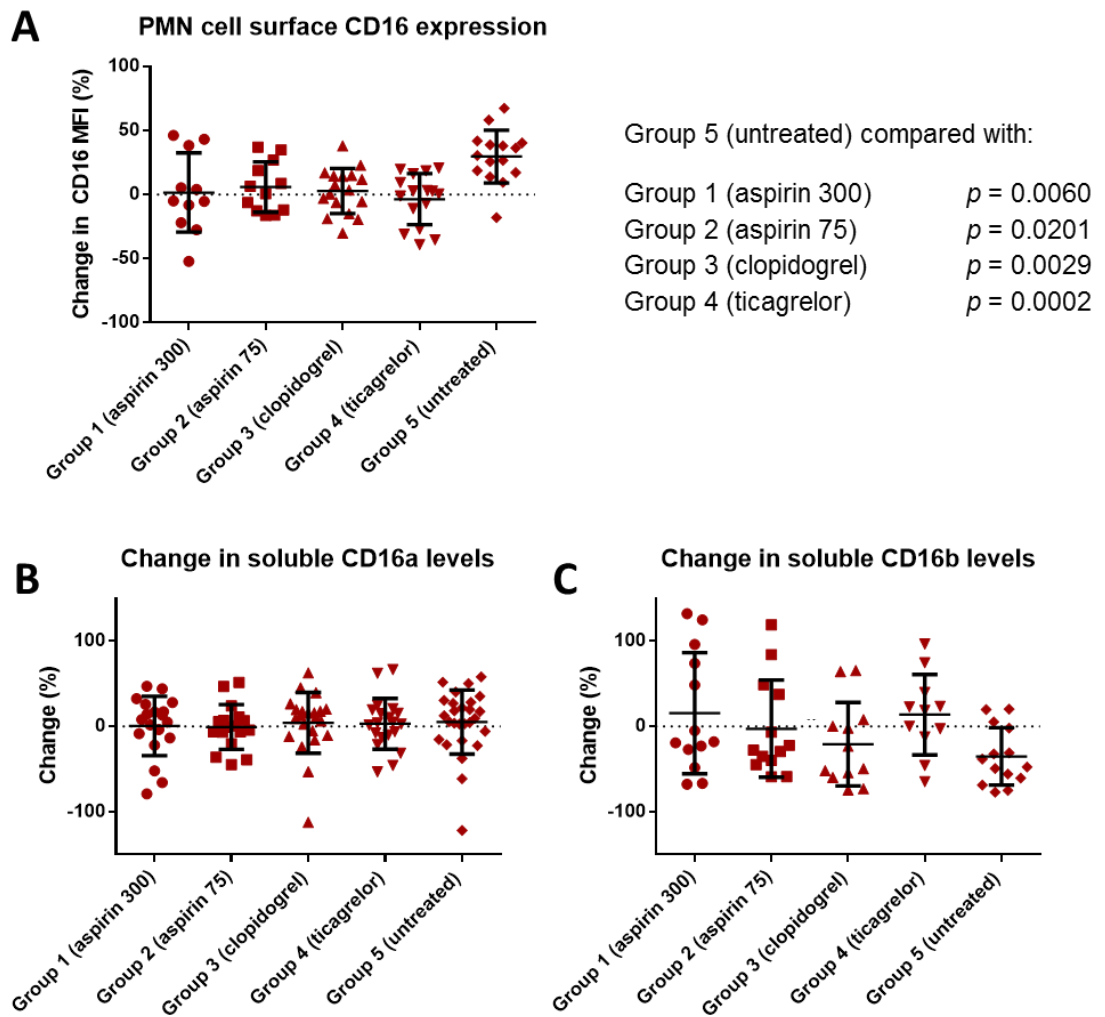
#### **2.4.5 As monocytes acquire a CD14<sup>high</sup>CD16<sup>+</sup> phenotype, there is no change in soluble CD16a but a reduction in soluble CD16b levels**

As CD16 undergoes shedding, I measured levels of soluble CD16 in serum, using ELISA kits that specifically detect either monocyte-derived CD16a or PMN-derived CD16b isoforms. Soluble CD16a levels remained unchanged in all groups; Table 2.3 and Figure 2.10 B. Conversely, soluble CD16b concentrations fell from  $89.90 \pm 11.29$  ng/ml to  $55.93 \pm 7.259$  ng/ml post-vaccination in Group 5 (untreated;  $p = 0.0143$ ). This response was counteracted with similar efficacy by all anti-platelet drugs; see Table 2.3 and Figure 2.10 C.



**Table 2.4: A comparison of CD16 levels between visits 1 and 2**

	PMN surface CD16 expression (nMFI)			Monocytic surface CD16 expression (nMFI)			Soluble CD16a (ng/ml)			Soluble CD16b (ng/ml)		
	Visit 1	Visit 2	p value	Visit 1	Visit 2	p value	Visit 1	Visit 2	p value	Visit 1	Visit 2	p value
<b>Group 1 (aspirin 300)</b>	241.00 ± 20.80	139.00 ± 32.10	0.0105	2.42 ± 0.21	2.35 ± 0.17	0.5040	35.18 ± 2.44	33.14 ± 2.40	0.4030	72.06 ± 7.37	70.28 ± 7.45	0.8571
<b>Group 2 (aspirin 75)</b>	216.80 ± 13.13	195.02 ± 39.53	0.6110	2.11 ± 0.13	2.16 ± 0.19	0.7903	34.96 ± 2.52	35.64 ± 2.40	0.8069	73.01 ± 10.15	64.48 ± 7.87	0.4251
<b>Group 3 (clopidogrel)</b>	241.00 ± 13.20	174.60 ± 28.24	0.0308	2.01 ± 0.14	2.01 ± 0.14	0.9715	36.78 ± 2.35	33.35 ± 2.32	0.1490	82.89 ± 11.21	62.76 ± 9.25	0.1296
<b>Group 4 (ticagrelor)</b>	282.20 ± 22.60	207.40 ± 46.55	0.0397	1.96 ± 0.23	1.75 ± 0.17	0.3545	36.53 ± 2.66	33.70 ± 2.64	0.3064	59.71 ± 6.47	69.22 ± 9.37	0.3086
<b>Group 5 (untreated)</b>	203.50 ± 17.93	332.50 ± 54.90	0.0262	1.90 ± 0.06	2.40 ± 0.16	0.0100	34.93 ± 2.16	32.84 ± 2.14	0.2808	89.90 ± 11.29	55.93 ± 7.26	0.0143



**Figure 2.10: A comparison of CD16 levels between visits 1 and 2**

Figure 2.10 shows a comparison of CD16 levels between visits 1 and 2, including PMN cell surface CD16 expression (A), measured as MFI with flow cytometry, as well as soluble CD16a (B) and CD16b (C) in serum. A multiple comparison analysis is shown for graph A, where the mean of Group 5 (untreated) is compared with the anti-platelet therapy groups.

#### **2.4.6 Modulation of P-selectin is linearly related to the change in monocyte phenotype**

The level of P-selectin post-immunisation in Group 5 (untreated) increased by a median of +17.2 % ( $p = 0.039$  vs. baseline) (Figure 2.11, Table 2.4). A significant decrease in P-selectin was observed in participants on aspirin 300 mg (median percentage change from baseline was -30.7 % in Group 1;  $p = 0.003$  vs. baseline and  $p = 0.007$  vs. untreated) and aspirin 75 mg (-34.7 % in Group 2;  $p = 0.011$  vs. baseline and  $p = 0.002$  vs. untreated).

Treatment with clopidogrel led to a median change from baseline of P-selectin of 3.3 % ( $p = 0.463$  vs. baseline and  $p = 0.388$  vs. untreated). Conversely, there was a significant rise in P-selectin of 16.71 % ( $p = 0.0335$  vs. baseline and  $p > 0.9999$  vs. untreated) among participants taking ticagrelor, producing similar results to those in Group 5 (untreated). In a combined Spearman's correlation analysis incorporating all study variables, monocyte phenotype changes correlated only with changes in P-selectin. Indeed, the increase in P-selectin values was directly correlated with an increase in CD14<sup>high</sup>CD16<sup>+</sup> cell count ( $r = 0.5$ ;  $p = 0.0003$ ) and a reduction in CD14<sup>high</sup>CD16<sup>-</sup> absolute number ( $r = -0.4$ ;  $p = 0.001$ ) (Figure 2.12). No relationships emerged between P-selectin and any of the other variables analysed in the study population, including baseline characteristics of the participants, serum biochemistry and haematology results and cytokine levels.

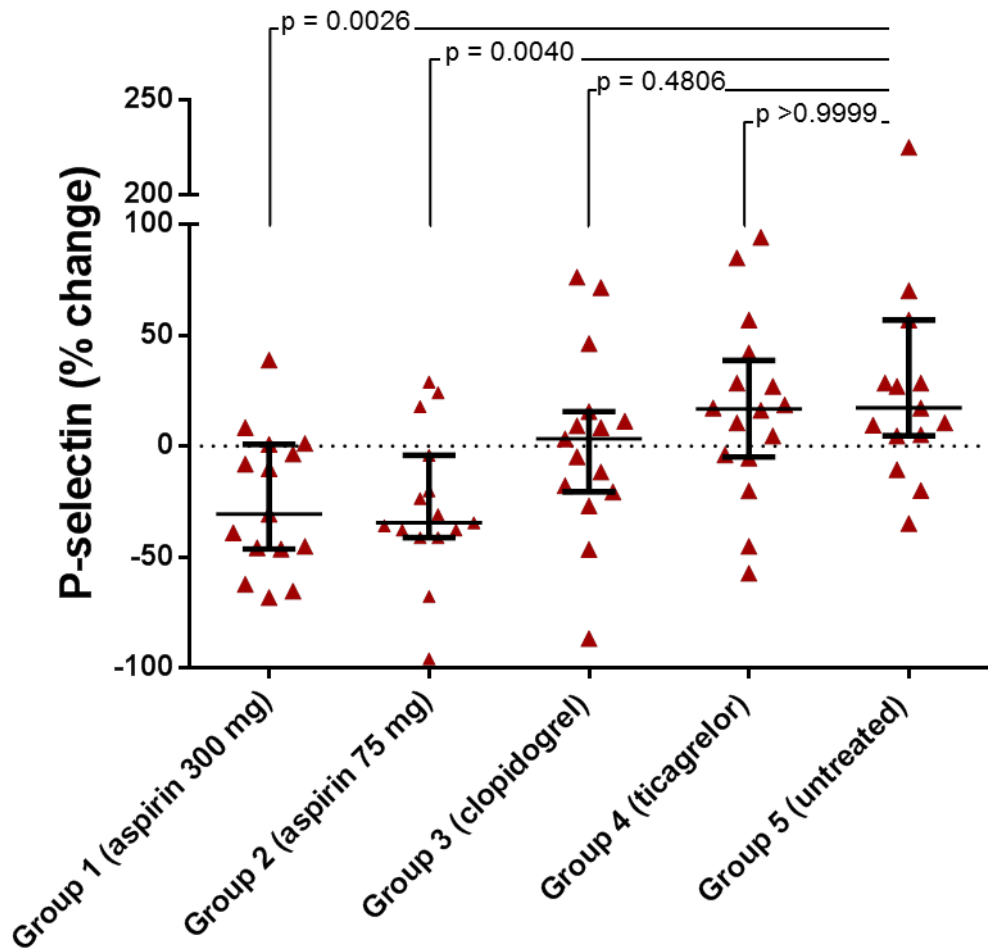
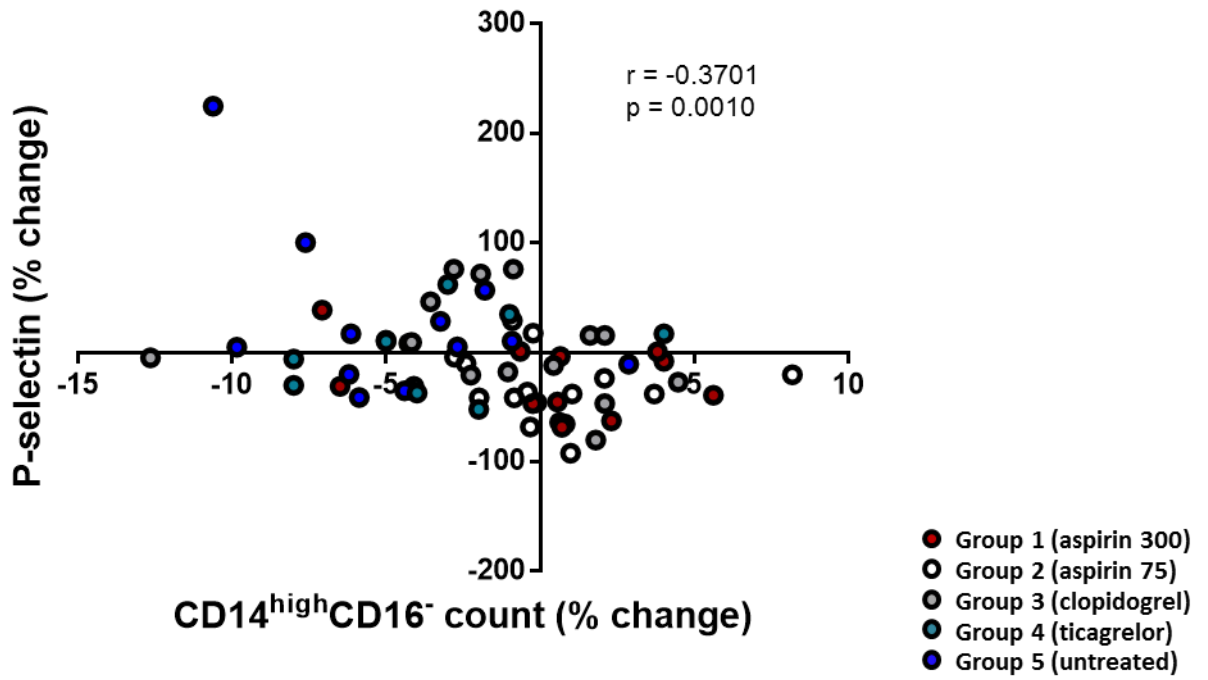


Figure 2.11: Post-immunisation change in P-selectin levels for each group

Figure 2.11 shows the percentage change from baseline of P-selectin in each group. The percentage changes of P-selectin in Groups 1-4 were compared with that of Group 5 (untreated).

A



B

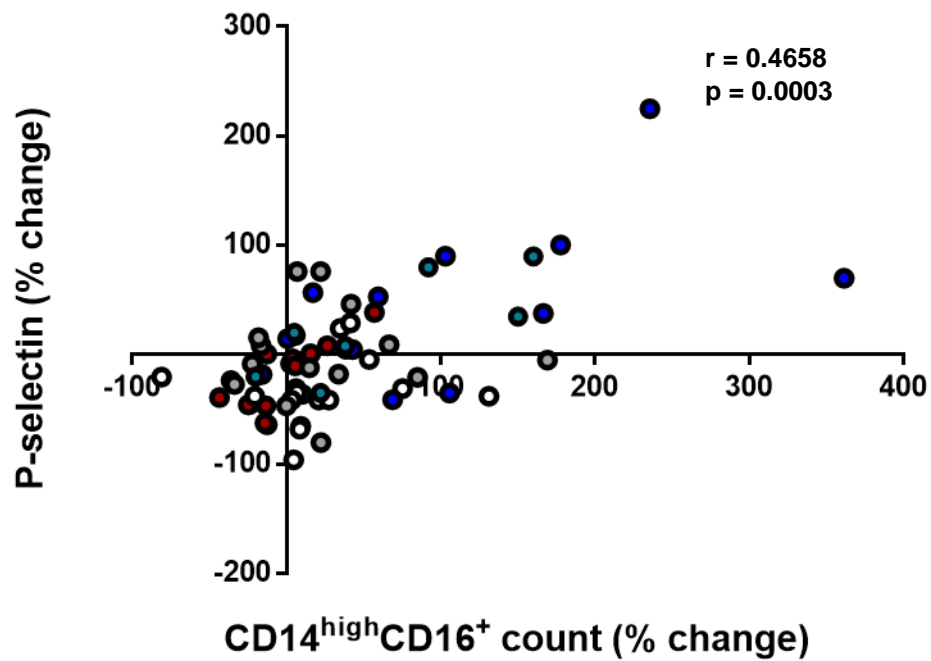


Figure 2.12: Modulation of P-selectin correlates with change in monocyte phenotype

Figure 2.12 shows the correlation between change in P-selectin and change in classical CD14<sup>high</sup>CD16<sup>-</sup> and intermediate CD14<sup>high</sup>CD16<sup>+</sup> monocyte cell count, respectively.

**Table 2.5: P-selectin values pre- and post-immunisation**

		<b>P-selectin (ng/ml)</b>				
		<b>Group 1 (aspirin 300)</b>	<b>Group 2 (aspirin 75)</b>	<b>Group 3 (clopidogrel)</b>	<b>Group 4 (ticagrelor)</b>	<b>Group 5 (untreated)</b>
<b>Baseline</b>		449.0 (135.4 – 761.1)	449.2 (204.7 – 701.8)	415.2 (320.1 – 552.2)	427.7 (331.3 – 654.7)	462.1 (241.2 – 659.7)
<b>Day 2 post vaccination p vs baseline</b>		247.9 (117.5 – 472.2) 0.0027	206.6 (149.8 – 524.1) 0.0105	371.7 (304.6 – 432.2) 0.3625	498.2 (346.9 – 847.8) 0.0335	531.6 (311.3 – 667.9) 0.0392

## **2.5 Discussion**

Atheroma development in humans is accompanied by systemic immunological abnormalities that share homologies with autoimmune diseases<sup>230</sup>. In the pathophysiology of atherosclerosis, the key contributing factors can be grouped into three main phases: (i) accumulation within the artery and in blood of immunogenic self-proteins, i.e. oxLDL derived from exposure to cardiovascular risk factors; (ii) consequent activation of innate immune mediators (primarily myeloid cells, but recent findings also point towards platelets playing a crucial role) through engagement of pattern recognition receptors; and (iii) a subsequent adaptive immunological response involving T and B cells<sup>231</sup>.

In this context, influenza immunisation provides a valuable experimental model to study the dynamics of monocyte phenotype in response to an immunogenic stimulus. In keeping with a previous study by this group<sup>31</sup>, the current data confirm that immunisation generates a reduction in the proportion of 'classical' CD14<sup>high</sup>CD16<sup>-</sup> monocytes, which mirrors the increase in the 'intermediate' CD14<sup>high</sup>CD16<sup>+</sup> subset, in peripheral blood samples from participants not receiving anti-platelet therapy. This shift in the profile of circulating monocytes towards a CD16<sup>+</sup> phenotype mimics the inflammatory response that is characteristically seen in patients with atherosclerosis<sup>22 188 189 224-227</sup>. Whether this shift in monocyte phenotype has functional implications as regards disease progression remains unclear.

It is worth mentioning, however, that CD16 is an important signalling molecule with a key role in the innate response to immunogenic stimuli, in light of its demonstrated involvement in the phagocytosis of IgG-opsonised external particles by myeloid cell types that subsequently stimulates the adaptive immune system<sup>19 232</sup>. Of note, polymorphism of CD16a, which is specifically expressed on monocytes, has been reported to influence susceptibility to, as well as severity of, CAD<sup>20</sup>. Indeed, CD16a genetic variants exhibit distinct affinities for IgG molecules, with consequent effects on the efficiency of clearance of circulating immunogenic / pro-atherogenic factors that would otherwise accumulate within the arterial wall to perpetuate inflammation<sup>20</sup>. On this background, an increase in circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes might

be regarded as a protective anti-atherogenic mechanism. On the other hand, 'intermediate' monocytes are known to more easily infiltrate the arterial wall compared with 'classical' CD14<sup>high</sup>CD16<sup>-</sup> cells thus suggesting a detrimental effect <sup>31</sup>. However, in order to test the pro-atherogenic relevance of 'intermediate' monocytes in human disease, interventional clinical trials would need to be conducted to establish whether therapeutic modulation of circulating monocyte profile impacts on plaque progression. To our knowledge, no such studies have been conducted to date.

The biomolecular mechanisms underlying CD16 up-regulation on circulating monocytes have yet to be established. In the current study we found that induction of CD16 also occurs on PMNs post-immunisation in untreated participants, although the level of soluble CD16b, which is the isoform specifically produced by PMNs <sup>19</sup>, decreased in the peripheral blood. This raises the possibility that expansion of the intermediate monocytic subset may be due to increased fixation of soluble CD16b (derived from PMNs) on the extracellular surface of circulating monocytes, rather than endogenous production of the CD16a isoform. Indeed, monocytes are known to absorb soluble CD16b on their extracellular membrane through engagement of CD11b/CD18 and CD11c/CD18 molecules <sup>233</sup>. Further work is required to characterise the mechanisms underlying CD16 upregulation in myeloid cells and to ascertain the functional consequences of this event in terms of monocyte biology.

The present data are the first to show a therapeutic modulation of the phenotype of circulating monocytes induced by therapies conventionally used in primary and secondary prevention of cardiovascular disease, whose potential effect on plaque progression would therefore merit consideration in future clinical trials. In our study, all the tested anti-platelet regimes, namely the COX-inhibitor aspirin, used at high (300 mg) and low (75 mg) doses, and the P2Y<sub>12</sub> antagonists clopidogrel and ticagrelor, were able to attenuate the expansion of 'intermediate' monocytes in the peripheral blood following immunisation. However, there appeared to be differences in efficacy between these treatments with respect to modulation of the phenotype of circulating monocytes that was found to be linearly correlated with their effectiveness in reducing soluble



P-selectin. We have previously shown that expression of P-selectin on activated platelets, and the consequent formation of MPA, is a key event in the acquisition of CD16 positivity by circulating monocytes<sup>31</sup>. In this current study, I did not perform MPA measurement due to logistical limitations. While the monocytic phenotype remains stable in blood collected in EDTA tubes for up to 4 hours post-venepuncture<sup>234</sup>, immediate blood processing is required for a reliable assessment of MPA in citrated plasma in order to avoid artefactual *in vitro* platelet activation<sup>192</sup>. In this study, plasma separation could be performed soon after blood sampling, while the staining for flow cytometric assays was delayed by 30 min following venepuncture. Hence, soluble P-selectin was chosen as a platelet marker to provide a more reliable evaluation of platelet activity and an indirect measure of monocyte–platelet interaction, particularly in light of prior *in vitro* work conducted by our group that showed a significant rise in soluble P-selectin during MPA formation<sup>31</sup> and recent clinical evidence describing a significant contribution of P-selectin to MPA formation *in vivo*<sup>235</sup>.

The different effects observed between treatments on the level of P-selectin, particularly between the two aspirin groups and the P2Y<sub>12</sub> inhibitors, might be attributed to either a different pharmacological target, i.e. COX-inhibition vs. P2Y<sub>12</sub> antagonism, or different pharmacokinetics. Indeed, aspirin achieves a dose-dependent maximum platelet inhibition within 2 hours post-dose while clopidogrel requires 5 hours to reach a level of platelet inhibition of 60 % following a loading dose of 300 mg<sup>86</sup>. These differences might have played a critical role particularly in a short-term study such as this. As regards to the two different aspirin groups, the slight apparent superiority of the 300 mg dose might be ascribed to dose-dependent inhibition of COX-activity with a consequent anti-inflammatory action greater than with low doses of aspirin (75 mg)<sup>236</sup>. However, aspirin 300 mg did not modify the inflammatory biomarkers measured in this study, consistent with published evidence demonstrating a clinically relevant anti-inflammatory effect of aspirin only occurring at doses of 1 g or greater<sup>237</sup>.

In the large randomised-controlled PLATO trial, ticagrelor was found to be more effective than clopidogrel in reducing the incidence of cardiovascular events and all-cause mortality<sup>122 238</sup>.

Although ticagrelor binds reversibly to the P2Y<sub>12</sub> receptor and has a plasma half-life of only around 7 hours, it has previously been proven to be highly effective at inhibiting platelet activation<sup>239</sup>. In our study, however, administration of ticagrelor did not achieve effective suppression of P-selectin, and indeed led to similar post-immunisation levels as in the untreated participants. The lack of efficacy of ticagrelor here may be related to the fact that we did not administer a loading dose (typically one dose of 180 mg, followed by maintenance 90 mg doses every 12 hours) in order to avoid further complicating the dosing regimen for this group, who already had a relatively high pill burden compared to the other groups. In retrospect, this may be responsible for the comparatively poor P-selectin inhibition observed in participants within this group, as it is likely that the 90 mg ticagrelor dose resulted in a slower onset of action and reduced inhibition of platelet activation stimulated by ADP compared with the loading 300 mg dose of clopidogrel.

Overall, this evidence points towards platelet activity as the main determinant of monocyte phenotype shift in these results; this is particularly supported by the direct relationship found between amplitude of P-selectin reduction and change in monocyte phenotype in response to anti-platelet therapy. Similarity between the two groups of aspirin appears of particular relevance in light of the fact that aspirin 75 mg daily is the dose currently recommended for cardiovascular prevention, and no superior antithrombotic efficacy has been reported for aspirin 300 mg daily<sup>240-245</sup>.

The lack of activity of anti-platelet therapy on the non-specific marker of inflammation, hs-CRP, suggests a targeted immunomodulatory action. Regarding production of pro-inflammatory cytokines, the phenotype of circulating monocytes did not influence the levels of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 in our study participants, at least when measured 48 hours post-immunisation. There were no significant differences between their baseline and post-immunisation values in any of the groups. This is consistent with previously published reports showing an increase in hs-CRP in healthy subjects 2 days post-influenza immunisation that can be partly ascribed to an early rise in IL-6, but not TNF- $\alpha$ , 1 day post-immunisation<sup>246,247</sup>. We cannot therefore exclude a change

in levels of inflammatory cytokines among groups at time points other than those analysed in the current study, particularly as a rise in IL-6 would be expected to precede the hs-CRP elevation. Due to logistical difficulties that resulted in a delay of up to 3 hours between taking blood samples and obtaining serum from these, levels of cytokines, and indeed P-selectin, may have been affected. Also, the effect of treatment on monocyte distribution was not analysed beyond 48 hours post-immunisation; thus, the total duration of this effect remains to be established.

## **2.6: Conclusion**

I have demonstrated in this proof-of-concept study that anti-platelet therapy can attenuate the development of a CD16<sup>+</sup> profile by circulating monocytes under pro-inflammatory conditions. Modulation of P-selectin levels seems to be a principal factor in determining the extent of this pharmacological action on monocytes, possibly linked to an interference in MPA formation; however, this remains to be confirmed. Further work is needed to better understand the underlying biomolecular mechanisms. A limitation of this study was the lack of inclusion of additional platelet biomarkers and functional assays, since these may have highlighted modulatory actions of anti-platelet drugs on specific platelet-dependent pathways. Further research is also needed to clarify whether the effects observed here with anti-platelet therapy translate to clinically useful therapeutic effects on atherosclerosis progression.

**CHAPTER THREE:**

**NETRIN-1 – A POTENTIAL CARDIOVASCULAR**

**TARGET**

### **3.1 Introduction**

Netrins are a class of laminin-like proteins, which were initially isolated within the central nervous system and identified as regulators of embryonic axonal guidance. Netrin-1, by far the best-characterised member of the group, has subsequently been found to have a wide spectrum of regulatory roles in numerous pathological conditions, given its broad expression in inflammatory, vascular and tumour cell types and ability to control their survival, apoptosis and migration. Netrin-1 has become particularly relevant within the field of oncology, where it has been shown to be of both diagnostic and prognostic value in many cancer subtypes<sup>248 249</sup>, and there is much interest in the development of therapeutic antibodies that interfere with netrin-1-dependent pathways as a potential novel chemotherapeutic approach<sup>250</sup>. Netrin-1 is also emerging as a therapeutic target in cardiovascular disease<sup>222</sup>, having been shown in pre-clinical studies to modulate atherogenesis via the control of arterial inflammation<sup>209</sup>, as well as exerting cardio- and renoprotective actions<sup>251 252</sup>, and in this context, enhancing netrin-1 signalling may be desirable.

To date, there has been limited characterisation of the *in vivo* pathways that regulate netrin-1 expression in humans, limiting the development of targeted therapeutic strategies to achieve either a stimulatory or an inhibitory effect on the synthesis of this important molecule in the clinical setting.

*In vitro* experiments in tumour cells have demonstrated that netrin-1 transcription is controlled by the NF- $\kappa$ B and that NF- $\kappa$ B-induced netrin-1 overexpression enhances cell survival<sup>221</sup>. Subsequently, the existence of two distinct isoforms of netrin-1 has been reported, namely a nuclear truncated protein that promotes cell survival and a full-length isoform that undergoes secretion and induces proliferation<sup>213</sup>.

Previous work on the vascular endothelium by our group has shown that NF- $\kappa$ B activation solely enhances expression of the nuclear isoform of netrin-1. On the contrary, pro-atherogenic vascular damage reduces the secretion of the endothelial-derived full-length isoform of netrin-1 that protects against arterial inflammation by repelling myeloid cells and their plaque

infiltration<sup>203</sup>. Administration of aspirin in a murine model of atherosclerosis counteracted the downregulation of secreted netrin-1 leading to a beneficial action in terms of plaque lipid content reduction. These experimental findings preceded the recently published clinical evidence showing reduced plasma levels of netrin-1 in patients with type 2 diabetes mellitus, thus suggesting that systemic pro-atherogenic inflammation downregulates circulating netrin-1 in patients with cardiovascular disease, possibly via a detrimental effect on the vascular endothelium<sup>253</sup>.

In the current study, I investigated the effect of systemic inflammation on the circulating level of netrin-1 in relation to vascular function, and explored the therapeutic potential of aspirin in modulating netrin-1 *in vivo* in healthy subjects.

### **3.2 Hypothesis and aim**

It has previously been demonstrated in animal models that aspirin preserves the synthesis of endothelial-derived netrin-1 under pro-inflammatory / pro-atherogenic conditions, thus reducing arterial inflammation. I explored the effect of aspirin on circulating netrin-1 levels in healthy volunteers and in the presence of endothelial dysfunction to test the hypothesis that anti-platelet therapy modulates netrin-1 levels in the presence of systemic inflammation.

### **3.3 Methods**

#### **3.3.1 Participant recruitment**

This was a *post hoc* study conducted on samples from 76 healthy volunteers previously recruited at Guy's and St Thomas' NHS Foundation Trust who underwent two separate clinical studies, and for whom serum samples were still available. All participants were aged 18 years or older (median: 31 years; IQR 25 - 38), had no significant past medical history, were not taking regular medications (aside from the combined oral contraceptive pill), and had not taken anti-platelet or anti-inflammatory drugs in addition to the study medications described below.

Cohort One comprised 36 participants from the clinical study described in Chapter Two of this thesis, wherein influenza immunisation was used as an experimental model of mild inflammation to evaluate the effect of different anti-platelet regimes on a number of inflammatory biomarkers, including cytokines, monocyte phenotype, and hs-CRP <sup>18</sup>. Serum samples were collected before and 48 hours after receiving the seasonal influenza vaccine, and in the presence of one of the following treatment regimens:

Group 1 aspirin 300 mg once daily (n = 9)

Group 2: aspirin 75 mg once daily (n = 9)

Group 3: clopidogrel with an initial loading dose of 300 mg followed by a further dose of 75 mg 24 hours later (n = 9)

Group 4: untreated participants (n = 9; serving as controls)

We did not include data from participants who had been treated with ticagrelor in the original study, due to relatively poor platelet inhibition with this anti-platelet agent, which was demonstrated by an inability to suppress P-selectin levels in response to inflammation (see Chapter Two).

Cohort Two comprised a separate group of 40 healthy volunteers. Participants were previously recruited into a clinical study which set out to identify novel biomarkers of aspirin resistance following 28 days of treatment with aspirin 300 mg once daily <sup>254</sup>. Table 3.1 shows the pooled baseline characteristics of all participants from Cohorts One and Two.

The clinical studies into which the participants were recruited had been reviewed and given favourable opinion by the NRES London—Dulwich Research Ethics Committee (ref. number 13/LO/1664; South London network study identification number 16644) and the Riverside Research Ethics Committee, London, UK (ref. number 07/Q0401/1) and registered on the UK Clinical Research Network Portfolio. All participants gave informed consent. The studies were performed conforming to the Declaration of Helsinki.

### **3.3.2 Collection of serum**

For Cohort One participants, 12 ml serum was collected using methods described in Chapter Two. For Cohort Two participants, 7 ml whole blood was collected into a tube containing a serum gel separator and 10 µg/ml indomethacin was added to the tubes to inhibit further activity of the COX enzymes. The whole blood collection and preparation for Cohort Two subjects was performed by Dr Timothy Goodman, whilst I collected and prepared all whole blood samples for Cohort One.

### **3.3.3 Enzyme-linked immunosorbent assays**

ELISAs were carried out to measure serum and urine levels of netrin-1 using commercially available kits (SEB827Hu, Cloud-Clone Corp.), as per manufacturer instructions (inter-assay variability was found to be <5 %).

Briefly, 100 µl netrin-1 standards (0 - 2000 pg/ml) and neat serum or urine samples were added in duplicate to a 96-multi-well plate pre-coated with an antibody specific to netrin-1. Following incubation for 2 hours at 37 °C, 100 µl biotin-conjugated polyclonal antibody specific for netrin-1 was added to each well and the plate was incubated for 1 hour at 37 °C. The plates were washed and subsequently incubated with 100 µl avidin conjugated to horseradish peroxidase for 30 minutes. The plates were washed again and 90 µl TMB substrate was added to the wells. After 10 minutes, 50 µl 0.16 M sulphuric acid was added to the wells to stop further colour change. The plates were analysed using a microplate optical density reader at 450 nm wavelength. A standard curve was constructed by plotting the optical densities of the standards against the corresponding known netrin-1 concentrations. Serum netrin-1 concentrations in the different samples were read from the standard curve on the basis of their optical density readings; Figure 3.1.

ELISA kits were also used to measure serum levels of PGE<sub>2</sub> (ID: MBS007171, MyBioSource, Inc., USA), TXB<sub>2</sub> (ID: CSB-E08046h, Cusabio, China), pepsinogen I (ID: CSB-E17538h; Cusabio, China), renin (ID: E-EL-H0119, Elabscience), VCAM-1 (ID: DVC00, R&D Systems, UK), ICAM-1 (ID:

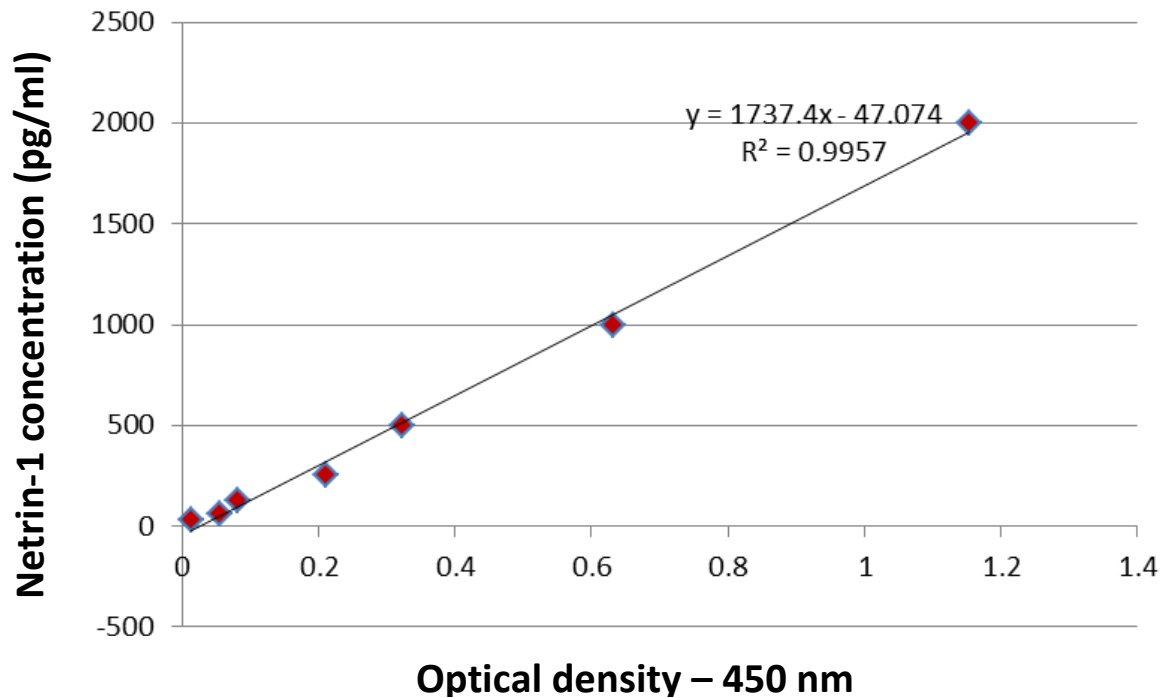


850.540.096, Diacclone, France), myeloperoxidase (MPO; ID: BMS2038INST; eBioscience, Austria), and E-selectin (ID: CSB-E04540h, Cusabio, China), in addition to urinary levels of cysteinyl leukotriene (ID: 10009291; Cayman Chemical Company, USA) using a similar technique to that stated above, with variations based on manufacturer instructions. Urinary TXB<sub>2</sub> levels were measured for Cohort 2 participants by Dr Tim Goodman, using a commercially available kit (ID: 501020; Caymen Chemical Company, USA).

	Gender (n)	Age (years)	Systolic blood pressure (mmHg)	BMI (kg/m <sup>2</sup> )	HDL cholesterol (mmol/l)	LDL cholesterol (mmol/l)	Triglycerides (mmol/l)	Hs-CRP (mg/l)
<b>Group 1: aspirin 300</b> <b>n = 9</b>	3 male 6 female	32.00 (30.00 - 37.00)	119 ± 6	23.114 (20.91 - 26.50)	1.831 ± 0.09	2.74 (2.39 - 3.50)	1.09 (0.81 - 1.59)	0.45 (0.0 - 0.90)
<b>Group 2: aspirin 75</b> <b>n = 9</b>	4 male 5 female	33.00 (29.99 - 41.00)	123 ± 6	25.26 (22.18 - 28.13)	1.774 ± 0.13	2.73 (2.22 - 3.38)	0.99 (0.69 - 1.57)	0.50 (0.40 - 1.00)
<b>Group 3: clopidogrel</b> <b>n = 9</b>	3 male 6 female	33.50 (32.00 - 43.75)	125 ± 4	25.00 (20.43 - 28.67)	1.707 ± 0.10	2.49 (2.26 - 3.14)	0.93 (0.77 - 1.53)	0.60 (0.20 - 0.93)
<b>Group 4: untreated</b> <b>n = 9</b>	4 male 5 female	37.00 (32.00 - 42.00)	124 ± 4	24.11 (21.73 - 28.69)	1.661 ± 0.09	2.52 (2.06 - 3.32)	1.06 (0.73 - 1.64)	0.45 (0.28 - 0.65)
<b>Unvaccinated group</b> <b>n = 40</b>	16 male 24 female	32.75 (30.25 - 39.75)	122 ± 5	22.80 (20.78 - 26.14)	1.880 ± 0.09	2.41 (2.17 - 3.01)	1.01 (0.78 - 1.33)	0.77 (0.34 - 1.60)

**Table 3.1: Baseline characteristics of study population**

Biometric data and cardiovascular risk profiles for the subjects in all groups are shown. Values are expressed as the mean ± SEM or median (IQR). BMI: body mass index; HDL: high density lipoprotein; LDL: low density lipoprotein; hs-CRP: high-sensitivity C-reactive protein



**Figure 3.1: A representative standard curve from the netrin-1 enzyme-linked immunosorbent assay**

The optical density reading (at 450 nm) was plotted against the netrin-1 concentration to obtain a standard curve, from which sample concentrations were inferred.

### **3.3.4 Serum biochemistry**

Methods for measuring serum renal profiles and lipid levels were described in Chapter Two of this thesis. Hs-CRP was measured by Quintiles Drug Research Unit laboratories, London.

### **3.3.5 Measurement of serum salicylate levels**

Serum salicylate levels were measured by Dr Timothy Goodman and Professor Anthony Wierzbicki using a Roche Cobas Fara automated analyser at St Thomas' Hospital, London. 5 µl of calibrator, control, blank (double-distilled water) or sample was added to a cuvette. Next 75 µl

nicotinamide adenine dinucleotide reagent was added to each sample or blank. The reaction was then activated through the addition of 150 µl salicylate hydroxylase and the absorbance at 340 nm of each reaction mixture was measured.

### **3.3.6 Calculation of creatinine clearance**

The Cockcroft-Gault equation,  $((140 - \text{age in years}) \times (\text{weight in kg}) \times (0.85 \text{ if female})) / (72 \times \text{creatinine in } \mu\text{mol/l})$  was used to calculate creatinine clearance.

### **3.3.7 Western blotting**

Western blotting of serum samples was performed with both rat anti-netrin-1 and goat anti-netrin-1 primary antibodies as previously described<sup>203</sup>. Prior to use, serum was treated, as per previously reported methodology<sup>255</sup>, for lipid, IgG and albumin depletion. Briefly, samples were centrifuged for 15 minutes at 15000 x g at room temperature to remove the lipid component; IgG depletion was subsequently performed on the delipidated serum with a Protein G Sepharose bead suspension (10278424, GE Healthcare Ltd, UK), and was followed by centrifugation in cold ethanol to remove the albumin-rich serum fraction<sup>255</sup>.

Proteins were subsequently re-suspended in radioimmunoprecipitation assay buffer and their concentration measured by bicinchoninic acid assay; section 3.3.8. 10 mcg of each protein sample was separated on a SDS-PAGE gel (10 % acrylamide), and transferred to a polyvinylidene difluoride membrane. After 1 hour blocking in phosphate buffer saline containing 5 % milk / 0.1 % Tween-20, the membranes were probed with either rat anti-netrin-1 or goat anti-netrin-1 antibodies (both 1:100 in blocking solution; R&D System), for 2 hours at room temperature. After washing, membranes were incubated with goat anti-rat or donkey anti-goat secondary antibodies as appropriate (1:2000; Cell Signalling, UK). Bands were detected with enhanced chemiluminescence reagent on Hyperfilm (Amersham Biosciences, UK).

### 3.3.8 Bicinchoninic acid protein assay

BSA standards were prepared from 2 mg/ml solution (ID: 500-0206, Bio-Rad) as follows:

BSA mg/ml	0.0	0.1	0.3	0.6	0.9	1.2	1.5	1.8	2.0
Volume of 2 mg/ml stock ( $\mu$ l)	0	25	75	150	225	300	375	450	400
Volume of RIPA buffer	500	475	425	350	275	200	125	50	0

10  $\mu$ l of either standards or serum samples were added to each well of a 96-well plate. Bicinchoninic acid working solution was prepared by combining Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) and Reagent B (4 % cupric sulphate) in a 50:1 ratio (reagents from Thermo Scientific), and 200  $\mu$ l of this was pipetted into each well. The plate was incubated for 30 minutes at 37 °C and then analysed using a microplate optical density reader at 490 nm wavelength. A standard curve was constructed and the protein concentrations from the serum samples were calculated using this.

### 3.3.9 Whole blood flow cytometry

Whole blood was immunostained using the technique described in Chapter 2, with a peridinin-chlorophyll-protein complex-cyanine 5 (PerCP-Cy5) goat anti-rat netrin 1 antibody (2BScientific, UK), in addition to the aforementioned PE-mouse anti-human CD14 and FITC-conjugated anti-human CD16 antibodies.

The samples were analysed using a BD FACSCalibur (BD Bioscience) flow cytometer to determine monocyte subset distribution. A total of 100,000 events were acquired and post-acquisition analysis was performed using FlowJo (version 10) software. Monocytes were identified on an FSC versus SSC plot and gated to distinguish the different subsets of monocytes based on CD14 and CD16 surface expression, and to identify whether netrin-1 was expressed on the monocytic cell surface.

### **3.3.10 Measurement of netrin-1 levels *in vitro***

Human umbilical vein endothelial cells (HUVEC) were isolated by Dr Virginia Tajadura-Ortega from umbilical cords donated by healthy mothers following uncomplicated deliveries using previously described methods<sup>256</sup>. HUVEC were incubated with PGE<sub>2</sub> (ID: 14010; Caymen Chemical Company, USA) re-suspended in dimethyl sulfoxide (DMSO) at concentrations of 10 nm and 100 nm for 4 hours and for 24 hours by Dr Virginia Tajadura-Ortega. HUVEC incubated with equivalent volumes of DMSO were used as control experiments. I subsequently measured netrin-1 levels in the cell supernatant from these experiments using an ELISA kit, as described in Section 3.3.3.

### **3.3.11 Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 6.0) software. Parametric data are expressed as the mean  $\pm$  SEM, whilst non-parametric data are expressed as the median with IQR. ANOVA was used to compare percentage variation of each study variable between the 4 treatment groups in the influenza immunisation study. Baseline and post-treatment values were compared within each group using a paired parametric or non-parametric test as appropriate. Netrin-1 correlation with the other study variables was analysed by Spearman correlation test (since netrin-1 was not normally distributed). A *p* value of <0.05 was taken as statistically significant.

## **3.4 Results**

### **3.4.1 Circulating levels of netrin-1 are reduced in the presence of endothelial dysfunction**

Influenza immunisation was previously reported to induce vascular dysfunction in healthy volunteers<sup>27 32</sup>. In accordance with these data, our study showed an increase in the serum level of VCAM-1, which rose from a baseline level of  $493.70 \pm 44.34$  ng/ml to  $542.30 \pm 49.60$  ng/ml post-immunisation in participants not treated with anti-platelet medication (Group 4; *p* = 0.0022 vs baseline); Figure 3.2. Levels of PGE<sub>2</sub>, which is known to have a protective effect on the vascular

endothelium under steady state conditions<sup>257-260</sup>, fell from  $320.90 \pm 55.04$  pg/ml to  $189.50 \pm 30.37$  pg/ml in the same group of participants ( $p = 0.0199$  vs baseline); Figures 3.3 – 3.4. Similarly, there was a significant reduction of 29.25 % in the circulating level of netrin-1, from  $311.90$  (IQR  $248.00 – 316.30$ ) pg/ml to  $220.20$  (IQR  $202.20 – 287.70$ ) pg/ml at baseline and post-immunisation respectively,  $p = 0.0017$ ; Figures 3.3 – 3.4.

All anti-platelet treatment regimens suppressed this rise in VCAM-1, with the highest degree of suppression observed in those taking the daily 300 mg dose of aspirin, although there were no statistically significant differences in efficacy between the treatment groups (Figure 3.2). The reduction in PGE<sub>2</sub> levels post-immunisation was attenuated by aspirin treatment, whilst in the clopidogrel-treated group a tendency towards an increase in PGE<sub>2</sub> was observed. The changes in netrin-1 in response to immunisation followed the same pattern as the PGE<sub>2</sub> changes in all three treatment arms. An increase in the level of netrin-1 reaching statistical significance was observed in the clopidogrel group (20.96 % change from baseline;  $p = 0.0033$  vs untreated), whilst aspirin counteracted the suppression of netrin-1 post-immunisation in what appeared to be a dose-dependent manner (% change from baseline in Group 1 and Group 2 were -3.06 and -17.03 respectively;  $p = 0.0465$  and  $p > 0.05$  vs untreated; Figure 3.3)

Levels of TXB<sub>2</sub>, a marker of platelet activation and aggregation, fell significantly from  $3416.0 \pm 285.7$  ng/ml at baseline to  $3130.0 \pm 244.0$  ng/ml post-immunisation in the group receiving aspirin 300 mg daily ( $p = 0.0296$ ). There was an apparent reduction in TXB<sub>2</sub> also in those participants on aspirin 75 mg daily, however this did not reach statistical significance ( $p = 0.2103$ ). Post-immunisation TXB<sub>2</sub> levels remained stable in the participants not treated with anti-platelet agents and in those taking clopidogrel; Figure 3.5. Of note, these TXB<sub>2</sub> measurements were higher than previously reported TXB<sub>2</sub> levels in healthy volunteers (16785341, 22098110), possibly due to a manufacturing issue with the standards used for the ELISA.

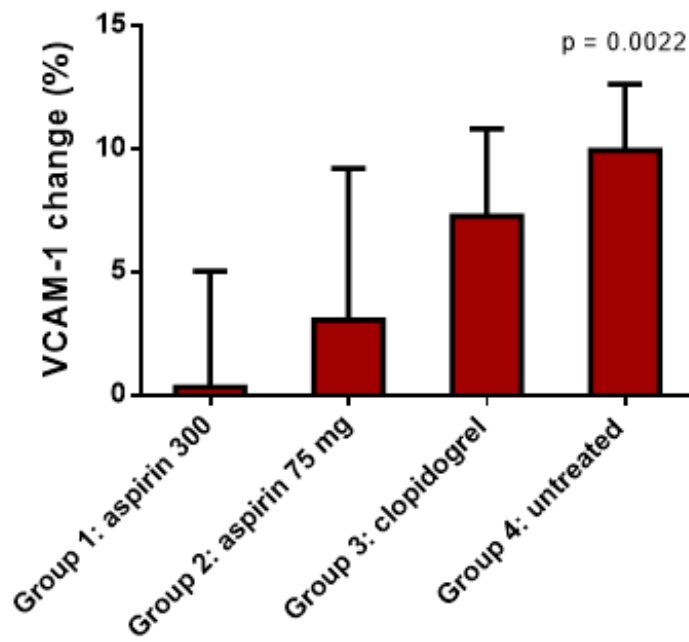
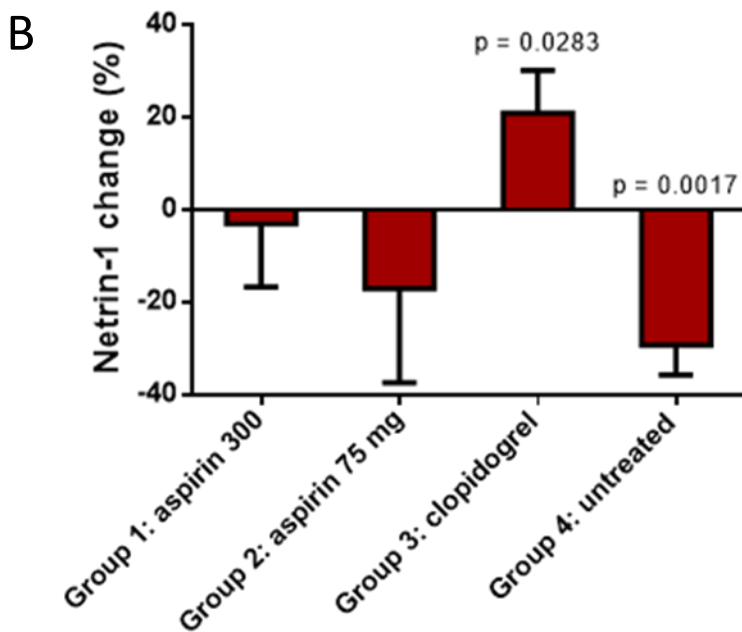
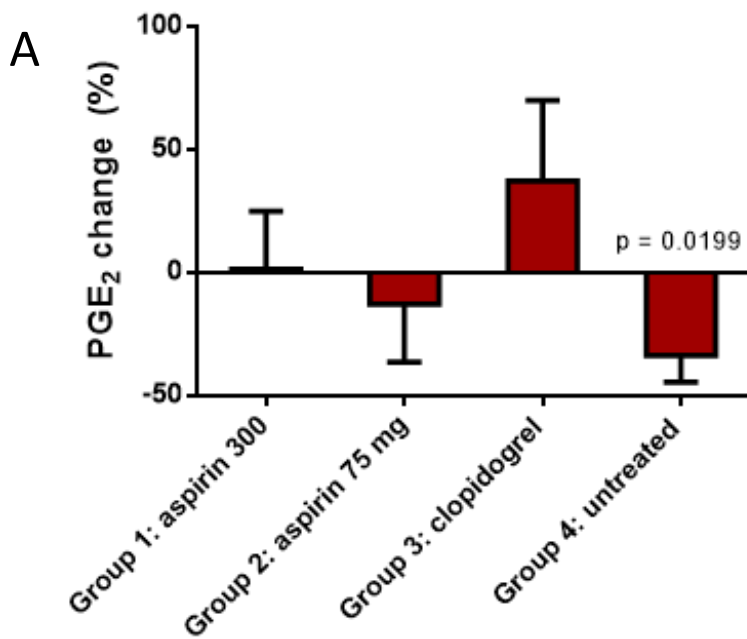


Figure 3.2: Percentage change in vascular cell adhesion protein 1-1 post-immunisation

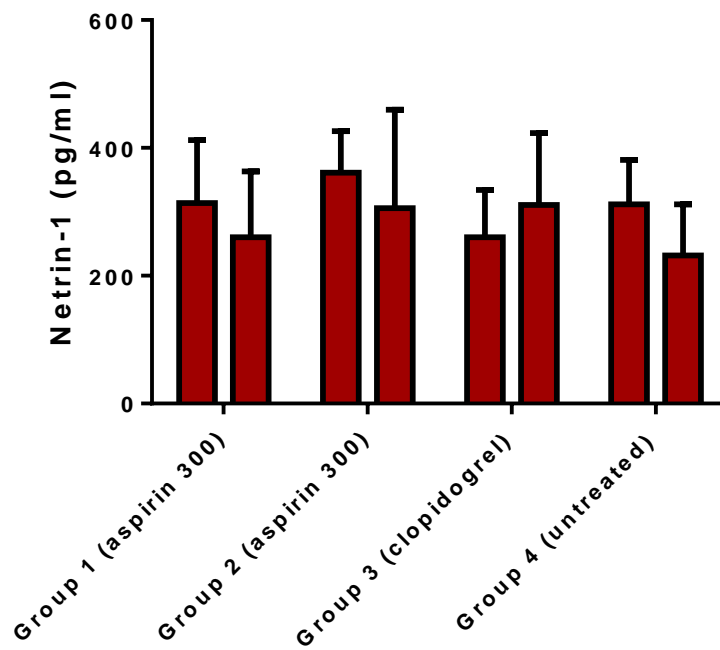
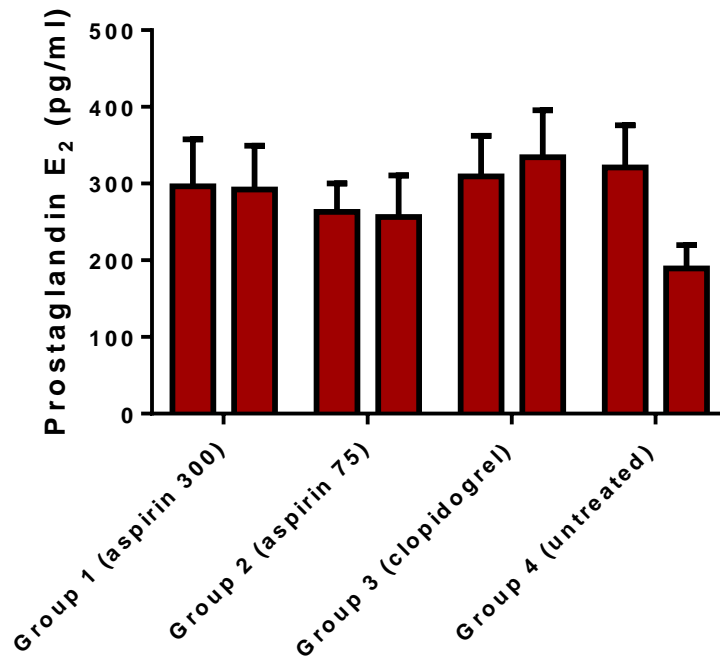
Graph to show the percentage change in VCAM-1 levels across groups.





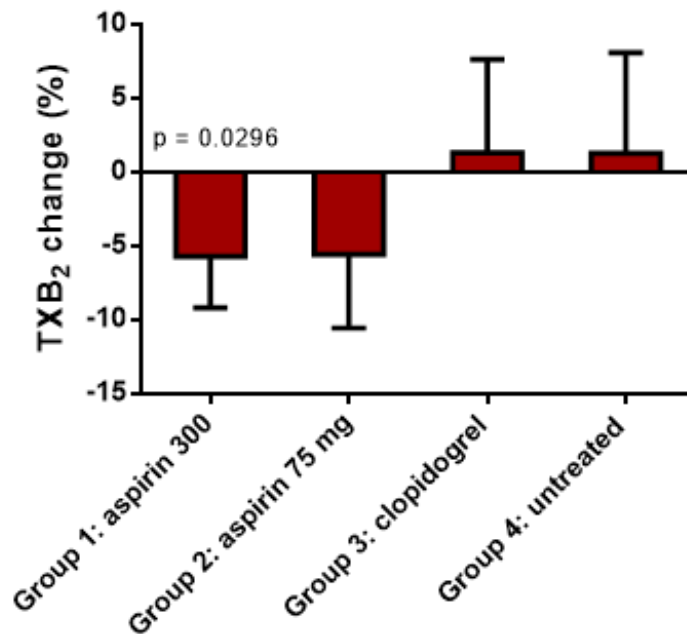
**Figure 3.3: Percentage change in prostaglandin E<sub>2</sub> and netrin-1 post-immunisation**

Panels A and B show the percentage change in PGE<sub>2</sub> and netrin-1 levels respectively across groups. P values are stated when the difference between pre- vs post-immunisation data is significant.



**Figure 3.4: Prostaglandin E<sub>2</sub> and netrin-1 absolute values pre- and post-immunisation**

Panels A and B show the absolute values of PGE<sub>2</sub> and netrin-1 levels respectively pre- and post-immunisation.



**Figure 3.5: Percentage change in thromboxane B<sub>2</sub> post-immunisation**

Graph to show the percentage change in TXB<sub>2</sub> levels across groups.

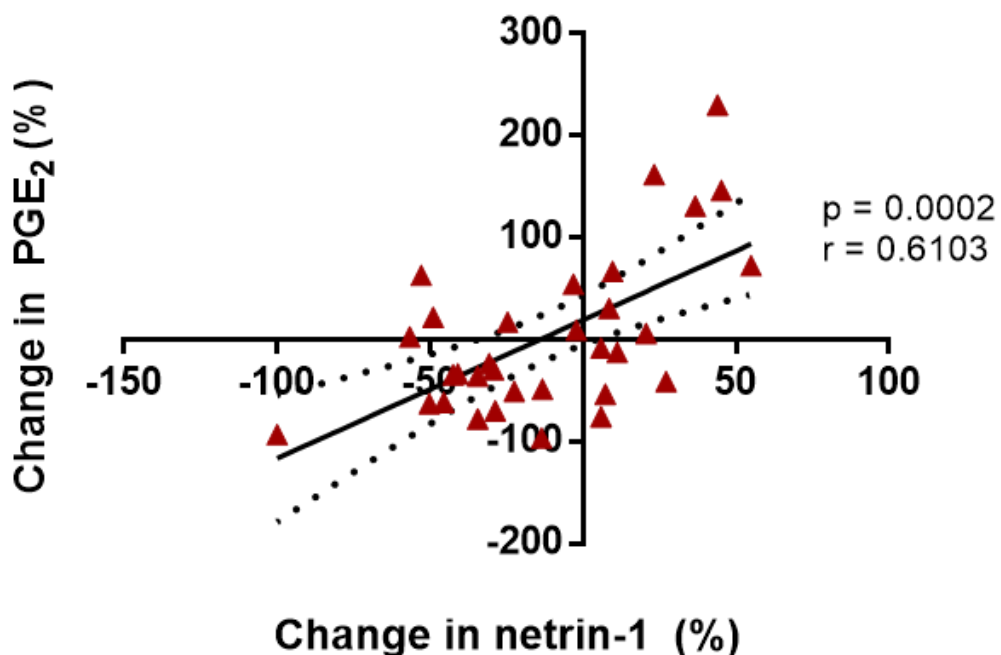
### 3.4.2 Netrin-1 levels are directly related to prostaglandin E<sub>2</sub> production

In a multiple regression analysis of the correlation between netrin-1 levels and all other study parameters, including TXB<sub>2</sub> and PGE<sub>2</sub>, as well as the previously measured hs-CRP, cytokines, P-selectin levels and monocyte cell subset count, I found that changes in netrin-1 levels in response to immunisation were linearly and directly related with changes in PGE<sub>2</sub> concentration only ( $r=0.6103$ ;  $p=0.0002$ ; Figure 3.6). No additional significant findings emerged.

In order to further delineate the regulatory relationship between PGE<sub>2</sub> and netrin-1 synthesis, I measured netrin-1 levels in Cohort Two – healthy volunteers following once daily treatment with 300 mg aspirin, which is known to exert an inhibitory action on both COX isoforms when used at this dose, for 28 days. Drug treatment in this cohort, as expected, led to a significant reduction

in both TXB<sub>2</sub> and PGE<sub>2</sub> levels (Figure 3.7). This latter fell from 331.9 ± 69.04 pg/ml at baseline to 89.99 ± 34.29 pg/ml post-aspirin therapy ( $p = 0.0114$ ).

There was a mild but consistent reduction in serum netrin-1 from a baseline level of 303.9 (IQR: 218.4 – 386.0) pg/ml to 246.9 (IQR: 193.2 – 316.2) pg/ml post-aspirin therapy in all study participants ( $p = 0.0012$ ), Fig 3.8. When combining baseline and post-treatment values of all study variables, a strong positive correlation was found between netrin-1 and PGE<sub>2</sub> levels ( $r = 0.3584$ ;  $p = 0.0015$ ) only; Figure 3.9. The percentage change in netrin-1 was found to be negatively correlated with serum salicylate levels ( $r = -0.5370$ ;  $p = 0.0100$ ), indicating that the reduction in netrin-1 was causally related to aspirin therapy; Figure 3.10. Of note, there was a wide variation in salicylate levels between participants, likely representing poor adherence with aspirin therapy in some individuals.



**Figure 3.6: Correlation between percentage change in prostaglandin E<sub>2</sub> and netrin-1 levels post-immunisation**

**There was a positive correlation between the percentage change in netrin-1 and PGE<sub>2</sub> levels**

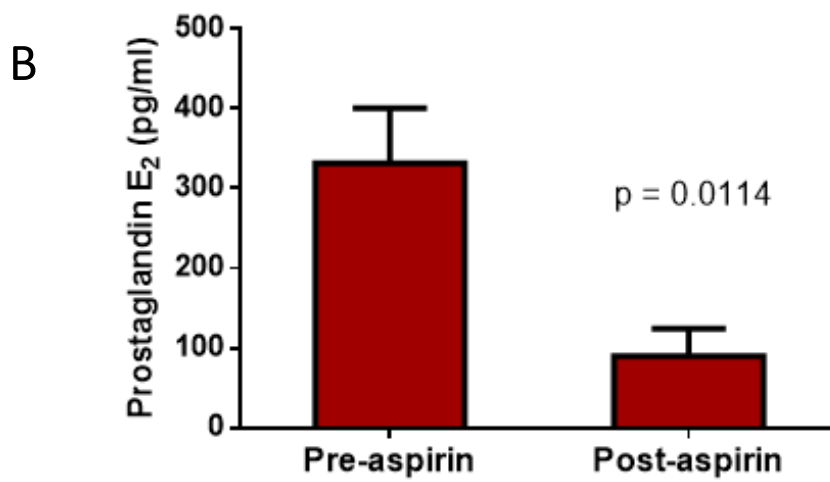
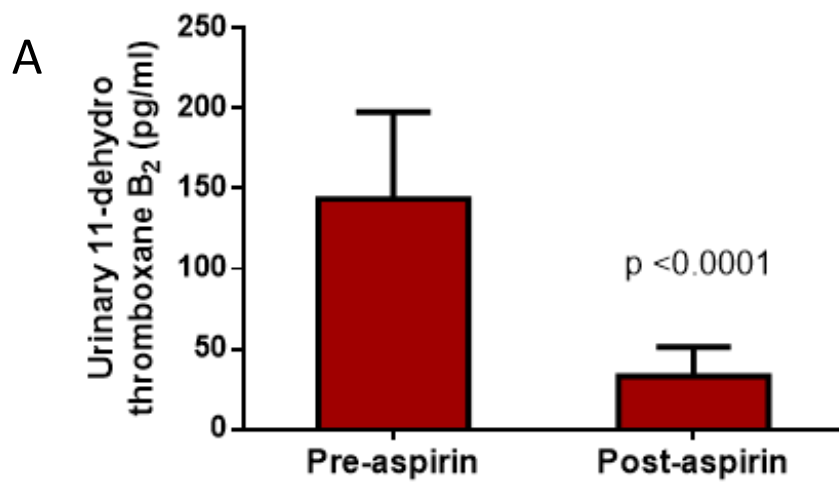


Figure 3.7: Thromboxane B<sub>2</sub> and prostaglandin E<sub>2</sub> levels pre- and post-aspirin

Graphs showing the reduction in both TXB<sub>2</sub> (A) and PGE<sub>2</sub> (B) following 28 days of treatment with aspirin 300 mg once daily.

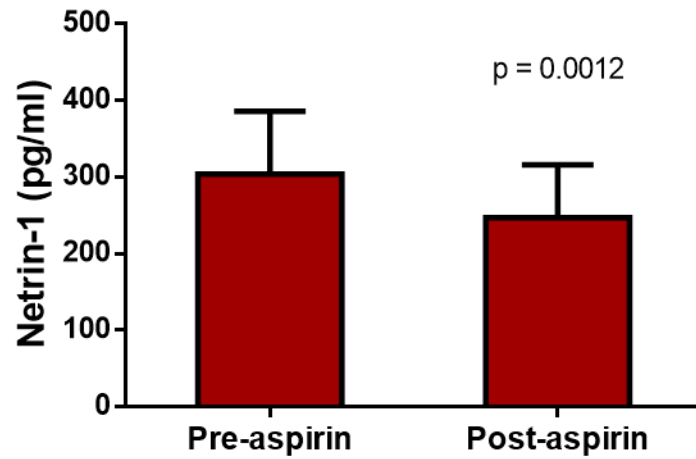


Figure 3.8: Serum netrin-1 levels pre- and post-aspirin

Graph showing the reduction netrin-1 levels following 28 days of treatment with aspirin 300 mg once daily.

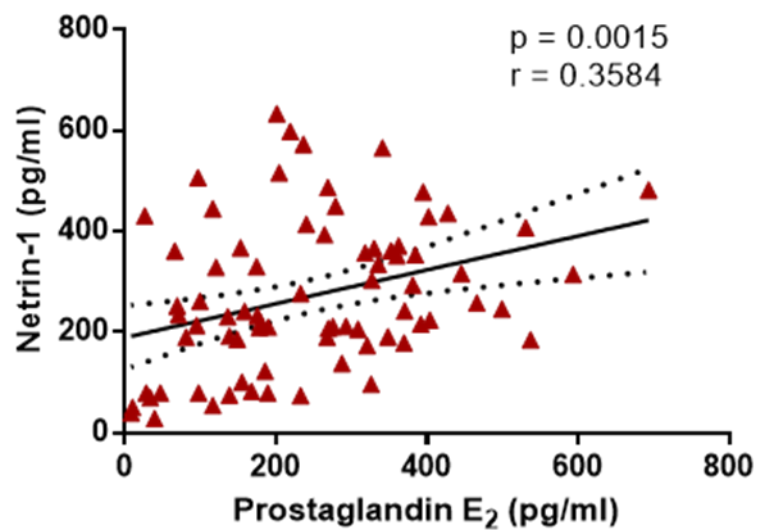
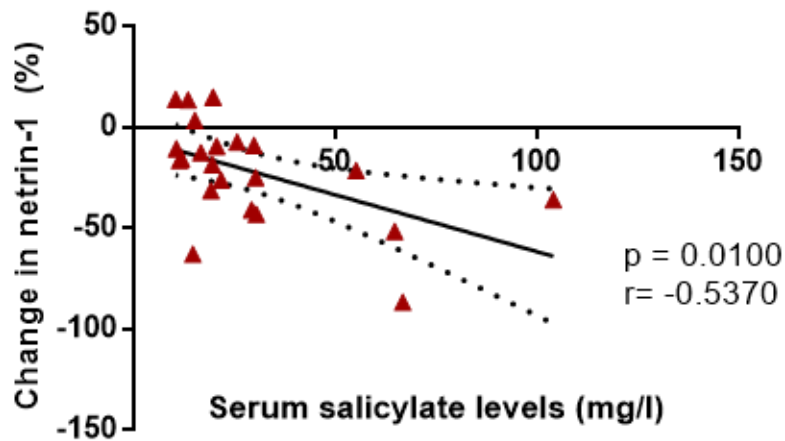


Figure 3.9: Correlation between netrin-1 and prostaglandin E<sub>2</sub> levels

There was a positive correlation between netrin-1 and PGE<sub>2</sub> levels.



**Figure 3.10: Correlation between percentage change in netrin-1 levels post-immunisation and serum salicylate levels**

The reduction in netrin-1 was associated with increased serum salicylate levels.

### **3.4.3 Aspirin-induced inflammatory changes are not responsible for the observed reduction in netrin-1 levels**

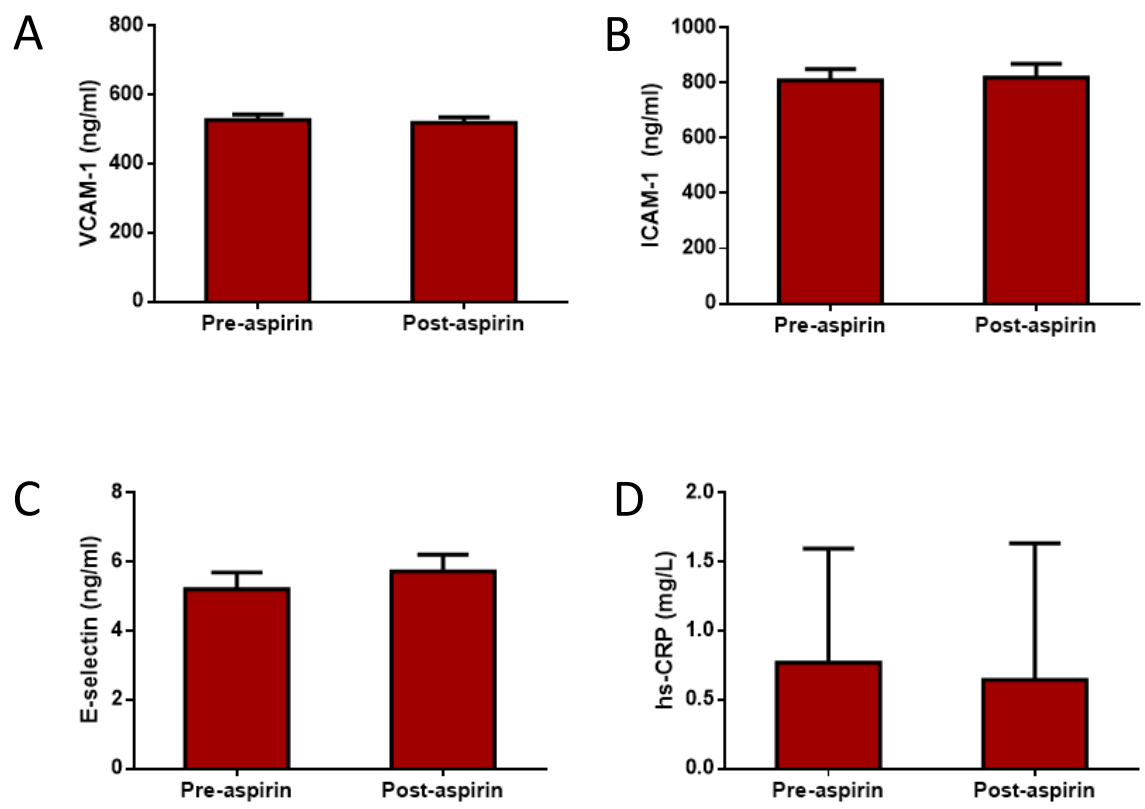
Serum VCAM-1 remained unchanged from baseline levels of  $527.32 \pm 15.46$  ng/ml to  $519.10 \pm 15.76$  ng/ml post-aspirin. ICAM-1 and E-selectin were also unmodified, indicating that there was no change in the inflammatory status of the endothelium in response to aspirin administration; Figure 3.8. Levels of hs-CRP were also not modified; Figure 3.11.

Leukotrienes are members of the eicosanoid family that are produced at the sites of inflammation by numerous cells, including monocytes, as a result of AA metabolism<sup>261</sup>. We measured urinary cysteinyl leukotriene levels in Cohort Two participants, as a mediator of the inflammatory response. Levels were unchanged post-aspirin treatment (baseline  $1081.0 \pm 110.1$  pg/ml to  $984.1 \pm 76.35$  pg/ml post-aspirin;  $p = 0.3645$ ), again confirming the lack of inflammatory response to aspirin therapy in these healthy subjects; Figure 3.12.

In view of the role that netrin-1 plays in monocyte trafficking and the pathophysiology of atherosclerosis, I measured MPO levels in Cohort Two participants pre- and post-aspirin therapy. MPO is a peroxidase enzyme that is found in the azurophilic granules of neutrophils and the lysosomes of monocytes, and plays a key role in their microbicidal activity. The enzyme may additionally mediate atherogenesis, via the oxidation of lipoproteins, and has been suggested to potentially represent a biomarker of coronary artery disease <sup>262</sup>, as well as inflammation. I identified no statistically significant change in MPO levels after 28 days of aspirin therapy (baseline 1739 (IQR: 1099 – 2715) pg/ml to 870 (IQR: 530 – 2910) pg/ml post-treatment;  $p = 0.4105$ ); Figure 3.13.

Lastly, as long term use of non-steroidal anti-inflammatory drugs, including aspirin, reduces prostaglandin synthesis, which is a key component in gastric mucosal defence, I wished to reasonably exclude gastric inflammation as a potential cause for the fall in netrin-1. I measured serum pepsinogen I levels, as a marker of gastritis that has been shown to rise following NSAID-induced gastritis <sup>263</sup> and found no significant change from pre- to post-aspirin (baseline 302.5 (IQR: 271.7 – 477.4) ng/ml to 325.6 (IQR: 260.4 – 413.7) ng/ml post-treatment;  $p = 0.6737$ ); Figure 3.14.





**Figure 3.11: Change in markers of endothelial dysfunction and inflammation**

There was no change in markers of endothelial dysfunction, VCAM-1 (A), ICAM-1 (B), or E-selectin (C). Hs-CRP levels remained unchanged post treatment (D).

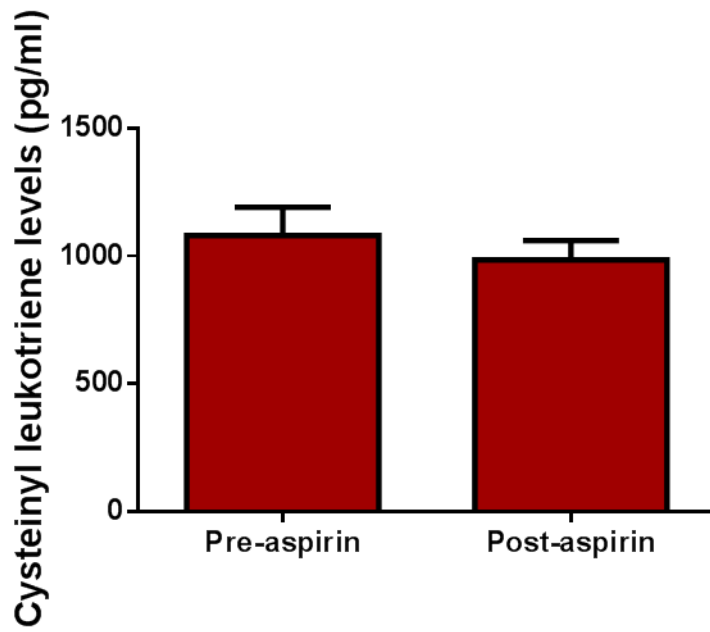


Figure 3.12: Cysteinyl leukotriene levels pre- and post-aspirin

There was no significant change in cysteinyl leukotriene levels following 28 days of aspirin 300 mg once daily.

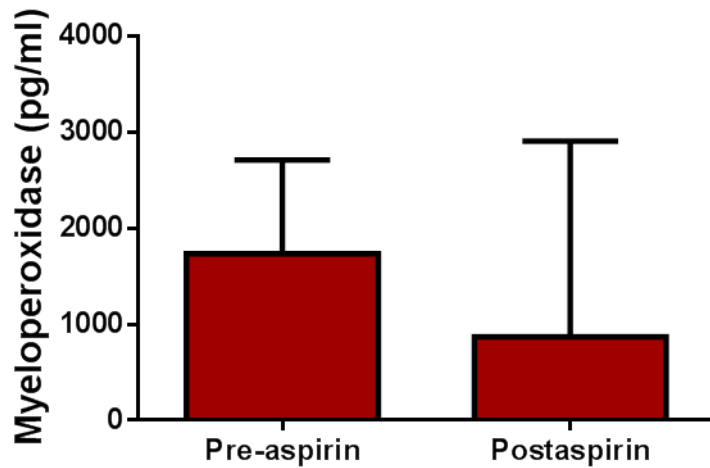
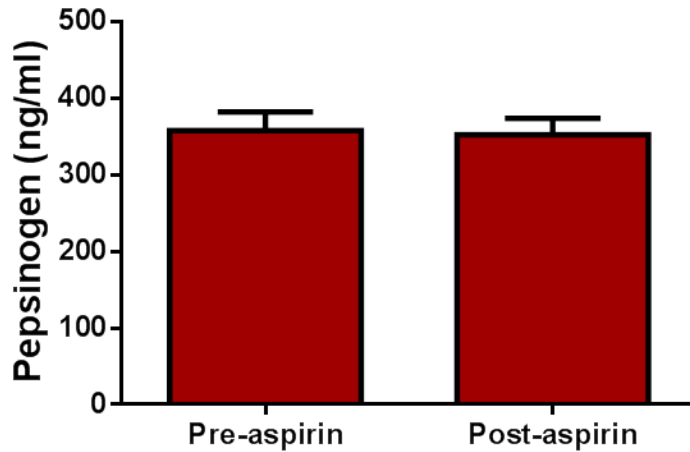


Figure 3.13: Myeloperoxidase levels pre- and post-aspirin

There was no significant change in myeloperoxidase levels following 28 days of aspirin 300 mg once daily.



**Figure 3.14: Pepsinogen I levels pre- and post-aspirin**

There was no significant change in pepsinogen I levels following 28 days of aspirin 300 mg once daily.

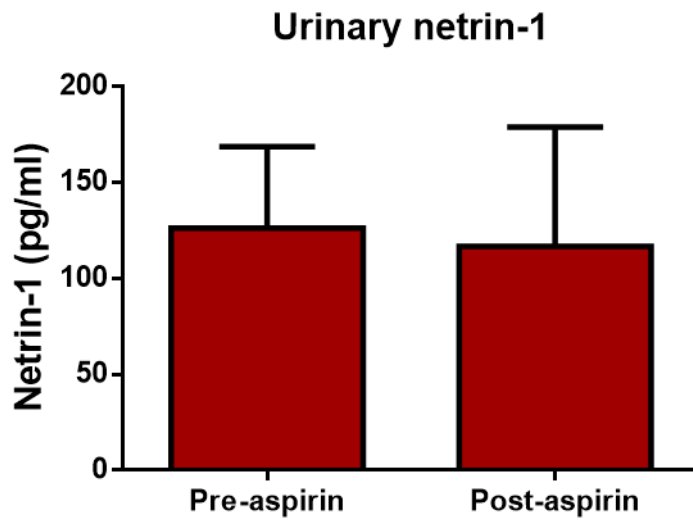
#### **3.4.4 The reduction in netrin-1 is not related to renal function**

In view of numerous reports of circulating netrin-1 levels falling in response to renal impairment<sup>214 264-266</sup>, in addition to a previous study showing that long-term use of 300 mg aspirin daily leads to a reduction in creatinine clearance in healthy young adults<sup>267</sup>, I assessed for potential changes in renal function from pre- to post-aspirin treatment in Cohort Two participants that may have contributed to the observed reduction in serum netrin-1 levels.

There were no significant changes in creatinine levels (baseline  $73.22 \pm 1.36 \mu\text{mol/l}$  to  $72.88 \pm 1.40 \mu\text{mol/l}$  post aspirin), creatinine clearance (baseline  $114.10 \pm 3.26 \text{ ml/min}$  to  $113.69 \pm 3.47 \text{ ml/min}$ ), or estimated glomerular filtration rate (baseline  $95.57$  (IQR:  $86.80 - 107.00$ )  $\text{ml/min}/1.73 \text{ m}^2$  to  $96.16$  (IQR:  $85.54 - 110.00$ )  $\text{ml/min}/1.73 \text{ m}^2$ ). There was also no correlation between netrin-1 levels and any of these measures of renal function.

Murine ischaemia-reperfusion injury models have shown that down-regulation of circulating netrin-1 occurs concomitantly with its up-regulation in tubular cells, leading to increased urinary netrin-1 secretion<sup>219 268</sup> and urinary netrin-1 levels are known to be directly correlated with renal function and indeed function as a biomarker for renal injury in humans<sup>264</sup>. I measured netrin-1

levels in the urine of Cohort Two participants and initially identified a reduction in urinary netrin-1 levels post-aspirin therapy. When these values were normalised for urinary creatinine, however, there was no significant change; Graph 3.12. There was also no correlation between serum and urinary netrin-1 levels.



**Figure 3.15: Urinary netrin-1 levels pre- and post-aspirin (normalised for urinary creatinine)**

There were no significant changes in urinary netrin-1 levels post-aspirin treatment. Baseline levels were 126.2 (IQR: 89.50 – 168.80) pg/ml and post-treatment levels were 116.8 (IQR: 100.6 – 178.9) pg/ml,  $p = 0.9664$ .

### 3.4.5 Aspirin administration causes a reduction in serum renin levels

It has previously been shown that plasma renin concentrations fall upon treatment with aspirin, albeit at higher doses than those used in our study<sup>269</sup>. A reduction in plasma renin levels has also been observed in response to nocturnal administration of low dose (100 mg) aspirin<sup>270</sup>. I measured serum renin levels in the Cohort Two participants and identified a significant reduction post-aspirin treatment. Renin levels fell by 26.97% from a baseline level of 339.7 (IQR: 95.91 – 519.4) pg/ml pre-aspirin, to 248.1 (IQR: 84.00 – 416.2) pg/ml post-aspirin,  $p = 0.0195$ ; Figure 3.12.

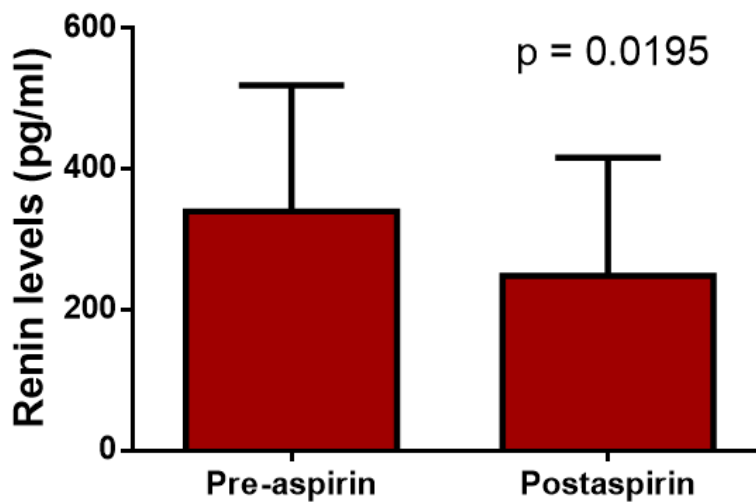


Figure 3.16: Renin levels pre- and post-aspirin

Graph showing the significant reduction in serum renin levels following 28 days of treatment with aspirin 300 mg once daily.

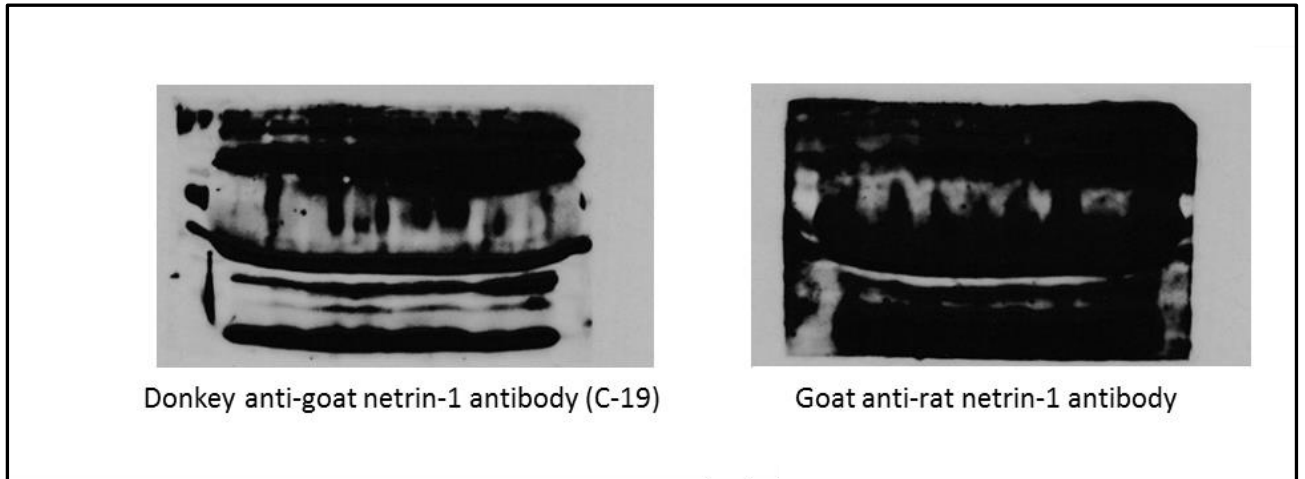
#### **3.4.6 Western blotting of serum netrin-1 isolated the truncated isoform only**

To characterise the netrin-1 isoform that was being measured in serum samples by ELISA, I performed Western blotting using a rat anti-netrin-1 antibody to specifically target the full length, 70 kDa protein, and a goat anti-netrin-1 antibody against the truncated, 55 kDa protein. Initially, I was unable to detect any antibody signal (Figure 3.13), however once I treated the serum to remove lipids, IgG, and albumin, the netrin-1 protein was detected. I was only able to detect the truncated isoform of netrin-1 in serum samples with either antibody (Figure 3.13).

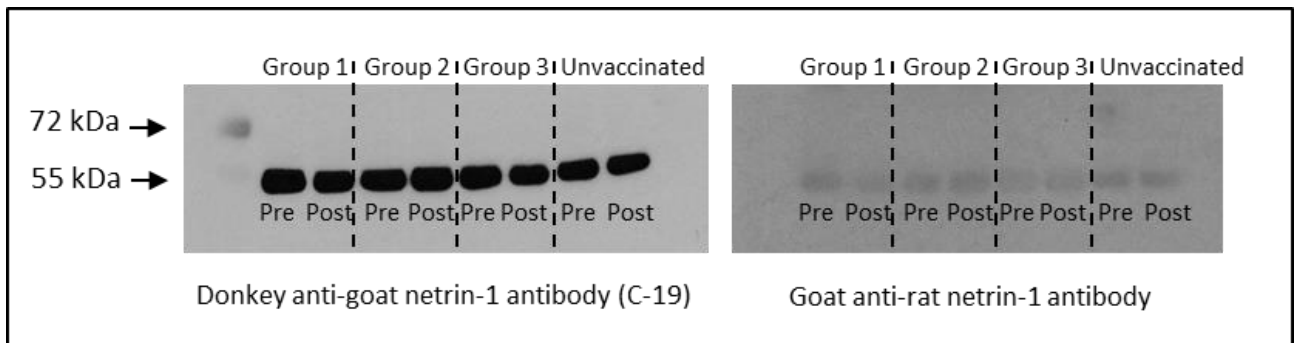
#### **3.4.7 Netrin-1 was not identified on the cell surface of monocytes**

Flow cytometry was performed to identify cell surface markers, including CD14, CD16, and netrin-1. There was no antibody signal from the PerCP-Cy5 goat anti-rat netrin-1 antibody, indicating that netrin-1 is not expressed on the monocytic surface.

A



B



**Figure 3.17: Western blotting of serum for netrin-1**

I performed Western blotting of serum to measure netrin-1 levels. Prior to treating the serum to remove lipids, IgG, and albumin, the results were uninterpretable (A). Once the serum had been treated to remove lipids, IgG and albumin, netrin-1 signal was detected, although only the truncated isoform was observed (B).

#### **3.4.8 Netrin-1 levels are not modified following exposure of endothelial cells to prostaglandin**

##### ***E<sub>2</sub> in vitro***

In view of the observed correlation between changes in netrin-1 and PGE<sub>2</sub>, netrin-1 was measured in cell supernatant from HUVEC at baseline and following either 4 or 24 hours of incubation with PGE<sub>2</sub> at 10 nM and 100 nM concentrations or DMSO. There was no detectable change from baseline in netrin-1 levels following exposure to PGE<sub>2</sub> or DMSO at either time point. Further concentrations of PGE<sub>2</sub> were not tested, as higher concentrations have been shown to stimulate cell proliferation, which would thus render the data uninterpretable <sup>271</sup>.

### **3.5 Discussion**

Prior *in vitro* work and animal experimentation have demonstrated that a dysfunctional endothelium markedly loses its ability to secrete netrin-1, and this compromises the integrity of the vascular barrier against inflammatory cell infiltration <sup>203</sup>. The current clinical study partly confirms these experimental data, whilst offering insight into the biomolecular mechanisms that regulate the synthesis of serum netrin-1 in humans.

Endothelial dysfunction, which is a systemic perturbation of vascular homeostasis characterised by impaired endothelium-dependent vasodilation and an associated state of endothelial activation, is not only triggered by pathological stimuli such as cardiovascular risk factors or immunological disorders but also occurs as part of physiological host defence mechanisms <sup>272</sup> <sup>273</sup>, such as occurs in the context of inflammation. The evidence provided by other authors demonstrating endothelial activation induced by influenza immunisation, were confirmed by the increased level of VCAM-1 observed in our participants 48 hours post-immunisation, thus supporting the validity of the influenza immunisation as an experimental model to study the relationship between endothelial dysfunction and circulating levels of netrin-1 in humans. In accordance with prior research performed in several animal models of inflammation, I found that endothelial activation, as driven by inflammatory stimuli, is also paralleled by a reduction in the serum concentration of netrin-1 in healthy subjects. This effect was abolished by all anti-



platelet treatments and was independent of their ability to inhibit platelet activation or to suppress systemic inflammation. Indeed, I have shown the lack of efficacy of both aspirin and clopidogrel in modulating the rise in hs-CRP with this experimental model of inflammation in data presented in Chapter Two. Moreover, whilst aspirin 300 mg exerted the strongest inhibition on platelet activity, as evidenced by a more effective suppression of P-selectin and TXB<sub>2</sub> in response to vaccination compared to clopidogrel, this latter was better at restoring netrin-1 synthesis in the peripheral blood of our immunised participants. The protective action of all anti-platelet agents on the vascular endothelium was similar, as demonstrated by their comparable effects in terms of suppression of the rise in VCAM-1 induced by immunisation. Aspirin alone, however, induced COX inhibition and this was found to account for the changes in the production of netrin-1 in both the immunisation study and the 28-day aspirin 300 mg interventional study.

These data demonstrate that netrin-1 reduction, as occurs in the presence of a dysfunctional endothelium caused by immunisation, occurs in parallel with PGE<sub>2</sub> reduction. In the absence of endothelial dysfunction, pharmacological inhibition of the COX enzymes, resulting in reduced PGE<sub>2</sub> production, also induced a small but significant decrease in netrin-1 levels. The TXA<sub>2</sub> pathway does not appear to be involved in netrin-1 regulation, as modulation of netrin-1, both in subjects receiving influenza immunisation and in those receiving high-dose aspirin for 28 days, was not related to changes in serum TXB<sub>2</sub>. Although I measured PGE<sub>2</sub> here, I cannot exclude the possibility that additional COX-dependent endothelium-derived prostanoids, such as PGI<sub>2</sub>, that generally follow the same expression pattern in healthy endothelium<sup>274</sup>, would show a similar association with netrin-1 under these experimental conditions. However, considering the prior demonstrated modulatory effect of netrin-1 on COX-2 expression and PGE<sub>2</sub> synthesis, it is intriguing to note that there is a regulatory link between COX-activity, COX-dependent prostaglandin production, and netrin-1 that may have important functional and therapeutic implications. Numerous studies have demonstrated that PGE<sub>2</sub> is protective against endotoxin injury, and promotes endothelial barrier enhancement<sup>257-260 275 276</sup> as well as cell survival<sup>277 278</sup>.

PGE<sub>2</sub> additionally has somewhat contradictory roles as both a direct mediator of early-stage inflammation and a suppressor of non-specific inflammation via modulation of IL-10 and pro-inflammatory cytokines<sup>279</sup>. The observed reduction in PGE<sub>2</sub> in response to immunisation was abolished by all anti-platelet therapy regimes, likely due to their beneficial effect on the vascular endothelium that, in turn, preserved its ability to synthesise protective vasoactive molecules and / or reduced PGE<sub>2</sub> consumption.

Local levels of PGE<sub>2</sub> derive from both its synthesis and degradation rates. Of note, there was a tendency towards an increase in PGE<sub>2</sub> levels in those taking clopidogrel, which may be related to the suppression of endothelial activation driven by the anti-platelet drug, without the additional effect of COX inhibition and subsequent reduced prostanoid production, which occurs with aspirin therapy.

The anti-inflammatory action exerted by anti-platelet agents on the vasculature that emerged in this study could partly be ascribed to their ability to suppress expansion of the pro-inflammatory monocytes that we have previously demonstrated in this human model of inflammation<sup>18</sup>. Several elements of discordancy were noted in this clinical study compared to previous research from our group in animal models of atherosclerosis treated with the same anti-platelet agents<sup>203</sup>. The superior effect of aspirin over clopidogrel in counteracting netrin-1 reduction in atherosclerotic mice was not confirmed in this clinical study; rather, clopidogrel demonstrated a better ability to maintain circulating levels of netrin-1 under pro-inflammatory conditions in humans. This discrepancy may be attributable to the distinct modulation of the tested anti-platelet agents on PGE<sub>2</sub> production, which has emerged as a potential factor implicated in the regulation of netrin-1 in human physiological settings.

Moreover, we were only able to detect the truncated isoform of netrin-1 in the peripheral blood of our healthy subjects, whilst previous *in vitro* experiments on endothelial cells reported a specific action of aspirin exerted on the full-length secreted isoform of the protein through an epigenetic modification of chromatin. The possibility that lack of expression of full-length netrin-1 is due to the phenomenon of protein truncation occurring in human serum cannot be

excluded. Furthermore, one of the limitations of the immunisation arm of this study was that serum samples were only available from two time-points, 48 hours apart, and we therefore have not established whether similar trends in netrin-1 and PGE<sub>2</sub> levels would have been present in an earlier stage of the inflammatory response and how the relationship between the two markers would have progressed until resolution of inflammation.

Further analysis of our group's previously published *in vitro* data from human endothelial cells in fact revealed that stimulation of cells with aspirin alone (in the absence of TNF as a co-stimulatory agent) showed a tendency, albeit not statistically significant, towards a reduction in netrin-1 release into serum supernatants compared to non-aspirin-treated cells. In light of the new data that have emerged from the current clinical study, it is possible that the aspirin-induced PGE<sub>2</sub> reduction, which was reported in the prior *in vitro* set of experiments, may have accounted for a subtle difference in netrin-1 that only became evident and statistically relevant in the *in vivo* human study here described. Furthermore, the cellular sources of both circulating netrin-1 and PGE<sub>2</sub> measured in our clinical study, remain to be established. Our primary focus was on the vascular endothelium, whose function was assessed by measuring classical endothelial-derived adhesion molecules. However, the lack of a direct relationship between either netrin-1 or PGE<sub>2</sub> and endothelial markers or monocyte subtype cell count implies a multicellular source for both molecules. Hence, PGE<sub>2</sub> and netrin-1 may also be derived from cell types other than endothelial cells, such as inflammatory cells, and these could have contributed to the modulation of systemic inflammation and/or activation of the vascular endothelium.

Interestingly, I observed a reduction in serum renin levels in response to aspirin treatment in the Cohort Two participants, who were treated with 28 days of aspirin 300 mg once daily, without any apparent effect of the drug on renal function. Renin is produced by the juxtaglomerular cells in the kidney, and is secreted as a homeostatic response to reduced arterial blood pressure. It has been suggested that aspirin treatment could lead to reduced renin secretion due to inhibition of prostaglandin-induced vasodilation, thus resulting in a higher baseline blood pressure<sup>267 270</sup>. It appears that, in this instance, prostaglandin inhibition by aspirin

may have resulted in a subclinical change in vascular tone and renal perfusion, thus reducing renin secretion. We also sought to establish whether the observed netrin-1 reduction in Cohort Two was related to a change in the status of endothelial activation induced by aspirin treatment. Netrin-1 is expressed abundantly throughout the endothelium where it is downregulated by pro-inflammatory cytokines and shear stress. The same stimuli induce ICAM-1 and E-selectin upregulation in endothelial cells<sup>218 220</sup>. We found no change in markers of endothelial activation or systemic inflammation (as measured by hs-CRP), nor in specific pathways that were linked to COX inhibition and netrin-1 suppression, thus effectively excluding netrin-1 reduction being explained by a change in inflammatory status.

PGE<sub>2</sub> possesses important immunomodulatory properties that are concentration-related, and its levels are regulated by both synthesis, mainly sustained by COX-2 activity, and degradation rates<sup>279</sup>. Hence, whilst PGE<sub>2</sub> upregulation, as driven by NF-κB inducers in the context of an acute inflammatory response, has detrimental pro-inflammatory effects on the vasculature by enhancing endothelial permeability, a reduced bioavailability of PGE<sub>2</sub> may also be damaging the endothelium by removing a pro-survival factor. In keeping with this, netrin-1 may represent the mediator of PGE<sub>2</sub>, particularly the truncated isoform that has been mainly related to cell survival, at least in cancer cells, further supporting the link between levels of PGE<sub>2</sub> and truncated netrin-1 that we have observed in this clinical study.

This biomolecular interplay between COX inhibition and netrin-1 may have important therapeutic implications. Previous investigators have found that netrin-1 regulates COX-2 expression within the kidney via NF-κB activation and thus directly modifies PGE<sub>2</sub> production in a murine model of renal inflammation<sup>221</sup>. The amplitude of PGE<sub>2</sub> reduction in our immunised participants, both treated and untreated with anti-platelet agents, was strongly related to the extent of reduction in netrin-1. Similarly, the baseline and post-treatment levels of both markers in serum from healthy subjects who took aspirin 300 mg daily for 28 days were strongly correlated with one another, confirming the existence of a direct relationship between these two parameters. However, our data point towards a potential regulatory role exerted by PGE<sub>2</sub>

on netrin-1 synthesis. The 28-day treatment period with aspirin 300 mg daily primarily targeted prostaglandin production through COX-inhibition. The concomitant reduction in circulating netrin-1 levels, which occurred in the absence of any inflammatory stimulus (as confirmed by stable levels of hs-CRP) and endothelial dysfunction (as demonstrated by unmodified levels of endothelial markers), is likely to be secondary to this. The COX-dependent modulation of netrin-1, with potential interactions with prostanoids, including PGE<sub>2</sub>, appears intriguing in consideration of the emerging role of netrin-1 as a cardiovascular target, as well as the recent evidence highlighting the pro-metastatic effect of PGE<sub>2</sub> in adenocarcinoma cells<sup>280</sup>, and the potential beneficial effect that was demonstrated with *in vitro* experiments using aspirin in this cancer subtype, for which netrin-1 overexpression was found to play important roles in terms of cell survival and proliferation as well as to have prognostic implications in clinical studies

### **3.6 Conclusion**

Our data suggest that circulating netrin-1 levels appear to be directly modulated by changes in COX-dependent vasoactive molecules, such as PGE<sub>2</sub>. Given the emerging role of netrin-1 in cardiovascular disease and as an oncological target, we believe that further exploration of the pathways that control netrin-1 production and its relationship with clinical outcomes may be important and may carry potential therapeutic benefit.

**CHAPTER FOUR:**

**THE ROLE OF CD16<sup>+</sup> MONOCYTES IN OXIDISED**

**LOW-DENSITY LIPOPROTEIN CLEARANCE**

#### **4.1 Introduction**

Atherogenesis is strongly associated with chronic inflammatory states<sup>8</sup> and patients with elevated markers of inflammation have an increased risk of subsequent cardiovascular events<sup>4</sup><sup>5</sup>, indicating that dysregulation of immune function may be one of the key factors involved in plaque formation. OxLDL accumulates at sites of atherosclerosis, inducing an immunological response with generation of anti-oxLDL autoantibodies by B cells<sup>281 282</sup>.

HMG-CoA reductase inhibitors (statins), which act to reduce circulating LDL levels, are among the most commonly prescribed therapies for patients with cardiovascular risk factors<sup>283</sup> although novel immunomodulatory drugs that reduce synthesis or increase clearance of LDL are currently in development<sup>11 12</sup>. IgG and IgM autoantibodies against oxLDL have been assessed as potential biomarkers of coronary artery disease and whilst associations have been found, there is no definitive evidence that they represent independent predictors of atherogenesis or cardiovascular events<sup>14 284</sup> and the pathways linking oxLDL to plaque generation remain unclear. As shown in Chapter Two, anti-platelet agents are capable of modifying the phenotype of circulating monocytes, as denoted by their surface expression of CD14 and CD16<sup>18</sup>. CD16 is a low affinity Fc  $\gamma$  receptor that is activated upon binding to IgG, thus initiating phagocytosis. Polymorphisms of Fc  $\gamma$  receptors for IgG may be associated with increased coronary atherosclerosis and it has been hypothesised that variations in these receptors may moderate the clearance of IgG antibodies by monocytes and macrophages and thus modify plaque formation<sup>20</sup>.

#### **4.2 Hypothesis and aim**

Patients with cardiovascular disease are known to have increased levels of circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes<sup>22 23</sup> but it has yet to be established whether this increase contributes directly to cardiovascular risk or is instead a protective mechanism that acts, for example, to modify oxLDL levels.

I evaluated the impact of CD16<sup>+</sup> monocyte reduction on the circulating levels of oxLDL and IgG anti-oxLDL in the context of a non-atherogenic immunological reaction to test the hypothesis that CD16 plays a critical role in the transduction of signalling pathways triggered by oxLDL.

### **4.3 Methods**

#### **4.3.1 Participant recruitment**

This was a *post hoc* study conducted on samples obtained from 40 healthy volunteers previously recruited at Guy's and St Thomas' NHS Foundation Trust, who participated in the clinical study described in Chapter Two and for whom serum samples were still available. All participants were aged 18 years or older (median: 30.5 years; IQR 24.5 - 38), had no significant past medical history, were not taking regular medications (aside from the combined oral contraceptive pill), and had not taken anti-platelet or anti-inflammatory drugs in the preceding fortnight.

Study volunteers received influenza immunisation, which is an established model of mild, systemic inflammation<sup>30</sup> and one of the 48 hour treatment regimens listed below. Serum samples were collected before and 48 hours after receiving the seasonal influenza immunisation, and in the presence of one of the following treatment regimens:

Group 1 aspirin 300 mg once daily (n = 10)

Group 2: aspirin 75 mg once daily (n = 10)

Group 3: clopidogrel with an initial loading dose of 300 mg followed by a further dose of 75 mg 24 hours later (n = 10)

Group 4: untreated participants (n = 10; serving as controls)

Once again, I did not include data from participants who had been treated with ticagrelor in the original study, due to relatively poor platelet inhibition with this anti-platelet agent, which was demonstrated by an inability to suppress P-selectin levels in response to inflammation (see Chapter Two).



#### **4.3.2 Collection of serum**

Serum was collected at baseline and 48 hours later using methods described in Chapter Two. Serum was stored at -80 °C for 6-9 months until it was utilised for the experiments described below.

#### **4.3.3 Enzyme-linked immunosorbent assays**

ELISAs were performed using commercially available kits as per manufacturer instructions, to measure serum levels of oxLDL (ID: STA-369; Cell Biolabs, Inc., USA), MPO (ID: BMS2038INST; eBioscience Ltd., UK), and bone morphogenetic protein-4 (BMP-4) (ID: E-EL-H0012; Elabscience, USA). Inter-assay variability of all kits was found to be <5 %.

Serum IgG anti-oxLDL levels were measured by coating a 96 well plate with copper-oxidised LDL at 5 µg/ml in PBS and incubating overnight at 4 °C on a rocker. The plate was subsequently washed four times with 200 µl PBS per well. The wells were blocked for 1 hour with a solution containing 5 % BSA (Fisher Scientific) and 0.1 % Tween-20 (Sigma) in PBS (300 µl per well). The plate was washed twice, as above, and 200 µl serum samples were added to the coated wells and incubated for 2 hours at 37 °C. The wells were washed three times with a PBS / 0.5 % BSA / 0.1 % Tween-20 solution. 200 µl horseradish peroxidase-conjugated rabbit anti-human IgG secondary antibody (Sigma-Aldrich, UK) was added to each well (1:2000 dilution in blocking buffer) and the plate was incubated for 30 minutes at 37 °C. The plate was washed 5 times and 90 µl TMB was added to each well, with a subsequent 20 minute incubation at 37 °C. The reaction was stopped by the addition of 0.16M sulphuric acid. Absorbance at 450 nm was measured using a multi-well plate reader (SPECTRAmax M5; Molecular Devices).

#### **4.3.4 Measurement of low-density lipoprotein levels**

Serum LDL measurements were conducted by the ViaPath laboratory at Guy's and St Thomas' NHS Foundation Trust. OxLDL levels stated in the results section of this chapter are expressed as the oxLDL/LDL ratio to adjust for serum LDL levels, as per commonly accepted practice<sup>285</sup>

<sup>286</sup>.

#### **4.3.5 Monocyte isolation:**

Monocytes were isolated from the peripheral blood mononuclear cells (PBMCs) of subjects using a commercially available pan-monocyte isolation kit for magnetic cell sorting (ID 130-096-537; Miltenyi Biotec, UK) and a magnet separator kit (MiniMACS Starting Kit ID: 130-090-312; Miltenyi Biotec, UK).

A buffer solution (pH 7.2) was prepared with PBS, 0.5 % BSA, and 2 mM EDTA, and this was kept at 4 °C. To prevent capping of antibodies on the cell surface and non-specific cell labelling, pre-chilled solutions were used throughout the experiment.

4 ml whole blood was collected in a vacuum-tube coated with EDTA. The blood was diluted with an equal volume of 0.9 % saline. 6 ml diluted blood was layered over 3 ml Lymphoprep™ solution (ID: 07851; STEMCELL Technologies, Canada) in a 15 mm centrifuge tube. The blood was centrifuged at 800 x g for 20 minutes at 20 °C to separate the blood components. The PBMC top layer was aspirated and transferred to a falcon tube. The cell suspension was centrifuged again at 300 x g for 10 minutes at 4 °C; the supernatant was removed and the cell pellet retained.

The cell pellet was re-suspended in 30 µL of buffer. 10 µL FcR Blocking Reagent was added, along with 10 µL of Biotin-Antibody Cocktail. The solution was vortexed and incubated for 5 minutes at 4°C. 30 µL of buffer was added, followed by 20 µL of Anti-Biotin MicroBeads. The solution was vortexed and incubated for a further 10 minutes at 4 °C.

The MACS magnetic column was attached to the MACS separator magnet. The column was rinsed with 500 µL of buffer and the cell suspension was applied onto the column. The flow-

through containing unlabelled cells, representing the enriched monocyte fraction, was collected.

The column was rinsed 3x with 500 µL buffer and the unlabelled cells were again collected and added to the previous yield.

#### **4.3.6 Stimulation of whole blood and isolated monocytes**

MDA-modified LDL (ID: STA-212; Cell Biolabs, Inc., USA) at a concentration of 100 µg/ml was used to stimulate whole blood and isolated monocytes.

A mouse monoclonal fragment antigen binding (F(ab')<sub>2</sub>) antibody directed against human CD16 cells (1mg/ml when reconstituted; Anti-CD16 human mAb 3G8 F(ab')<sub>2</sub>; ID: ANC165-520; Caltag, UK) was used at saturating concentrations<sup>287 288</sup> to specifically block CD16 Fc γ receptor activation and thus delineate CD16-dependent pathways.

Whole blood:

2 ml whole blood was collected in a vacuum-tube coated with EDTA. Four 250 µl aliquots of each blood sample were prepared as follows:

Tube 1: Control sample – 250 µl whole blood

Tube 2: 250 µl whole blood plus 12.5 µl MDA-LDL

Tube 3: 250 µl whole blood plus 2.5 µl F(ab')<sub>2</sub>

Tube 4: 250 µl whole blood plus 12.5 µl MDA-LDL and 2.5 µl F(ab')<sub>2</sub>

The tubes were incubated at 37 °C for 60 minutes and then immediately processed for Ribonucleic acid (RNA) isolation.

Isolated monocytes:

Monocytes were isolated, as per Section 4.3.5. Four monocyte suspension aliquots from each donor were prepared as follows:

Tube 1: Control sample – 250 µl monocyte suspension

Tube 2: 250 µl monocyte suspension plus 12.5 µl MDA-LDL

Tube 3: 250 µl monocyte suspension plus 2.5 µl F(ab')<sub>2</sub>

Tube 4: 250 µl monocyte suspension plus 12.5 µl MDA-LDL and 2.5 µl F(ab')<sub>2</sub>

The tubes were incubated at 37 °C for 60 minutes and then immediately processed for RNA isolation.

#### **4.3.7 Ribonucleic acid isolation**

RNA was isolated from whole blood samples (collected in vacutainer tubes coated with EDTA to prevent coagulation) using a commercially available QIAamp RNA Blood Mini Kit (ID 52304; Qiagen) as per the manufacturer's protocol. When isolated monocytes were used in place of whole blood, the protocol began from Step 2.

##### Step 1: Cell lysis

500 µl whole blood was mixed with 2500 µl erythrocyte lysis buffer in a falcon tube, and incubated on ice for 20 minutes. The tube was vortexed twice during incubation. The sample was then centrifuged at 400 x g for 10 minutes at 4 °C and the red cell supernatant was then discarded to retain the leucocyte pellet. 1000 µl erythrocyte lysis buffer was then added and the tube was vortexed to re-suspend the cells. Centrifugation was performed for the second time, at 400 x g for 10 minutes at 4 °C and the red cell supernatant was discarded.

350 µl RLT buffer (a buffer containing a high concentration of guanidine isothiocyanate to aid the binding of RNA to the silica membrane of the spin columns) was added to the pelleted leucocytes and repeatedly pipetted to re-suspend the cells.

#### Step 2: Homogenisation

The lysate was pipetted into a QIAshredder spin column in a 2 ml collection tube and centrifuged at 10,000 x g for 2 minutes at 4 °C. The spin column was discarded and the homogenised lysate was retained. 350 µl 70 % ethanol was added to the collection tube, and was mixed by repeated pipetting. The sample was pipetted into a new QIAamp spin column in a 2 ml collection tube and was centrifuged at 10,000 x g for 2 minutes at 4 °C.

#### Step 3: DNA digestion

Deoxyribonuclease (DNase) stock solution was prepared by dissolving lyophilized a DNase I (1500 Kunitz units) vial (ID 79254, Qiagen) in 550 µl nuclease-free water. 350 µl RW1 buffer (a wash buffer containing guanidine salt and ethanol that removes biomolecules from the silica membrane whilst retaining RNA molecules) was added to the QIAamp spin column. The tube was centrifuged at 10,000 x g for 15 seconds at 4 °C.

10 µl DNase I stock solution was added to 70 µl RDD buffer (a buffer that facilitates on-column digestion of DNA) in a 1.5 ml microcentrifuge tube. The tube was gently mixed and then centrifuged at 10,000 x g for 5 seconds to collect residual fluid from the sides of the tube. The 80 µl DNase I mix was pipetted directly onto the QIAamp spin column membrane and placed on the benchtop for 15 minutes at room temperature.

350 µl RW1 buffer was added to the spin column, and this was then centrifuged at 10,000 x g for 15 seconds at 4 °C. The flow-through was discarded and the membrane was retained.

#### Step 4: RNA extraction

The spin column was placed in a new 2 ml collection tube. Ethanol was added to the RPE buffer (a buffer to remove residual traces of salts from previous buffers) that was provided with the kit. 500 µl of RPE buffer was pipetted into the spin column, which was then centrifuged at 10,000 x g for 15 seconds at 4 °C. The flow-through and collection tube were discarded.

500 µl RPE buffer was added to the spin column, which was then centrifuged at 20,000 x g for 3 minutes at 4 °C. The collection tube and filtrate were discarded and the spin column was placed in a new collection tube, which was then centrifuged at 20,000 x g for 1 minute at 4°C to prevent any RPE buffer from being retained on the membrane. The spin column was transferred into a 1.5 ml microcentrifuge tube and 50 µl nuclease-free water was pipetted directly onto the membrane. The tube was centrifuged at 8000 x g for 1 minute at 4 °C to obtain the RNA yield.

RNA content and purity were assessed using a spectrophotometer (NanoDrop, Thermo Scientific, UK).

#### **4.3.8 Copy deoxyribonucleic acid synthesis**

cDNA was synthesised from the RNA samples by using a RevertAid H Minus First Strand cDNA Synthesis Kit (ID: K1631; Thermo Fisher Scientific, UK) as per the manufacturer's protocol.

#### Step 1: First strand cDNA synthesis

Aliquots of each RNA sample were diluted with nuclease-free water to achieve a uniform concentration in a sterile, nuclease-free microcentrifuge tube, at a total volume of 11.5 µl and were kept on ice. 1.5 µl random hexamers were subsequently added to anneal to complementary sites on the RNA, thus serving as primers for cDNA synthesis.

4 µl 5x reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl<sub>2</sub>, 50 mM DTT) was added to the tube, followed by 1 µl RiboLock RNase Inhibitor (20 U/ µl). 2 µl of 10 mM

deoxynucleotide mix was then added, followed by 1  $\mu$ l RevertAid H Minus M-MuLV reverse transcriptase (200 U/ $\mu$ l), bringing the total volume in each tube to 20  $\mu$ l. The tubes were mixed gently and subsequently centrifuged.

A 96 well thermal cycler (2920 Thermal Cycler; Applied Biosystems, UK) was used to conduct the polymerase chain reaction. The cycler was set to incubate the RNA for 5 minutes at 25 °C followed by 60 minutes at 42 °C. The cDNA synthesis reaction was terminated by heating the tubes to 70 °C for 5 minutes.

#### Step 2: Real-time quantitative polymerase chain reaction amplification of cDNA

A panel of genes related to biomolecular pathways involved in the activity of the adaptive immune system and synthesis of osteogenic molecules were studied, including BMPs, matrix metalloproteinases (MMPs) and the NLRP3 gene component of the inflammasome oligomer. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primer sequences are listed in Table 4.1.

**Table 4.1: Sequence of primers used for polymerase chain reaction**

<b>Gene</b>	<b>Forward primer (5' – 3')</b>	<b>Reverse primer (5' – 3')</b>
<b>GAPDH</b>	CAAGGTCATCCATGACAACTTTG	GGCCATCCACAGTCTTCTG
<b>CD16</b>	GACAGTGTGACTCTGAAG	GCACCTGTA CTCTCCAC
<b>IL-1<math>\beta</math></b>	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT
<b>IL-8</b>	ACCGGAAGGAACCATCTCAC	GGCAAACTGCACCTTCACAC
<b>Inflammasome (NLRP3)</b>	TGAAGAAAGATTACCGTAAGAAGTACAGA	GCGTTTGTTGAGGCTCACACT
<b>BMP-2</b>	CCCCCTACATGCTAGACCTGT	CACTCGTTTCTGGTAGTCTTCC
<b>BMP-4</b>	TGGTCTTGAGTATCCTGAGCG	CACTCGTTTCTGGTAGTCTTCC
<b>Caspase-1</b>	TGCCTGTTCTGTGATGTGG	CTGGGAAGAGGTAGAAACATC
<b>c-Myc</b>	GGCGAACACACAACGTCTTG	TCCGTTTTAGCTCGTTCCTC
<b>Cyclin D1</b>	GATGGCGATCGTCTGT CAT	ACAGGCCGCTACAAGAAACA
<b>Beta catenin</b>	GTCAGCTCGTGCCTGTGAA	TTCAGGTACCCTCAGGCC
<b>Netrin-1 (full length)</b>	CCCGGACTTTGTCAATGC	GTTGTTGAGGTCGGRGAGGA
<b>Netrin-1 (truncated)</b>	ACTATGCCGTCCAGATCCAC	TCTTGAGGGGCTTGATTTTG
<b>MMP-2</b>	TATTTGATGGCATCGCTCAG	GCCTCGTATACCGCATCAAT
<b>MMP-4</b>	AGGTGGACCGGATGTTCC	GGCACTGCAGGATGTCATAG

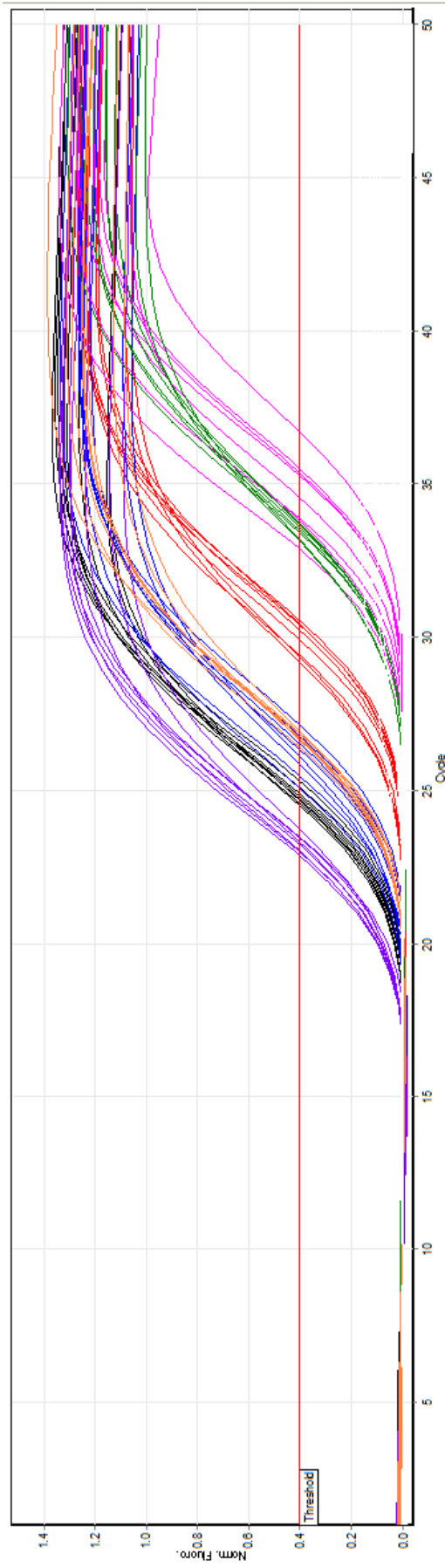


2  $\mu$ l of the first strand cDNA synthesis reaction mixture (to be used as template for the subsequent polymerase chain reaction (PCR) step) was pipetted directly into each of the wells of a Rotor-Disc 100 plate (Qiagen). An 8  $\mu$ l mixture containing 5  $\mu$ l SYBR green mix (a nucleic acid fluorophore stain), 2.6  $\mu$ l nuclease-free water, 0.2  $\mu$ l of the relevant forward primer, and 0.2  $\mu$ l of the relevant reverse primer was subsequently added to each well. The disc was sealed using a Rotor-Disc Heat Sealer (Qiagen).

DNA was amplified using a real-time quantitative PCR cycler (Rotor-Gene Q; Qiagen), with 50 cycles of the following thermal protocol, following an initial 10 minutes held at 95  $^{\circ}$ C:

- 1) 95  $^{\circ}$ C for 10 seconds
- 2) 58  $^{\circ}$ C for 20 seconds
- 3) 72  $^{\circ}$ C for 10 seconds

Data were analysed using Rotor-Gene Q (Qiagen) software. A typical analysis is shown in Figure 4.1.



**Figure 4:1: A typical analysis of deoxyribonucleic acid amplification using a real-time polymerase chain reaction cycle**

Each coloured line represents the amplification of a particular marker, such as IL-1 $\beta$ , in a DNA sample from either whole blood or isolated monocytes.

### **4.3.9 Evaluation of monocyte lipid content**

Monocyte subsets were identified according to their surface expression of CD14 and CD16 using fluorochrome conjugated antibodies, as per methodology described in Chapter Two. Dr Gabriella Passacquale characterised the lipid content within each subset of monocytes by flow cytometry, using Nile red (9-diethylamino-5-benzo[ $\alpha$ ]phenoxazinone) staining to localise and quantify intracellular lipid droplets.

### **4.3.10 Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 6.0) software. Parametric data are expressed as the mean  $\pm$  SEM, whilst non-parametric data are expressed as the median with IQR. ANOVA was used to compare percentage variation of each study variable between the 4 treatment groups in the influenza immunisation study. Baseline and post-treatment values were compared within each group using a paired parametric or non-parametric test as appropriate. ANCOVA was used to compare percentage variation of each study variable between the 4 groups in the immunisation study. A  $p$  value of  $<0.05$  was accepted as statistically significant.

## **4.4 Results**

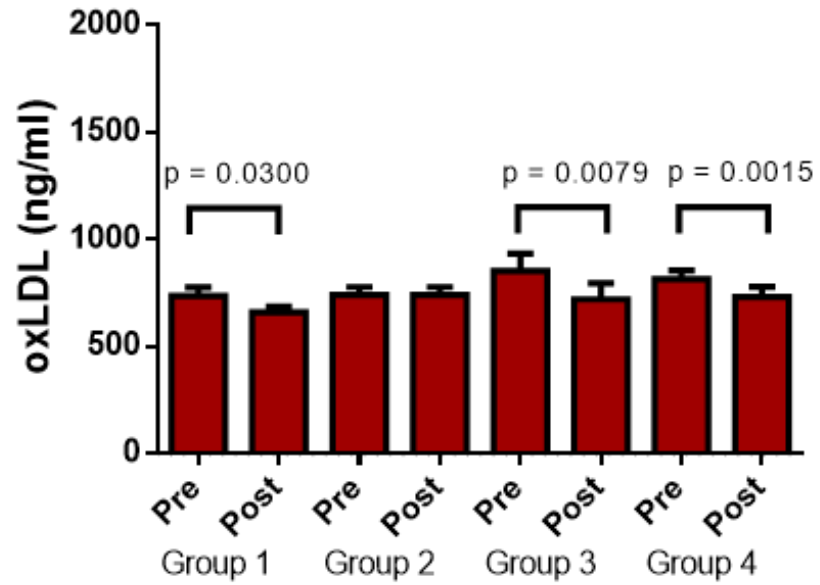
### **4.4.1 There is an inverse relationship between circulating oxidised low-density lipoprotein and IgG anti-oxidised low-density lipoprotein levels**

A highly significant reduction in baseline circulating oxLDL levels of 10.75 % post-immunisation, from  $819.00 \pm 38.68$  ng/ml to  $730.20 \pm 49.37$  ng/ml ( $p = 0.0015$ ) was observed in participants who were not treated with anti-platelet agents. In those taking aspirin 300 mg (Group 1), circulating oxLDL levels fell by 10.50 %, from  $735.8 \pm 39.99$  to  $658.5 \pm 27.68$  ( $p = 0.03$ ), whilst remaining unchanged in those taking aspirin 75 mg (Group 2). In participants taking clopidogrel (Group 3), oxLDL levels exhibited a non-significant reduction of 15.37 % from  $852.70 \pm 82.16$  ng/ml to  $721.60 \pm 74.10$  ng/ml ( $p = 0.079$ ), Figure 4.2. It is worth noting that although the

baseline level of oxLDL in this group was apparently higher than other groups, there were no statistically significant differences between groups for baseline levels of either oxLDL, IgG anti-oxLDL.

There were no significant changes in levels of IgG anti-oxLDL among all groups following immunisation (Figure 4.3). The levels of circulating IgG anti-oxLDL measured at baseline (before immunisation) in the total population were inversely related to baseline oxLDL levels ( $p = 0.0011$ ,  $r = -0.4207$ ); Figure 4.4. This inverse relationship was preserved post-immunisation, with a negative linear correlation between the percentage change in oxLDL and the percentage change in IgG anti-oxLDL;  $p = 0.0396$ ,  $r = -0.3268$  (Figure 4.4).

A



B

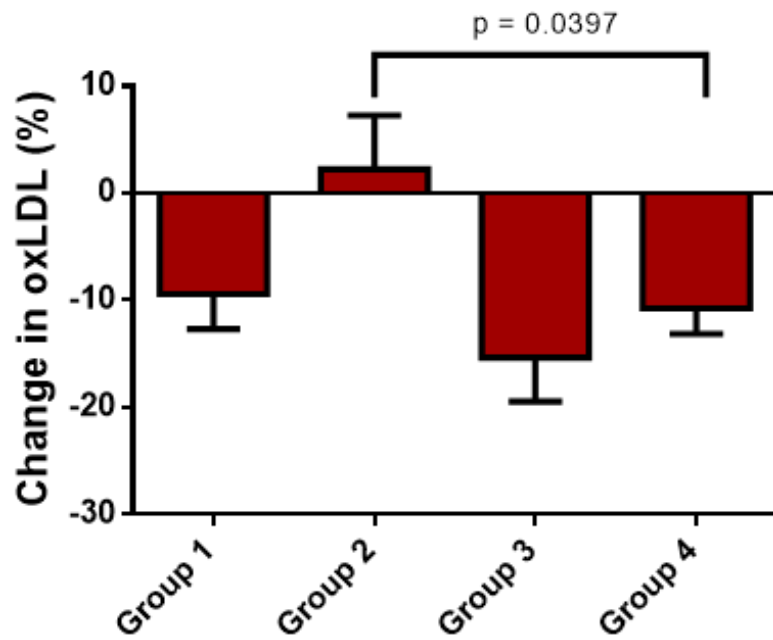
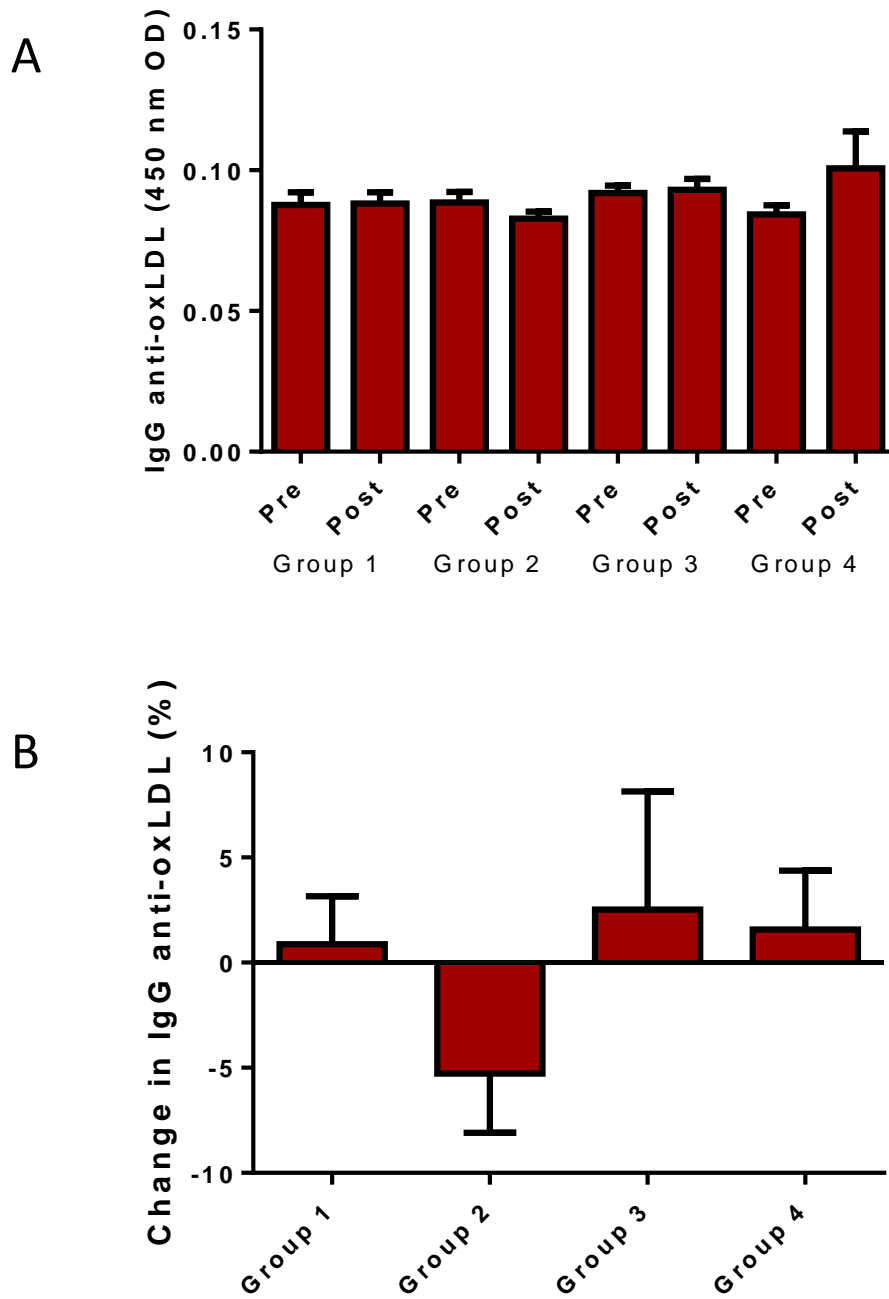


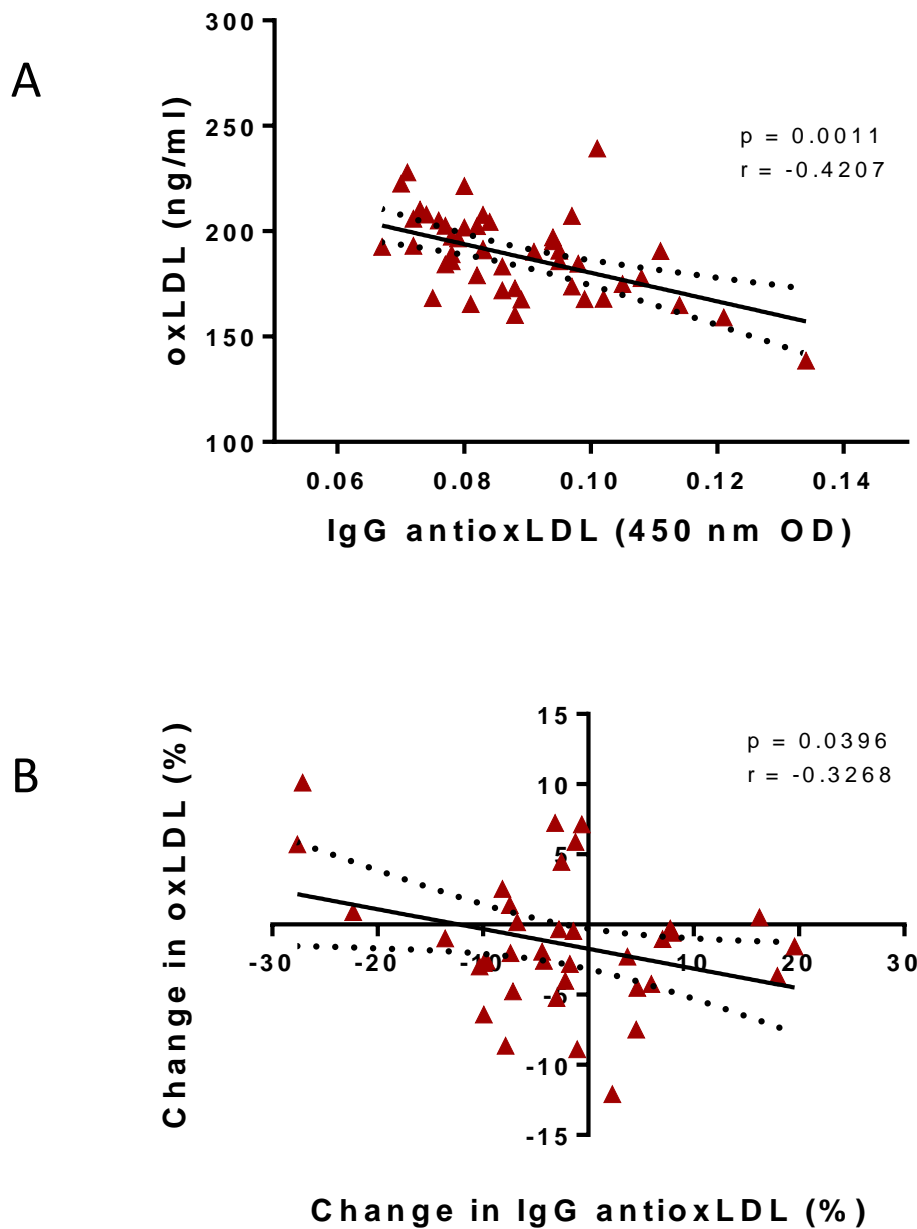
Figure 4.2: Change in oxidised low-density lipoprotein levels post-immunisation

Panel A shows the absolute values of oxLDL pre- and post-immunisation. Panel B shows the percentage change in oxLDL across groups.



**Figure 4.3: Change in IgG anti-oxidised low-density lipoprotein levels post-immunisation**

Panel A shows the absolute values of IgG anti-oxLDL pre- and post-immunisation. Panel B shows the percentage change in IgG anti-oxLDL across groups.



**Figure 4.4: Correlation between percentage change in oxidised-low density lipoprotein and IgG anti-oxidised low-density lipoprotein levels**

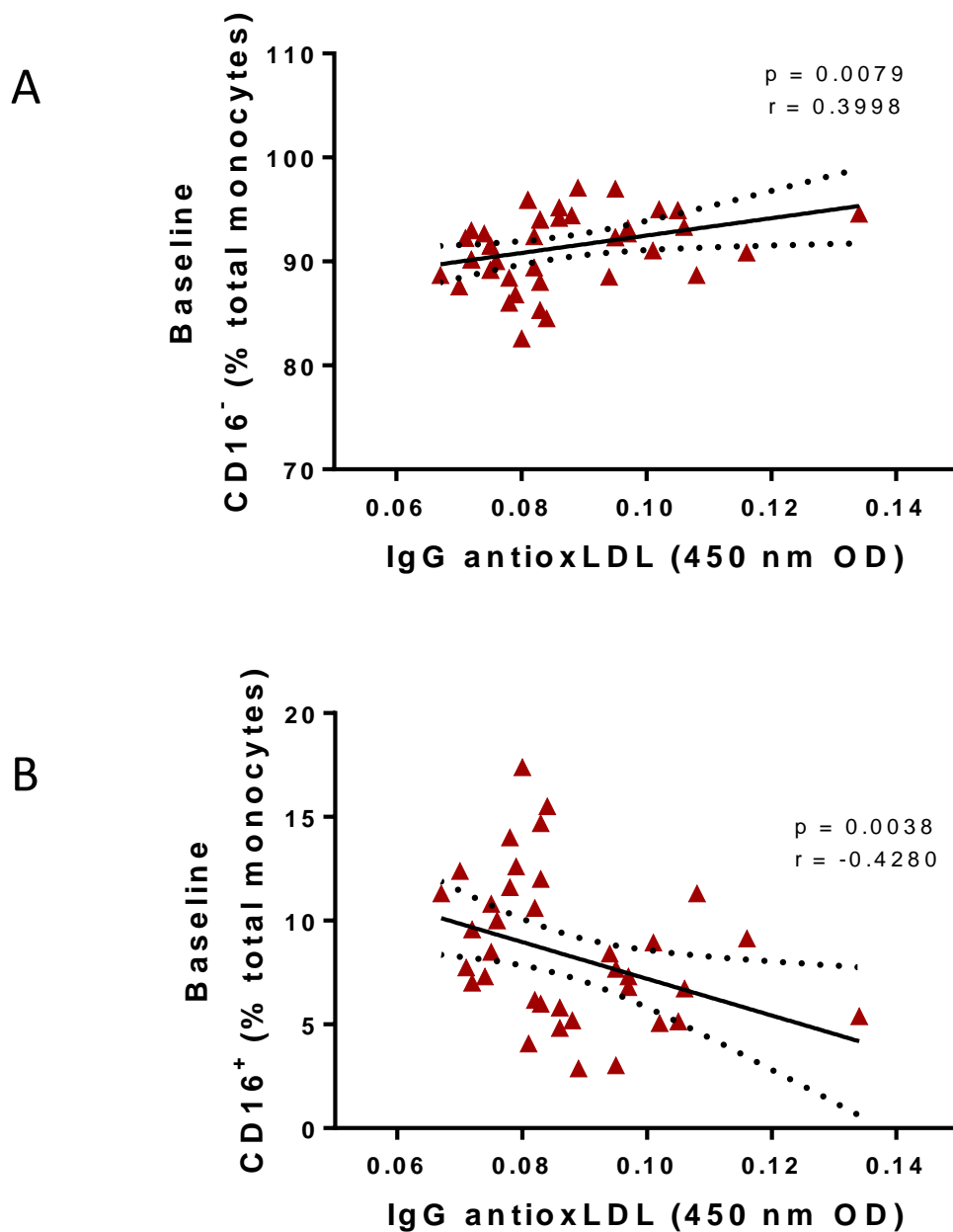
Panel A shows the correlation between baseline levels of oxLDL and anti-oxLDL IgG. Panel B shows the correlation between the percentage change in oxLDL and IgG anti-oxLDL post-immunisation.

#### **4.4.2 In the absence of inflammation, circulating IgG anti-oxidised low-density lipoprotein levels are inversely related to the percentage of CD16<sup>+</sup> monocytes**

As described in Chapter Two, the percentage of CD16<sup>+</sup> monocytes (specifically, the CD14<sup>high</sup>CD16<sup>+</sup> subtype) increased significantly post-immunisation in non-anti-platelet treated (Group 4) participants. There was a strong negative correlation between baseline IgG anti-oxLDL levels and the baseline total percentage of CD16<sup>+</sup> monocytes in the total population ( $p = 0.0038$ ,  $r = -0.4280$ ), whilst anti-oxLDL IgG was positively correlated with the percentage of CD16<sup>+</sup> monocytes ( $p = 0.0079$ ,  $r = 0.3998$ ); Figure 4.5. No correlation was found between either oxLDL or anti-oxLDL IgG and the marker of platelet activation, P-selectin, however.

Baseline levels of oxLDL were not related to monocyte subtype percentage. There was no significant correlation between post-immunisation oxLDL levels and CD16<sup>+</sup> monocytes in the untreated group ( $p = 0.8929$ ,  $r = -0.103$ ); nor was there any correlation between oxLDL levels and CD16 monocyte distribution in the total population.





**Figure 4.5: Correlation between levels of IgG anti-oxidised low density lipoprotein and CD16<sup>-</sup> / CD16<sup>+</sup> monocytes**

Panels A and B show the correlation between baseline IgG anti-oxLDL levels and CD16<sup>-</sup> and CD16<sup>+</sup> monocytes respectively

#### **4.4.3 CD14<sup>high</sup>CD16<sup>+</sup> monocytes have a higher intracellular lipid content than CD16<sup>-</sup> monocytes**

Evaluation of the *in vivo* lipid content of the different subsets of monocytes revealed that CD14<sup>high</sup>CD16<sup>+</sup> cells are those with the higher amount of intracellular lipid droplets, thus suggesting their more avid phagocytosis of oxLDL; see Figure 4.6.

#### **4.4.4 Exposure of monocytes to oxidised low-density lipoprotein leads to induction of the inflammasome / IL-1 $\beta$ pathway**

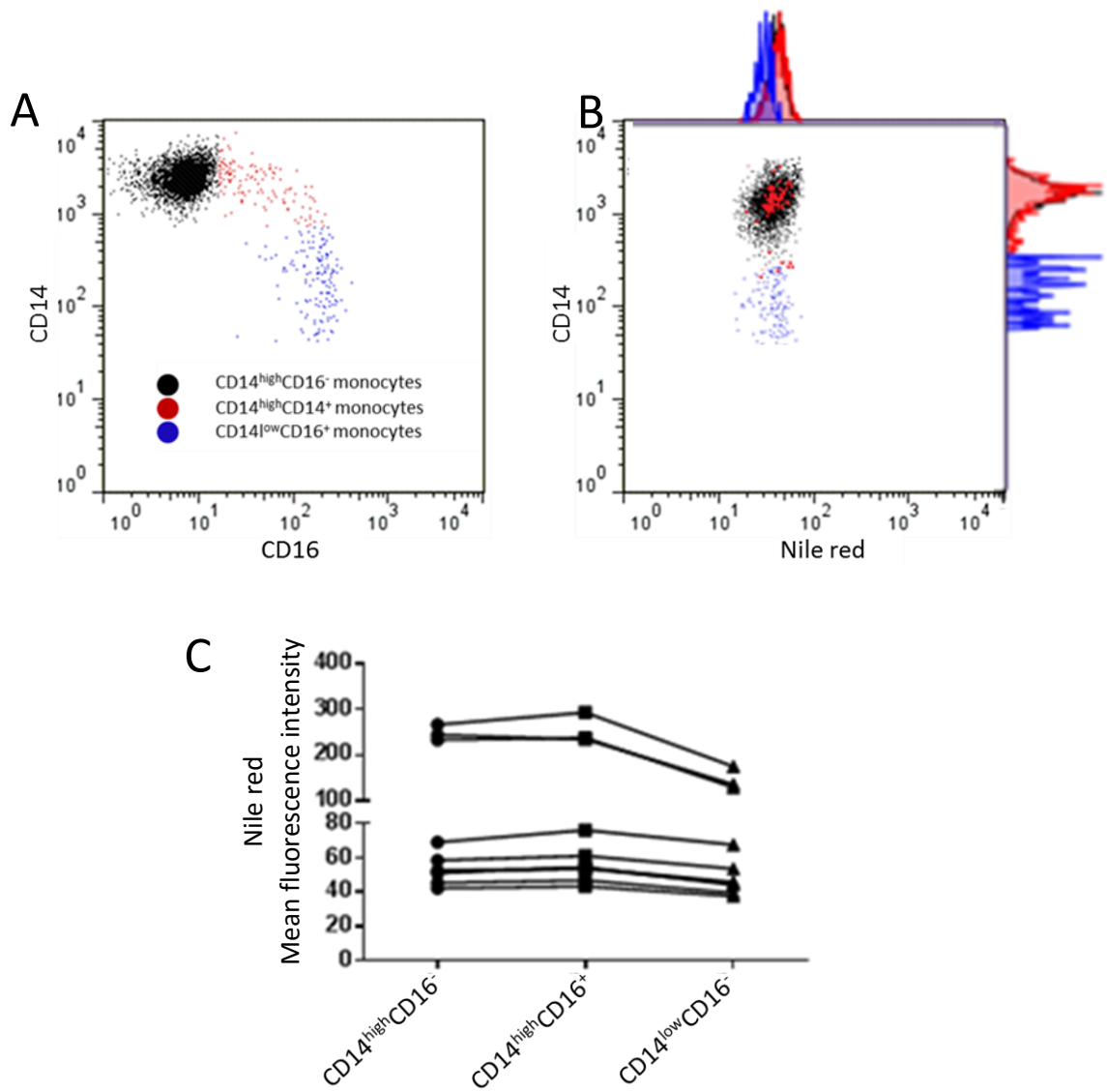
In view of the fact that CD16 is expressed on both monocytes and CD56<sup>dim</sup> peripheral blood natural killer cells, I performed PCR analysis of RNA from both isolated monocytes and whole blood in each sample. Results did not differ significantly between the two methods, indicating that the observed CD16-dependent outcomes were primarily related to monocytic CD16.

No association was identified between levels of circulating anti-oxLDL IgG and hs-CRP<sup>18</sup>, nor was there a relationship between anti-oxLDL IgG and MPO, a peroxidase enzyme that is stored in neutrophil granules and is a key part of the inflammatory response. Baseline levels of IgG anti-oxLDL were, however, linearly correlated with levels of the pro-inflammatory, inflammasome-mediated cytokines, IL-1 $\beta$  ( $p = 0.0016$ ,  $r = 0.4532$ ) and IL-6 ( $p = 0.0044$ ,  $r = 0.4902$ ), as measured using the Luminex<sup>®</sup> technique; Figure 4.7. The relationship between anti-oxLDL IgG and the inflammasome pathway was further explored by stimulating isolated monocytes with MDA-LDL, which led to induction of IL-1 $\beta$  and NLRP3 inflammasome genes (at a rate of  $2.30 \pm 0.55$  and  $1.94 \pm 0.36$  times that of monocytes not exposed to MDA-LDL respectively). An amplification in IL-1 $\beta$  synthesis occurred in response to MDA-LDL in the presence of the anti-CD16 F(ab')<sub>2</sub>; Figure 4.8.

#### **4.4.5: Malondialdehyde-modified low-density lipoprotein stimulates CD16-dependent gene induction of osteogenic molecules**

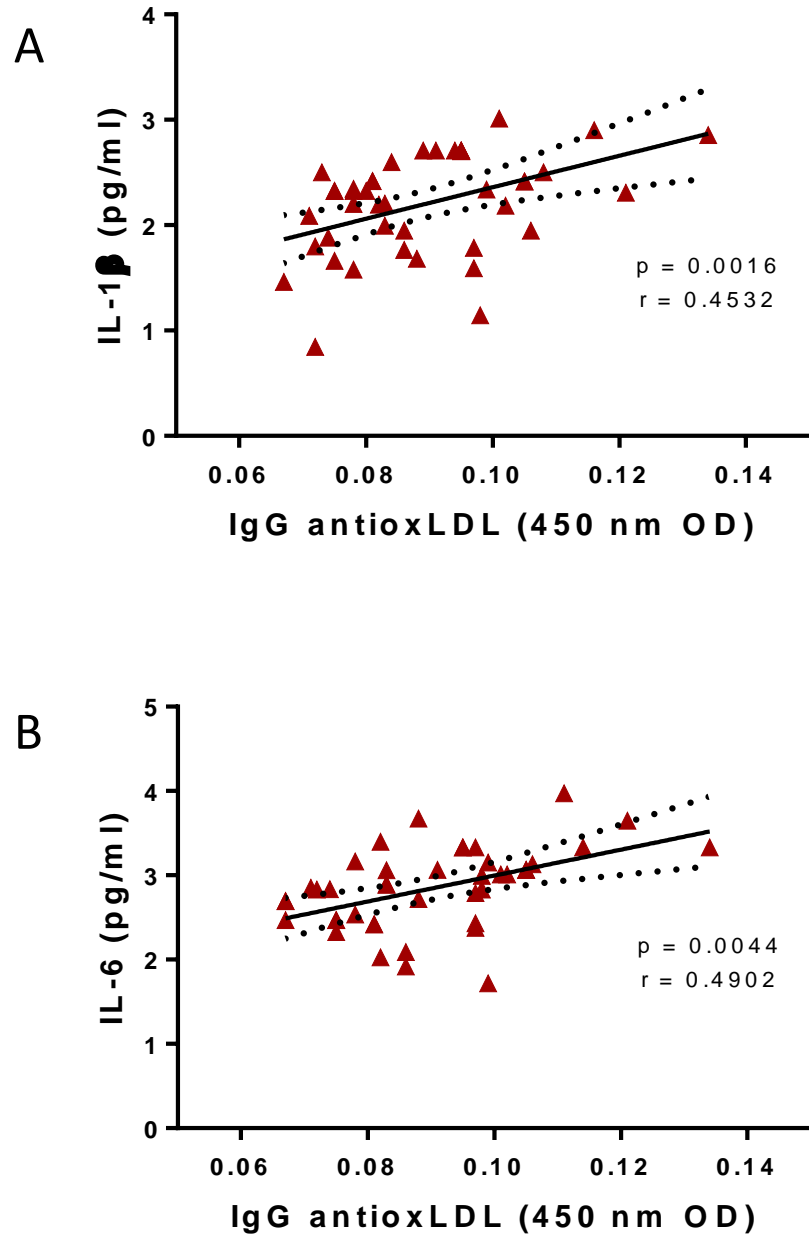
Vascular calcification is directly related to plaque burden<sup>289,290</sup> and many studies have associated LDL levels with the presence and severity of vascular calcification<sup>289,291</sup>. It has been widely demonstrated that inflammasome activation is required in the formation of intra-vessel calcium deposits<sup>292,293</sup> and calcified arteries express various regulatory factors, including BMPs<sup>289</sup>. To further assess the immunomodulatory action of CD16 in the signal transduction pathways triggered by MDA-LDL, we stimulated isolated monocytes with MDA-LDL and measured levels of osteogenic markers. We found that exposure to MDA-LDL led to induction of BMP-4 RNA expression in monocytes at a rate of more than 4 times ( $4.05 \pm 1.95$ ) that of monocytes that were not exposed to MDA-LDL. Blockade of CD16 using the anti-CD16 F(ab')<sub>2</sub> inhibited BMP-4 induction, indicating that the pathway was CD16-dependent; Figure 4.8.

In light of this finding, BMP-4 protein levels were measured in the stored serum samples obtained from the vaccination study using the ELISA technique. The rise in CD16 and reduction in oxLDL in the untreated group post-immunisation was found to also be paralleled by a significant decrease in BMP-4 levels by 13.5 %, from 1270 (IQR: 1175 – 1563) pg/ml to 1151 (IQR 852 – 1341) pg/ml ( $p = 0.0110$ ). All anti-platelet therapy regimens tested counteracted this reduction in BMP-4; Figure 4.9.



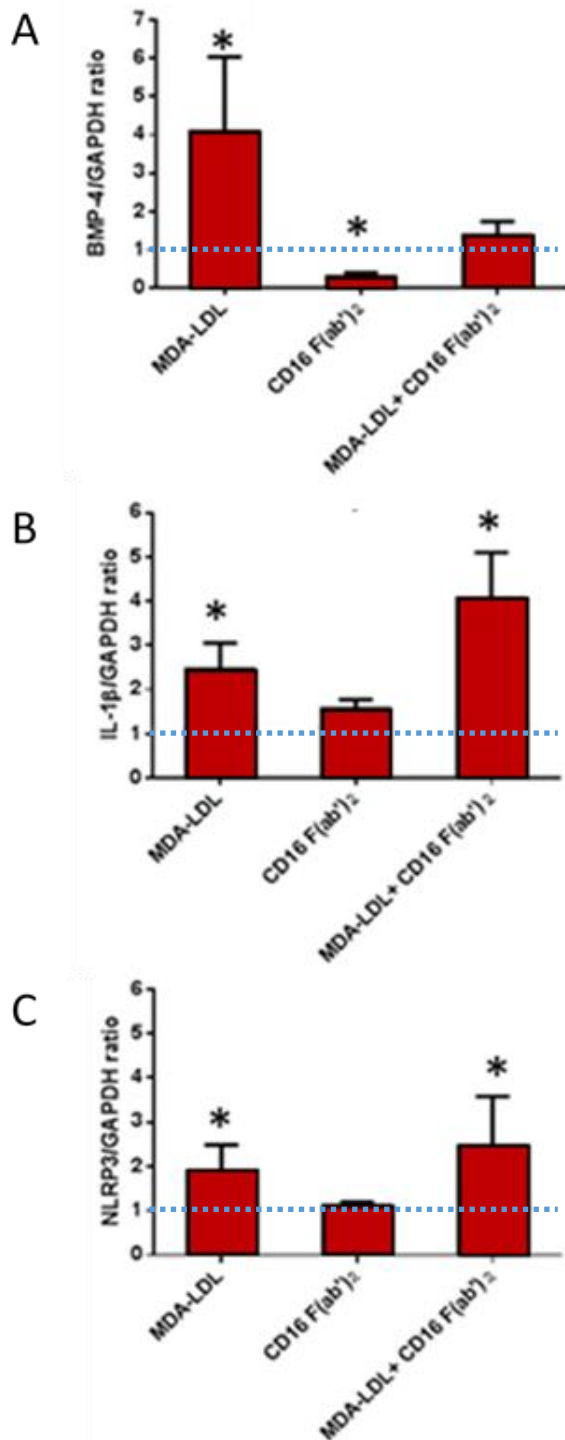
**Figure 4.6: Characterisation of lipid content within monocytes**

Panel A shows a typical monocyte phenotype profile using flow cytometry to sort the cells based on their CD14 and CD16 surface markers. Panel B shows the same cells now sorted based on their uptake of Nile red stain. Panel C shows the MFI of the Nile red fluorochrome for each monocyte subtype.



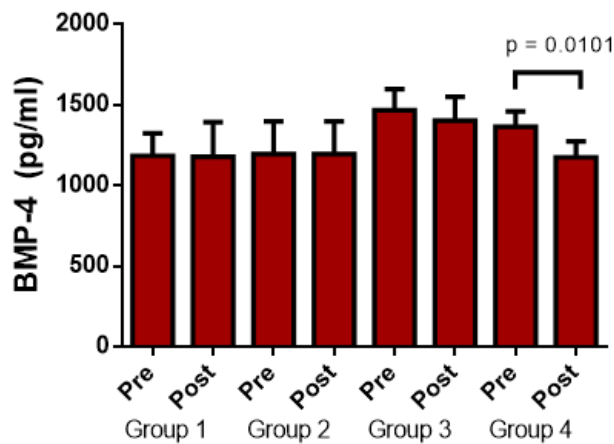
**Figure 4.7: Correlation between levels of IgG anti-oxidised low-density lipoprotein and inflammasome-mediated cytokines**

Panels A and B show the correlation between IgG anti-oxLDL and IL-1 $\beta$  and IL-6 respectively, as measured by ELISA.



**Figure 4.8: Gene induction following *in vitro* stimulation of monocytes with malondialdehyde-modified low density lipoprotein**

Stimulation of isolated monocytes with MDA-LDL *in vitro* led to induction of BMP-4 (A), IL-1β (B) and NLRP3 (C). Blockade of CD16 using anti-CD16 F(ab')<sub>2</sub> (10 μg/ml) inhibited gene induction of BMP-4 only, either with or without MDA-LDL. Data were obtained by quantitative real-time quantitative PCR and expressed as fold changes compared with unstimulated cells (dotted line) (n=3; \* p < 0.05 vs untreated cells).



**Figure 4.9: Bone morphogenetic protein 4 levels pre- and post-immunisation**

This graph shows the absolute values of BMP-4 pre- and post-immunisation.

#### **4.5 Discussion**

It has been hypothesised that the FC $\gamma$  receptor, CD16, may regulate oxLDL phagocytosis, as part of the homeostatic immunological response<sup>294</sup>. I have explored the potential role of CD16<sup>+</sup> monocytes in modulating levels of oxLDL and IgG anti-oxLDL autoantibodies in the context of an immunogenic response to influenza immunisation, which is known to generate systemic inflammation<sup>30</sup>. Immunisation has been shown earlier in this thesis (see Chapter Two) to induce a rise in CD16<sup>+</sup> monocytes, specifically the CD14<sup>high</sup>CD16<sup>+</sup> subset, in the absence of lipid profile modifications.

I found that in healthy subjects, the circulating level of IgG anti-oxLDL was inversely related to both circulating oxLDL concentration and the percentage of C16<sup>+</sup> monocytes, in keeping with the hypothesis that C16<sup>+</sup> cells act as scavengers for anti-oxLDL IgG antibodies, forming immunocomplexes with modified lipoproteins.

In the presence of mild, systemic inflammation induced by influenza immunisation, there was an expansion of the CD16<sup>+</sup> monocyte subset that was paralleled by a downregulation of oxLDL in the non-anti-platelet treated participants, possibly indicating that a higher burden of inflammation leads to greater recruitment of CD16 cells to mediate the clearance of pro-immunogenic molecules, such as oxLDL. Although there was no significant correlation between CD16 positivity within monocytes and oxLDL levels post-immunisation in this untreated group, the fact that the sample size was small (n = 10) and the reduction in oxLDL, although consistent, was small may account for this lack of significance.

Of note, the post-immunisation attenuation in oxLDL levels observed in the untreated participants (Group 4) was also present in those taking aspirin 300 mg daily (Group 1) and clopidogrel (Group 3), but not in those on aspirin 75 mg daily (Group 2) despite all treatment groups having stable levels of CD16<sup>+</sup> monocytes. The observed changes in oxLDL among all groups were contrasted by apparently opposite changes in anti-oxLDL IgG levels, although the increase in IgG anti-oxLDL did not reach significance. Despite the absence of a significant increase in IgG anti-oxLDL post-immunisation, the percentage reduction in circulating oxLDL levels was linearly related to the change from baseline of IgG antibodies post-immunisation, supporting the theory that IgG anti-oxLDL mediates the clearance of immunogenic lipoproteins in the presence of inflammation, and this process may be linked to CD16.

Furthermore, the reduction in oxLDL observed in two treatment groups (aspirin 300mg daily and clopidogrel), may be attributed to the anti-platelet effect of the drugs inhibiting activated platelet-induced oxidation of LDL, in the context of acute inflammation<sup>295</sup>. Activated platelets oxidise LDL molecules via NOX2 activation in the presence of oxidative stress<sup>296</sup> and the observed reduction in oxLDL in participants taking either aspirin 300 mg daily or a clopidogrel regime (including a loading dose) may represent effective platelet inhibition and thus reduced oxidation of lipoproteins. The fact that there was no reduction in oxLDL in the participants taking aspirin 75 mg daily (Group 2) could point towards a reduced effectiveness in achieving platelet inhibition with the lower dose of aspirin. Indeed, I previously discussed, in Chapter Two of this



thesis, the fact that aspirin 75 mg may be less efficacious than aspirin 300 mg both at reducing soluble the platelet-activation marker, P-selectin, and at modulating the phenotype of circulating monocytes in this model of inflammation <sup>18</sup>.

Evaluation of the *in vivo* lipid content of the different subsets of monocytes identified that CD14<sup>high</sup>CD16<sup>+</sup> cells are those with the higher amount of lipid droplets, suggesting their more avid phagocytosis of oxLDL compared with other monocyte subtypes - as other authors have previously observed and reported to be independent from conventional scavenger receptors <sup>294</sup>. As there is a higher number of circulating CD16<sup>+</sup> monocytes present in acute inflammation, this could also explain the reduction in oxLDL in the untreated group.

I subsequently assessed the immunomodulatory action of CD16 in the signal transduction pathways triggered by MDA-LDL. This latter was found to directly activate the CD16 Fc $\gamma$  receptor and to stimulate CD16-dependent gene induction of the osteogenic molecule, BMP-4. In the setting of immunisation-induced inflammation, the rise in CD16<sup>+</sup> monocyte percentage that was associated with the reduction in oxLDL in the untreated group was paralleled by a reduction in BMP-4, whilst anti-platelet therapy counteracted the changes in both CD16 and BMP-4. These data suggest that CD16 plays a role in the regulation of monocyte/macrophage osteogenic pathways in response to MDA-LDL. Given the emerging role of BMPs, particularly BMP-2 and BMP-4, in promoting vascular calcification <sup>297 298</sup>, modulation of these pathways via CD16 may provide a target in the prevention of chronic calcific arteriopathy.

MDA-LDL also activated the inflammasome pathway, which controls IL-1 $\beta$  and thus IL-6 production, although this was found to be in a CD16-independent manner. Of note, a slight amplification of the IL-1 $\beta$  gene occurred in the presence of CD16 blockade, indicating that CD16 can cross-block MDA-LDL from binding to other receptors. In Chapter Two of this thesis, I previously identified a tendency towards a reduction in circulating IL-1 $\beta$  levels in the presence of CD16<sup>+</sup> monocyte expansion, although this did not reach statistical significance, which was not present in those taking anti-platelet therapy <sup>18</sup>. CD16 is a natural ligand of the CR3 (CD11b/CD18) receptor <sup>299</sup> that mediates the activation of the inflammasome pathway induced

by cholesterol crystals and modified LDL, which is not only implicated in foam cell formation, but is also required for T and B cell stimulation and consequent antibody production<sup>300-302</sup>. Further research is required to establish whether similar changes in inflammasome activation are present in chronic inflammatory disease, such as atherosclerosis, and whether reduced CD16 activity in this context can modulate the accumulation of oxLDL and IgG anti-oxLDL autoantibodies and thus modify the amplification of the adaptive immune response mediated by the inflammasome pathway.

#### **4.6 Conclusion**

CD16 is an FC  $\gamma$  receptor that is activated upon binding to IgG and may mediate the clearance of IgG antibodies against oxLDL. The present data indicate that CD16<sup>+</sup> monocytes may regulate the clearance of oxidised lipoproteins and their systemic accumulation, possibly through the internalisation of circulating oxLDL / IgG immunocomplexes mediated by Fc  $\gamma$  receptors, including CD16. Furthermore, CD16 is directly activated by MDA-LDL and up-regulation of this biomolecular pathway (consequent to either reduced clearance of immunocomplexes via Fc  $\gamma$  receptors and/or abnormal synthesis of oxLDL) may be implicated in the induction of osteogenic signalling cascades, potentially leading to arterial calcification.

## **CHAPTER FIVE:**

## **DISCUSSION**

## **5.1 Introduction**

Until recent years, the pathophysiology of atherosclerosis has principally been described as a disorder of lipid storage, and pharmacological therapies have thus primarily been targeted at reducing serum cholesterol. As the underlying cellular mechanisms of atherogenesis have been gradually elucidated, it has become apparent that chronic inflammation is a key mediator of the processes that lead to plaque formation.

This thesis has explored the potential role of several novel inflammatory biomarkers in the pathways governing the development of atherosclerosis and its complications, and provides evidence that anti-platelet agents may be beneficial in limiting further disease progression, through both their anti-platelet and anti-inflammatory actions.

## **5.2 Intermediate monocytes as a potential therapeutic target**

The primary focus of this PhD was to assess the role of CD14<sup>high</sup>CD16<sup>+</sup> monocytes in the context of an underlying inflammatory process and to ascertain whether the COX inhibitor, aspirin, or P2Y<sub>12</sub> receptor inhibitors are able to modify the circulating levels of these pro-inflammatory monocytes.

An important finding from my work was that anti-platelet therapy, namely aspirin, clopidogrel or ticagrelor, was capable of preventing a rise in circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes in the presence of an inflammatory stimulus. The data obtained from this study was in accordance with previous work demonstrating an expansion in the intermediate monocyte subset in the presence of acute, systemic inflammation<sup>31</sup>, and I have been able to show that all of the tested anti-platelet agents were able to counteract this response.

Part of the rationale behind choosing both P2Y<sub>12</sub> inhibitors and aspirin was to ascertain whether attenuation of the CD14<sup>high</sup>CD16<sup>+</sup> subgroup was occurring primarily due to an anti-platelet effect, an anti-inflammatory effect, or a combination of these factors. The fact that all three treatments were effective in suppressing the rise in intermediate monocytes suggests that the

effect was primarily related to inhibition of platelet function, particularly as there was no reduction in hs-CRP levels post-immunisation in any of the treatment groups.

Many studies have linked CD14<sup>high</sup>CD16<sup>+</sup> monocytes to increased cardiovascular events<sup>22 225</sup> and previous work has shown a correlation between a reduction in this monocytic subset, and a decrease in subclinical atherosclerosis in obese patients undergoing rapid weight loss<sup>189</sup> but there is no evidence to date that a reduction in circulating CD14<sup>high</sup>CD16<sup>+</sup> monocyte levels, without modifying cardiovascular risk factors, will result in attenuation of atherosclerosis. Having shown that anti-platelet therapy can suppress the rise in intermediate monocytes in the context of acute inflammation, the next step in this area of our research will be to assess whether anti-platelet therapy is capable of directly attenuating circulating intermediate monocytes in patients with atherosclerotic plaque disease, which could potentially indicate a role for these drugs in the long term management of atherosclerosis.

### **5.3 The role of anti-platelet therapy in modifying biomarkers of cardiovascular risk**

Following on from the above research where I demonstrated that anti-platelet therapy can attenuate pro-inflammatory monocyte levels, I went on to assess whether these agents can similarly modify other novel biomarkers of cardiovascular risk. Data from the last 15 years suggests that traditional indicators of cardiovascular risk, such as LDL, may soon be superseded by measures of inflammation<sup>303</sup>. Hs-CRP, as a non-specific marker of inflammation, now forms part of the standard assessment of cardiovascular risk in asymptomatic adults in the USA<sup>304</sup>.

In view of the emerging role of netrin-1 in both inflammatory pathways and as a novel therapeutic target in cardiovascular disease, we selected this protein for further assessment. A clinical study previously undertaken by other members of our research group involved administering aspirin for 28 days to healthy subjects<sup>254</sup> – there was frozen serum available from this cohort and I measured netrin-1 levels in these samples. I identified a small but consistent decline in netrin-1 levels in these healthy people following aspirin therapy. Previous work had shown that netrin-1 levels fall in the presence of acute inflammation<sup>217</sup> and thus these findings

were somewhat unexpected. I subsequently confirmed a reduction in netrin-1 levels in participants exposed to an inflammatory stimulus and was ultimately able to establish the presence of an interaction between netrin-1 and COX, possibly through COX-derived prostaglandin E<sub>2</sub>. Netrin-1 plays a complex role in leucocyte recruitment and, whilst this work has shown that it is possible to modify netrin-1 levels using anti-platelet therapy, whether pharmacological up- or downregulation of netrin-1 levels could have potential benefits in a clinical setting has yet to be determined.

Having demonstrated the ability to manipulate the circulating levels of intermediate monocytes with anti-platelet agents, I then performed further experiments to determine whether this could induce changes in the levels of circulating oxLDL and IgG anti-oxLDL. Although I was unable to show a direct link between levels of these two key biomarkers of atherogenesis and monocytic CD16 positivity, the data did indicate that CD16<sup>+</sup> monocytes may regulate the clearance of oxidised lipoproteins, and I have speculated that this may be via the internalisation of circulating oxLDL / IgG immunocomplexes mediated by CD16. In view of the differing effects of low-dose (75 mg daily) aspirin compared with higher doses of aspirin and with clopidogrel, I hypothesised that the latter two therapies were capable of inhibiting activated platelet-induced oxidation of LDL but a direct link was not proven and further work will need to be done to ascertain whether oxLDL levels can be modified by these drugs in the context of cardiovascular disease.

#### **6.4 Future work**

In view of the fact that atherosclerosis is a chronic, inflammatory process, my PhD has thus far investigated whether anti-platelet agents are able to modify the progression of atherogenesis via their anti-inflammatory effects. Having established further information regarding some of the complex pathways involved in mild inflammation in healthy people, our next objective will be to determine whether the hypothesis that platelet inhibition, achieved via administration of anti-platelet drugs, reduces the activation of monocytes and the upregulation of pro-

inflammatory CD14<sup>high</sup>CD16<sup>+</sup> monocyte expression in patients with cardiovascular risk factors or indeed with established cardiovascular disease is correct.

We are currently in the early stages of conducting a clinical study with the aim of identifying whether anti-platelet therapy is capable of attenuating the expansion of circulating CD14<sup>high</sup>CD16<sup>+</sup> monocytes in patients with chronic inflammation related to atherosclerosis and, if so, whether this might be a potentially useful therapeutic strategy. The study design and preliminary data are discussed in Appendix One.

Once the study proceeds, I hope to produce data that further elucidates the mechanisms underlying the attenuation of monocyte CD16<sup>+</sup> acquisition following anti-platelet therapy by measuring additional biomarkers and conducting platelet function assays. If anti-platelet therapy is indeed capable of modifying the circulating monocyte phenotype in patients with cardiovascular risk factors, this could have important implications for the pharmacological management of atherosclerosis.

Furthermore, when conducting the clinical study described above I will measure levels of netrin-1 in patients with established cardiovascular risk factors with the aim of establishing whether their baseline levels differ from that of the general population and, if so, whether anti-platelet agents are capable of normalising this. Measurement of additional markers of COX function may enable a better understanding of how netrin-1 interacts with prostanoids and, given the proven role of netrin-1 in monocyte trafficking, could contribute to the development of future targeted therapies with the aim of modulating netrin-1 and thus leucocyte migration.

Additionally, samples from patients with cardiovascular risk or with established atherosclerosis will allow us to establish whether alterations in baseline inflammasome activation are present in these subjects and to further study the interactions between CD16 and oxLDL / IgG anti-oxLDL antibodies. Such data could facilitate the ultimate development of techniques to modify the amplification of immune responses that lead to atherogenesis.

In the future, it is hoped that the work described here may lead to a large-scale long-term longitudinal study to definitively ascertain the effect of anti-platelet therapy on atherosclerosis

progression. Identification of the molecular mechanisms underlying inflammation-driven monocytic CD16<sup>+</sup> acquisition and their subsequent interactions with other markers of inflammation, including netrin-1 and oxLDL, may contribute to our understanding of the pathophysiology underlying atherogenesis as well as novel pharmacological options to interrupt the immunological pathways that lead to atheroma formation.



## **CHAPTER SIX:**

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## **APPENDIX ONE**

### **Appendix 1.1 Study design**

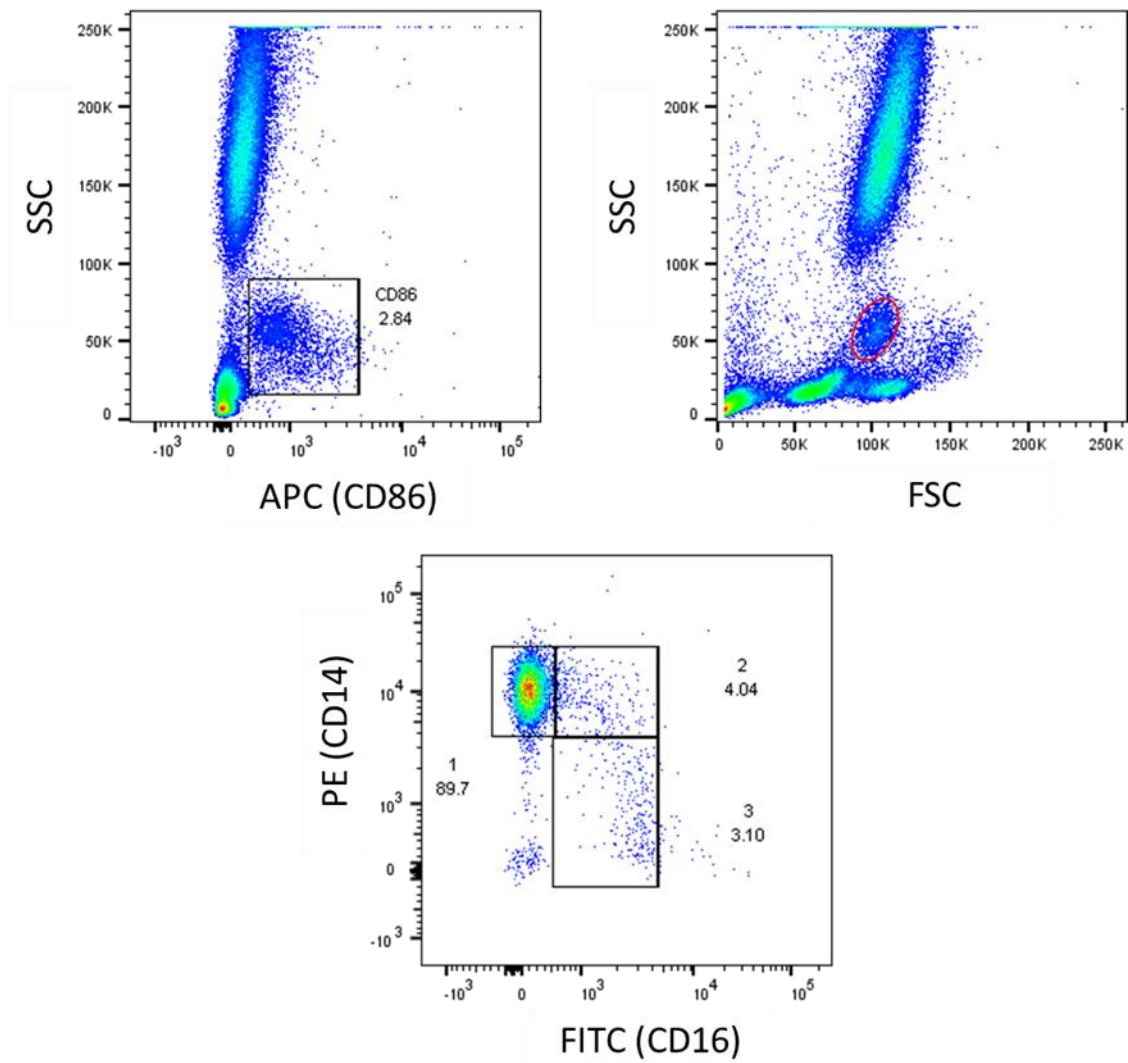
This study will aim to recruit up to 60 patients with cardiovascular risk factors but no prior cardiovascular events from hypertension clinics at Guy's and St Thomas' NHS Foundation Trust. Those with underlying cardiovascular risk factors, otherwise healthy, with no prior cardiovascular event and asymptomatic for atherosclerotic disease will be screened for recruitment.

Patients will have blood samples taken at baseline and then receive 28 days of treatment with aspirin 75 mg once daily, at which point they will undergo further blood sampling. Participants will additionally be assessed for increased carotid IMT and/or carotid atherosclerotic plaque disease as diagnosed on carotid ultrasound. Those with evidence of carotid plaques will be advised to continue on lifelong aspirin and their general practitioners will be informed.

Carotid ultrasonography will be performed by Dr Ben Yu Jiang at the Clinical Research Facility of St Thomas' Hospital. Carotid IMT will be measured by ultrasonography (Accuson Sequoia 512 machine with an 8-MHz transducer). IMT is taken as the distance between the blood / intima borderline and the media/adventitia borderline, and evaluated on the wall of the common carotid arteries bilaterally, specifically in the distal 1 cm of the artery just proximal to the bulb. Mean carotid IMT will be measured using a semi-automated computer analysis system, and confirmed by taking the mean of three manual readings at 20mm intervals along the CCA. Mean values of IMT in the anterior and posterior walls of both arteries are to be recorded for analysis.

Blood samples will be collected as described in Section 2.3.3. A whole blood sample in EDTA will be sent to the Viapath laboratory at Guy's and St Thomas' NHS Foundation Trust to measure HbA1c levels and a full blood cell count. Similarly, a serum sample will be sent to Viapath to measure renal function, liver function, a cholesterol profile and CRP. I will retain serum and plasma aliquots for future use, the preparation of these samples is detailed in Section 2.3.3.

In order to assess monocyte phenotype, whole blood will be immunostained, as detailed in Section 2.3.4, with the following antibodies: PE-mouse anti-human CD14 (BD Bioscience), 5 FITC-conjugated anti-human CD16 (BD Bioscience), APC-mouse anti-human CD86 (BD Bioscience). The immunostained samples will be analysed using a BD FACSCalibur (BD Bioscience) flow cytometer to determine monocyte subset distribution. A total of 50,000 leucocyte events are to be acquired and post-acquisition analysis will be performed using FlowJo (version 10) software. Cells that are positive for CD86 (a pan-monocytic marker) staining will be used to gate for cells with a typical monocytic FSC versus SSC profile, and this population will then be analysed based on CD14 and CD16 expression, as previously described in Section 2.3.5; Figure 5.1. The minor change in the flow cytometry methodology utilising CD86 to improve the quality of monocytic gating has been implemented following feedback from experts in the flow cytometry field, who explained that by gating for monocytes using CD14 and CD16 only, we would risk including natural killer cells.



### Appendix Figure 1.1: Flow cytometry analysis

Panel A shows the gating of cells that were positive for the pan-monocytic stain, CD86. These cells were then selected from the monocytic group, as identified on the typical FSC vs SSC plot shown in Panel B. Finally, this population of cells were then gated into the respective monocyte populations (CD14<sup>high</sup>CD16<sup>-</sup> - marked as 1, CD14<sup>high</sup>CD16<sup>+</sup> - marked as 2, and CD14<sup>low</sup>CD16<sup>+</sup> - marked by 3).

ELISA kits to measure the following markers have been purchased: netrin-1 (SEB827Hu, Cloud-Clone Corp.), PGE<sub>2</sub> (ID: MBS007171, MyBioSource, Inc., USA), TXB<sub>2</sub> (ID: CSB-E08046h, Cusabio, China), VCAM-1 (ID: DVC00, R&D Systems, UK), and ICAM-1 (ID: 850.540.096, Diaclone, France).

All ELISA experiments will be carried out according to the manufacturer specifications.

The standard deviation for total pro-inflammatory monocytes measured within a population of patients with cardiovascular risk factors and either increased IMT or carotid plaque disease was 6 % in our pilot data. We therefore calculated a sample size of 20 patients per group for the clinical study described above, taking a minimum detectable difference in means between pre- and post-treatment of 4 %, with power 0.9 and significance level 0.05. All statistical analyses will be performed using GraphPad Prism (version 7.3) software. Parametric data will be expressed as the mean  $\pm$  SEM, whilst non-parametric data will be expressed as the median with IQR. ANOVA will be used to compare percentage variation of each study variable between the 3 treatment groups (aspirin, clopidogrel or untreated) in the study. Baseline and post-treatment values will be compared within each group using a paired parametric or non-parametric test as appropriate.



Participant demographics for the initial four patients recruited into the study are reported in Appendix Table 5.1.

<b>Participant Demographics (n = 4)</b>	
<b>Age (years)</b>	<b>62.00 (53.00 – 73.25)</b>
<b>Sex</b>	<b>2 male, 2 female</b>
<b>Smoking history</b>	<b>3 ex-smokers, 1 non-smoker</b>
<b>BMI (weight kg/ height m<sup>2</sup>)</b>	<b>32.30 (26.85 – 37.22)</b>
<b>Systolic blood pressure (mmHg)</b>	<b>158 (140 – 165)</b>
<b>Diastolic blood pressure (mmHg)</b>	<b>95 (84 – 100)</b>
<b>WCC (x10<sup>9</sup>/l)</b>	<b>5.25 (4.43 – 6.60)</b>
<b>CRP (mg/l)</b>	<b>0.5 (0.0 – 1.8)</b>
<b>Triglycerides (mmol/l)</b>	<b>1.20 (0.51 – 2.34)</b>
<b>HDL (mmol/l)</b>	<b>1.53 (1.16 – 2.08)</b>
<b>LDL (mmol/l)</b>	<b>2.44 (1.67 – 2.58)</b>
<b>HbA1c (mmol/mol)</b>	<b>39.5 (37.5 – 49.0)</b>
<b>Mean IMT (mm)</b>	<b>0.79 (0.18 – 1.01)</b>

**Appendix Table 1.1: Participant demographics**

	<b>CD14<sup>high</sup>CD16<sup>-</sup> %</b>	<b>CD14<sup>high</sup>CD16<sup>+</sup> %</b>	<b>CD14<sup>low</sup>CD16<sup>+</sup> %</b>
Hypertension patients (n = 4)	<b>88.70 (83.70 – 92.35)</b>	<b>5.84 (2.34 – 11.84)</b>	<b>1.22 (0.67 – 1.44)</b>

**Appendix Table 1.2: Preliminary data on monocyte subsets in hypertension patients**

Monocyte counts are also expressed in percentages rather than absolute cell counts due to a discrepancy with the data (results from the first participant that was studied show higher cell count values for the three monocytic subtypes due to larger total number of events being recorded for analysis).