Platelet Function in Inflammatory Arthritis

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Supervisors

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Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a Medical Doctorate (MD) is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Table of Contents

Pages:

Abbreviations 10

Chapter 1: Introduction

1.1 Background 16
1.2 Rationale for current investigation 17
1.3 Investigation of platelet ADP pathway in inflammatory arthritis 18
1.4 Study of the inhibitory effect of sulfasalazine on platelet function 18
1.5 Examination of the effect of anti-TNFα on platelet function 20
1.6 Research Collaboration 20
1.7 Relevant Publications 21

Chapter 2: Subjects and Methods

2.1 Patients with Inflammatory Arthritis 23
2.2 Healthy Volunteers 24
2.3 Patients with cardiovascular disease 24
2.4 Ethical Approval 24
2.5 Disease activity 25
2.6 Clinical Data 25
2.7 Phlebotomy and Platelet Preparation 25
2.8 Platelet Function Assay Sampling and Flow 26
Chapter 3: The Influence of Disease Activity on Platelet Function in Patients with Inflammatory Arthritis

3.1 Background

3.2 Rationale

3.3 Patients and Methods
   3.3.1 Disease Activity
   3.3.2 Platelet Function
   3.3.3 Patient Groups
   3.3.4 Statistics

3.4 Results
   3.4.1 Demographic Data:
   3.4.2 Clinical Data:
   3.4.3 Platelet Data:

3.5 Conclusion

Chapter 4 Pro-inflammatory cytokines and platelet ADP reactivity

4.1 Background

4.2 Rationale

4.3 Patients and Methods
   4.3.1 Patient clinical data
Chapter 5 Investigation of the inhibitory effect of sulfasalazine on arachidonic acid mediated platelet aggregation.

5.1 Background 55
5.2 Rationale 55
5.3 Patients and Methods 56
5.3.1 Clinical data 57
5.3.2 Phlebotomy and platelet preparation 57
5.3.3 Platelet Function Assay 57
5.3.4 Platelet function with sulfasalazine and metabolites in vitro 58
5.3.5 Statistics 59
5.4 Results 60
5.4.1 Patient data 60
5.4.2 Patient characteristics 60
Chapter 6 The impact of anti-TNF therapy on platelet function, insulin metabolism, and lipid profile in patients with inflammatory arthritis

6.1 Background
6.2 Rationale
6.3 Patients and Methods
6.3.1 Patient clinical data
6.3.2 Phlebotomy and platelet preparation
6.3.3 Platelet function assay
6.3.4 Statistics
6.4 Results
6.5 Conclusion

Chapter 7 Discussion

7.1 Inflammation and platelet function in patients with IA.
7.2 Pro-inflammatory cytokines and the platelet ADP pathway
7.3 Inhibition of platelet function by Sulfasalazine
7.4 The impact of anti-TNF therapy on platelet function, insulin metabolism, and lipid profile.
7.5 Conclusion

REFERENCES
List of Tables

3.1 Patient Demographics 35
3.2 Clinical Data 37
4.1 Clinical Data 50
5.1 Patient Characteristics 61

6.1 Demographics 76
6.2 Disease Activity & Serological Data 78
6.3 Platelet Function Data 80
List of Figures

3.1: Platelet aggregation is enhanced in the Active group in response to submaximal concentrations of ADP. 38

3.2 There is no difference in platelet aggregation between the Active and Control groups in response to the other agonists 39

4.1 Effect of pro-inflammatory cytokines on ADP-induced platelet aggregation. 47

4.2 P-Selectin expression of resting vs ADP stimulated PRP samples following cytokine incubation, as measured by flow cytometry. 48

4.3 Disease activity indices pre and post treatment with the IL 6 inhibitor, tocilizumab 51

4.4 Log dose-response curves of platelet aggregation in response to various agonists (collagen, epinephrine, arachidonic acid, and ADP) in samples taken from RA patients (n=6), pre-and post treatment 52

5.1 Platelet aggregation in response to arachidonic acid is significantly inhibited in both the Sulfasalazine and Aspirin group compared to those on Other DMARDs 64

5.2 ADP induced platelet aggregation is significantly greater in both groups of IA patients compared to CVD patients taking aspirin. 66

5.3 Maximal aggregation responses reveal a uniquely enhanced ADP response in patients with IA compared to CVD patients taking aspirin. 67

5.4 Platelet aggregation in response to Arachidonic Acid is inhibited by sulfapyridine, 5-ASA, and sulfasalazine 69

6.1 Pre and post-treatment platelet aggregation responses (clockwise from top left) to Arachidonic Acid (AA), Collagen, TRAP, and epinephrine are similar across the Entire Cohort. 81

6.2 Platelet reactivity to ADP is decreased following treatment with Anti-TNF therapy, p<0.05. 82

6.3 There is a significant reduction in ADP induced platelet aggregation among Responders to Anti-TNF therapy only. 83
ABBREVIATIONS

AA=arachidonic acid
ADP=adenosine diphosphate
Epi=epinephrine
Coll=collagen
TRAP=thrombin receptor activating peptide
ESR=erythrocyte sedimentation rate
CRP=C-reactive protein
Fib=fibrinogen
DAS=disease activity score
VAS=visual analogue scale
VASDA=visual analogue scale of disease activity
EMS=early morning stiffness
TJC=tender joint count
SJC=swollen joint count
TNF=tumour necrosis factor
DMARD=disease modifying anti-rheumatic drug
NSAID=non-steroidal anti-inflammatory drug
SSZ=sulfasalazine
MTX=methotrexate
HCQ=hydroxychloroquine
IL=interleukin
CVD=cardiovascular disease
IA=inflammatory arthritis
RA=rheumatoid arthritis
PsA=psoriatic arthritis
SpA=spondyloarthritis
AS=ankylosing spondylitis
OA=osteoarthritis
PRP=platelet rich plasma
DMSO=dimethyl sulfoxide
mm=millimolar
nm=nanomolar
\( \mu m = \) micromolar
\( g/l = \) grammes per litre
\( g/dl = \) grammes per decilitre
\( agg = \) aggregation
LTA = light transmission aggregometry
ACR = American College of Rheumatology
EULAR = European league against arthritis
MMUH = Mater Misericordiae University Hospital
DAMC = Dublin Academic Medicine Centre
RCSI = Royal College of Surgeons of Ireland
Summary

Patients with inflammatory arthritis (IA) are at increased risk of adverse cardiovascular events. While traditional risk factors are important, recent evidence highlights the key role of inflammation in cardiovascular disease (CVD). Platelets play a key patho-physiological role in the complex chain of events leading to the occlusion of a coronary artery. To date, platelet function in the IA population has been poorly investigated.

We examined platelet function in patients with IA and assessed the influence of disease activity on platelet reactivity. Our data demonstrate an enhanced platelet response, unique to ADP stimulation, in patients with inflammatory arthritis whose disease is poorly controlled. Remarkably there was no difference in platelet reactivity noted in response to any of the other agonists tested (arachidonic acid, collagen, epinephrine, and TRAP).

Using both ex vivo measures of platelet function and in vitro assays we then investigated the amplifying effect of several pro-inflammatory cytokines (IL-1β, IL-6, anti-TNFα and IL17α) on the platelet ADP pathway and also found a decreased platelet ADP response in patients with previously refractory disease who were treated with the IL-6 inhibitor tocilizumab.

Following our observation of an, heretofore unknown, inhibitory effect of sulfasalazine therapy on platelet aggregation, we subsequently demonstrate that this is due to both the parent compound and its metabolites, and confined to the arachidonic acid pathway.

Finally, we prospectively assessed the impact of improved disease control with anti-TNFα agents on platelet function, insulin metabolism, and cholesterol levels in patients with IA. We found patients who respond
to anti-TNFα therapy also achieve a normalization of platelet function and decreased insulin resistance, with no change in lipid profile.

Taken together, these findings offer novel mechanistic insights into how inflammation and its treatment influences platelet function and CVD risk in this vulnerable patient population, for whom anti-platelet guidelines are currently lacking.

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**Dedication**

To Alison, my beautiful wife, confidant, and friend, thanks for being you.
Chapter 1: Introduction
1.1 Background

Patients with inflammatory arthritis (IA) are at increased risk of adverse cardiovascular events (1). Moreover, patients with IA have increased mortality rates following a first acute cardiovascular event (2). Following a diagnosis of IA (rheumatoid, psoriatic, ankylosing spondylitis and the seronegative spondyloarthropathies) the 10 year risk of myocardial infarction is more than twice that of the general population and equivalent to that of patients with Type 2 diabetes mellitus (3). While traditional risk factors are known to play a role in the pathogenesis of cardiovascular events, recent work has highlighted the key role of inflammation in atherothrombosis (4) and as a risk factor for cardiovascular disease (CVD) in patients with rheumatoid arthritis (5). A significant body of research in CVD in rheumatoid arthritis (RA) has focused on the prevalence of conventional risk factors, abnormalities of lipid and glucose metabolism, endothelial cell dysfunction, and medication effects.

Myocardial infarction results from a complex chain of events leading to the thrombotic occlusion of a coronary artery. Platelets are central to the development of these thrombotic events (4). It is well established that inflammation activates platelets (6) and that platelet-derived mediators in turn potentiate the inflammatory response (4). To date, platelet function in the IA population has not been well characterised. Previous studies have shown that patients with inflammatory rheumatic conditions exhibit increased \textit{in vitro} platelet aggregation (7). However, these patients were not characterized in terms of disease activity. More recent studies have shown a strong correlation between elevated markers of disease activity (CRP, ESR) and markers of platelet activation (CD62P/P-selectin, CD63) in RA (8). However, the functional responsiveness or reactivity of these platelets was not assessed and therefore a significant gap in the medical scientific literature was identified, regarding an association or otherwise between platelet function and disease activity in patients with IA, and any potential mechanisms which might underpin same.
1.2 Rationale for initial investigation

Research into the underlying pathophysiology of the increased risk in inflammatory arthritis has expanded in recent years and substantial interest has been directed into endothelial dysfunction in inflammation. Despite this intensive research, cardiovascular mortality is still unacceptably high in this group. Moreover recent clinical data especially from studies where Cox2 inhibitors were used have added a degree of complexity to managing these patients. Finally, there are no agreed guidelines on specific anti-platelet therapy in patients with inflammatory arthritis. While previous studies have demonstrated abnormalities of platelet activation markers, platelet turnover and platelet count in patients with IA (7, 8), platelet function has not been evaluated critically in these patients with specific attention to disease activity. Moreover, assays of platelet function have a degree of complexity that calls into question the limited number of studies to date.

Platelet reactivity is a measure of how easily platelets will adhere to one another to form thrombus. Several lines of evidence suggest that platelet reactivity is useful in clinical practice to predict the risk of future adverse cardiovascular events (9-12).

Therefore, we decided to examine platelet reactivity in the IA population and to assess the influence of disease activity on platelet function.

Standard platelet function assays using light transmission aggregometry typically assess the platelet response to single agonists at maximal concentrations and have limited clinical applicability (13, 14). Platelet aggregation in vivo is complex and involves multiple agonists, receptor-effector mechanisms and signaling pathways (4). Several lines of evidence suggest that increased platelet reactivity as assessed by the aggregation response to agonists such as arachidonic acid (AA), adenosine diphosphate (ADP), collagen, epinephrine and thrombin receptor activating peptide (TRAP) is associated with recurrent ischaemic
events (15). We developed an assay that measures the response of platelets to all of these agonists at multiple concentrations, simultaneously (16). We used this novel assay of platelet reactivity and compared the *ex vivo* platelet function of patients with active inflammatory disease to that of patients with no clinical evidence of inflammation. We found an enhanced platelet response, particular to the adenosine diphosphate pathway, in those patients with objective evidence of active inflammation compared to those in a state of disease remission. The results of this initial investigation are detailed in Chapter 3.

1.3 Investigation of platelet ADP pathway in inflammatory arthritis

Given the main finding of our initial cross-sectional study, we then examined in vitro the effect of a range of pro-inflammatory cytokines known to be involved in the pathogenesis of IA, namely interleukin 1 beta (IL1β), tumour necrosis factor alpha (TNFα), interleukin 17 alpha (IL17α), and interleukin 6 (IL6) (17, 18), on the platelet ADP pathway in samples from healthy volunteers. We found that three of these cytokines (IL1β, TNFα, and IL6) significantly increased the platelet response to ADP, with IL6 demonstrating the greatest amplificatory effect.

Interestingly IL-6 plays a key pathogenic role in both RA and CVD (19), and previous evidence also demonstrates that IL6 increases platelet reactivity, particularly in response to ADP (20). It is not known if treatment targeted against the IL-6 pathway alters platelet behaviour in patients with RA. Therefore, we also investigated the impact of IL6 receptor inhibition with tocilizumab therapy on platelet function in patients with refractory RA and demonstrate that improved disease control with tocilizumab is accompanied by a reduced platelet response to ADP in these patients. Both of these findings are described in Chapter 4.

1.4 Study of the inhibitory effect of sulfasalazine on platelet function

One of our earliest observations from our initial investigation was that patients who were receiving sulfasalazine therapy had a poor platelet aggregation response to arachidonic acid. However, this observation was confounded by concomitant NSAID use, so we chose to examine the
potential anti-platelet effect of sulfasalazine in patients who were
definitely avoidant of all NSAID use and, in vitro, in samples from healthy
volunteers.

Sulfasalazine is an older generation anti-rheumatic drug first synthesized
in the 1940’s as a combination of an antibiotic (sulfapyridine) and an
NSAID (5-aminosalicylic acid) (21). Patients with rheumatoid arthritis (RA)
who regularly take sulfsalazine have a significantly reduced risk of
myocardial infarction (MI), independent of disease severity, (hazard ratio
0.82, p<0.05) (5). The mechanisms underlying this cardiovascular risk
reduction with sulfasalazine therapy are not entirely understood.
Sulfasalazine is a pro-drug composed of two moieties, sulfapyridine and
5-aminosalicylic acid (5-ASA), linked by an azo bond (21). Approximately
10-15% of the parent compound is absorbed from the small intestine, with
10% of the 5-aminosalicylate moiety and 40-60% of the sulfapyridine
component absorbed from the large intestine following cleavage of the
azo bond by colonic bacteria (22). Plasma concentrations of all three
substances in RA patients receiving stable doses of sulfasalazine have
recently been reported (22). Sulfasalazine inhibits platelet thromboxane
generation (23), but the effect of sulfasalazine on platelet function is not
known. Therefore we decided to assess the influence of sulfasalazine
therapy on platelet function in patients with inflammatory arthritis (IA).

Our hypothesis was that sulfasalazine would have an inhibitory effect on
platelet function in patients with IA and that this would be less than the
inhibition achieved by standard low-dose aspirin in patients with CVD.
Somewhat surprisingly, patients taking sulfasalazine had equivalent
inhibition of arachidonic acid (AA) induced platelet aggregation to patients
taking standard doses of aspirin. Therefore, we also examined the
effect(s) of sulfasalazine and its individual components (sulfapyridine and
5-aminosalicylic acid) on AA-induced platelet aggregation in vitro, in
blood samples obtained from healthy volunteers. We demonstrate a
synergistic, dose-dependent inhibition of the platelet arachidonic acid
pathway by the parent compound (sulfasalazine) and its metabolites
(sulfapyridine and 5-aminosalicylic acid), at drug concentrations
equivalent to those present physiologically in patients receiving sulfasalazine therapy. These findings are reported in Chapter 5.

1.5 Examination of the effect of anti-TNFα on platelet function

Biologic therapy with anti-TNFα agents has revolutionised the treatment of IA (24) and data from the British Biologics Registry demonstrates a clear reduction in the incidence of MI in patients with RA who respond to anti-TNFα therapy (25). The mechanisms underlying this CVD risk reduction in RA patients receiving anti-TNFα drugs are unclear, with some studies indicating possible effects on lipid profile (26) and insulin metabolism (27, 28). The effects of anti-TNFα therapy on platelet function are unknown. Interestingly, one of the few differences between the patients in our initial cross-sectional study reported in Chapter 3 was that significantly more patients in the remission group (who failed to demonstrate platelet hyper-reactivity) were receiving anti-TNFα agents. Therefore we decided to investigate the impact of improved disease control with anti-TNFα therapy on platelet function, cholesterol levels, and insulin resistance in patients with IA.

Thus, in Chapter 6, we report a prospective demonstration that improved disease control with anti-TNFα therapy in patients with previously active IA is accompanied by decreased platelet reactivity to ADP, improved insulin sensitivity, and preservation of a favourable anti-atherogenic lipid profile.

1.6 Research Collaboration

All the work contained in this thesis represents a close collaboration between the rheumatology department of the Mater Misericordiae University Hospital (MMUH) and the cardiovascular biology laboratory at the department of molecular and cellular therapeutics (MCT) of the Royal College of Surgeons of Ireland (RCSI).
MMUH is a major tertiary referral centre serving the catchment area of northeast Leinster, but also accepts rheumatology referrals from all over Ireland. It is staffed by three consultant rheumatologists, four non-consultant hospital doctors at various stages of training, two clinical nurses and a dedicated research nurse. MMUH is one of the two major university teaching hospitals for University College Dublin (UCD) Medical School and along with its counterpart, St Vincents University Hospital (SVUH), forms the recently established Dublin Academic Medical Centre (DAMC) for the advancement of translational research. The DAMC laboratories are located on-site at the MMUH campus in the Catherine McCauley postgraduate education centre.

The Cardiovascular Biology Lab of the MCT department at RCSI employs state of the art techniques to examine platelet function in a variety of disease states and has an international reputation for research excellence. The collaboration across these sites and results of the various studies reported herein are an example of the opportunities afforded by high volume clinical practice and dedicated laboratory investigation in the generation of meaningful findings and potential stimulation of important translational research.

1.7 Relevant Publications

All of the work contained herein has been presented at local, national, and international scientific meetings and a detailed list of abstract citations is outlined in Appendix 1. Furthermore, Chapters 3 and 5 have also been peer reviewed and published as original research manuscripts in international rheumatology journals (Rheumatology and Clinical Rheumatology, respectively) (29, 30). A complete copy of the former and the title page of the latter (for copyright reasons) are made available in Appendices 2 and 3, respectively. Finally, Chapter 6 has been submitted for publication in a leading rheumatology journal and is under review, while the investigations reported in Chapter 4 are currently being prepared as a single manuscript.
Chapter 2: Subjects and Methods
2.1 Patients with Inflammatory Arthritis

Patients attending the rheumatology outpatient department of the Mater Misericordiae University Hospital (MMUH) were invited to participate. Only patients with an established diagnosis of IA (rheumatoid, psoriatic, or ankylosing spondylitis/spondyloarthritis) as per 1987 ACR criteria (31) and aged between 18 and 70 years were considered for inclusion. For all patients with IA in whom platelet function analyses were carried out in each of the four individual investigations of this thesis (described separately in Chapters 3 through 6), those with a prior history of ischaemic heart disease, diabetes mellitus, and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine, or dipyrimadole), or thromboembolic prophylaxis (heparin or warfarin) were excluded. Patients with a serum creatinine > 140mmol/l, a platelet count < 120,000/mm³, or who were pregnant, or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

Patients recruited for the initial cohort reported in Chapter 3 comprised a convenience sample for a cross-sectional study investigating the association or otherwise between inflammatory disease activity and platelet function. Patients using NSAIDs, conventional DMARD agents (methotrexate, hydroxychloroquine, sulfasalazine, steroid, azathioprine, cyclosporin, leflunomide) and anti-TNF therapy, alone or in combination, were included.

Patients recruited for the clinical component of Chapter 4, (investigating the impact of IL6 receptor inhibition on platelet function), had to have failed conventional DMARDs and biologic therapy with at least one anti-TNF agent, in addition.

To assess the impact of sulfasalazine treatment on arachidonic-acid mediated platelet aggregation in Chapter 5, those patients who were
receiving concomitant NSAIDs were excluded from participation due to the confounding effect of these agents on this pathway.

Finally, for the prospective investigation of the effects (or otherwise) of improved disease control with anti-TNF therapy on platelet function, insulin metabolism, and lipid profile carried out in Chapter 6, patients with IA who had previously failed treatment with, or were intolerant of, methotrexate and/or whose disease, in the opinion of their attending consultant rheumatologist, warranted treatment with an anti-TNFα agent were considered for inclusion.

For all patients with IA, sampling and subsequent platelet function testing was carried out immediately on-site at MMUH in the DAMC laboratory.

2.2 Healthy Volunteers

For those additional subjects recruited for the in vitro analyses described in Chapters 4 and 5, these were all aged between 18 and 30 years, had no underlying medical conditions and were receiving no prescribed medications, over the counter drugs, or dietary supplements. Sampling of these subjects and subsequent testing was carried out in the department of MCT at RCSI. Essentially, these subjects comprised a convenience sample of staff and students at the institution and were obviously demographically unmatched to specific disease groups.

2.3 Patients with cardiovascular disease

Stable CVD patients with no history of IA and who were receiving standard anti-platelet monotherapy with low-dose aspirin (75mg) were also recruited as positive controls for the clinical component of the sulfasalazine study in Chapter 5. Sampling for these patients was also performed on site at MMUH.

2.4 Ethical Approval

For all patients investigated, ethical approval was obtained from the Mater Misericordiae University Hospital Ethics Committee and the study complied with the Declaration of Helsinki.
Ethical approval for the healthy volunteer sampling required for the in vitro studies in Chapters 4 and 5 was conferred by the Ethics Committee of the RCSI as per the operational policy of the department of MCT. Informed consent was obtained from all patients and healthy volunteers prior to phlebotomy.

2.5 Disease activity

Several methods were used to accurately determine disease activity. An initial Evaluator Global Assessment (EGA) based on history and clinical examination was performed on all patients by the same clinician (the author).

All patients were then examined by the same clinical nurse specialist (Research Nurse, Ms Ann Madigan MSc). Tender joint count, swollen joint count, duration of early morning stiffness (EMS), visual analogue scale of disease activity (VASDA), and visual analogue scale of pain (VASPAIN) were recorded. Serum inflammatory markers (erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), and fibrinogen (Fib) ) were obtained as outlined below. In addition, the internationally validated DAS-28 score (32) was calculated for those patients with a diagnosis of RA and PsA.

2.6 Clinical Data

Demographic data and traditional cardiovascular risk factors were also recorded. A detailed and comprehensive medication record was also obtained from each patient. Routine serological data (full blood count, renal, liver and bone profiles) and fasting samples of traditional cardiovascular risk factors (blood glucose, lipids and insulin level) were also collected. Insulin resistance was calculated using the established HOMA-IR method (33), where appropriate. Total cholesterol, HDL, triglycerides, and LDL levels were obtained as per standard laboratory assessment and the HDL/LDL ratio calculated for the relevant participants (34).
2.7 Phlebotomy and Platelet Preparation

Blood was drawn from all patients by the same phlebotomist. All samples were obtained uncuffed. Blood was collected through a 19-gauge Butterfly needle and the first 20 ml was sent for baseline serological data and inflammatory markers. A further 40ml was collected into a syringe containing 3.2% sodium citrate. Blood was then centrifuged for 10 min at 150g. Platelet-rich plasma (PRP) aspirated from the supernatant was placed in a reagent reservoir. Using a multichannel pipette, the PRP was dispensed across wells in a 96-well plate (black isoplate with clear flat-bottomed wells, Perkin Elmer) containing different concentrations of the five agonists arachidonic acid, collagen (type 1 soluble calf skin), adenosine diphosphate (ADP), epinephrine and thrombin receptor activating peptide (TRAP).

2.8 Platelet Function Assay

To assess platelet function for each of the four patient studies in Chapters 3 through 6, we used a novel platelet function assay based on a modification of light transmission aggregometry, that has since been validated in several publications (16, 35), (36).

The detailed description of the exact protocol and procedure for this micro-titre array is provided in Appendix 4. In brief, 180 \( \mu \)l of PRP was added to each well of a 96-well plate containing the different agonists. Light absorbance was measured at standard times. To characterise platelet aggregation, increasing concentrations of the agonists were tested. Platelet aggregation measured as a percentage of absorbance from baseline, using a 572nm filter, was assayed at 0,3,6,9,12,15 and 18 mins. Between each of the standardised times, the plate was rotated at 1000 r.p.m. through a 0.1mm orbit. The concentrations of the agonists used were (500,375,188,83.8,46.9,23.4,11.8,5.86) \( \mu \)g/ml for arachidonic acid; (190,143,71.3,35.6,17.8,8.9,4.45,2.23) \( \mu \)g/ml for collagen; (20,10,5,2.5,1.25,0.625,0.313,0.156) \( \mu \)M for ADP and TRAP; and (20,5,1.25,0.313,0.078,0.0195,0.00488,0.00122) \( \mu \)M for epinephrine. The agonist volumes used were 50 \( \mu \)L of arachidonic acid, 50 \( \mu \)L of collagen,
40 μL of ADP, 40 μL of epinephrine and 40 μL of TRAP. The 96-well plate was then read using a Victor 3 Multilabel plate reader (Perkin Elmer, Wellesley, MA, USA). The time from blood draw until the end of the assay protocol was recorded.

The reproducibility of this novel platelet function assay has been established recently and is described in detail elsewhere (16). In brief, the between assay precision of each agonist is; 12% for arachidonic acid, 10% for collagen, 8% for ADP, 11% for epinephrine, and 11% for TRAP. A value of <12% for between assay precision is regarded as acceptable for assay validation (37). The within assay precision is <1% for all agonists, indicating excellent reproducibility (16).

2.9 Sampling and Flow Cytometry of Healthy Volunteers in Chapter 4

Uncuffed fasting samples were obtained as per the patient protocol above and the initial 5ml were discarded. As baseline serological data was not required, only 40 ml of 3.2% citrated blood was collected from each volunteer (n=6). PRP was obtained as per the patient studies, following which samples were aliquoted and incubated for 15min at 37°C with either vehicle control or 10ng/ml of each of the following pro-inflammatory cytokines; TNF alpha, IL1 beta, IL 6, and IL17a. Following incubation, the PRP samples were assayed to determine their effects or otherwise on the platelet ADP pathway using a modification of the Platelet Function Assay protocol described above, whereby only increasing concentrations of ADP (20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156) μM were tested.

In addition, to investigate whether there was a direct effect of these pro-inflammatory cytokines on platelet activation, platelet surface P-Selectin (CD62P) expression in resting and ADP-stimulated PRP samples was measured by an established flow cytometry assay using the fluorescently labelled anti–CD62P antibody (Becton Dickinson, Palo Alto, CA, USA). In brief, using a total reaction volume of 100 μl, 10 μl of each PRP sample was further incubated with 10 μl of 1μM ADP or 0.9% NaCl in the presence of 10 μl of FITC-labelled ant-CD62P or an appropriate FITC labelled isotype control (1.25 μg/ml). All incubations were performed at
room temperature for 10 minutes. The reaction was terminated by the addition of 1 ml of buffer A (130 mM NaCl, 10 mM sodium citrate, 9 mM NaHCO3, 6 mM D-glucose, 0.9 mM MgCl2, 0.81 mM KH2PO4, 10 mM Tris, pH 7.4). Samples were analysed using a BD FACS Calibur (Becton Dickinson, Palo Alto, CA, USA) within 1 hour. The instrument was set to measure size (forward scatter, FSC), granularity (side scatter, SSC) and cell fluorescence. Using a log FSC vs. log SSC dot plot, a two-dimensional analysis gate was drawn around the platelet population, and a fluorescence histogram (log APC/FITC vs. count) was obtained for 10000 platelet events for each sample. Data was analysed using CellQuest Pro software. P-Selectin expression was calculated as the percentage of platelets positive for anti-CD62P binding relative to the isotype control.

2.10 Sampling and testing of Healthy Volunteers in Chapter 5

To assess the specific effect of sulfasalazine and its individual components (5-ASA and sulfapyridine) on the platelet thromboxane pathway (with aspirin as a positive control), blood samples were obtained from healthy volunteers (n=18, 9 male and 9 female) who were not taking any medication. Sulfasalazine, 5-ASA, sulfapyridine, and acetylsalicylic acid (aspirin) were purchased from Sigma laboratories. All drugs were reconstituted with dimethyl sulfoxide (DMSO) and diluted to their respective concentrations with deionised water. The final concentration of DMSO in each solution tested was <1%. Phlebotomy and platelet preparation were carried out as described above. The PRP obtained was divided into aliquots of 1ml and these were incubated (for 45mins at 37°C) with increasing concentrations (40μM, 50μM, and 100μM) of either sulfasalazine, 5-ASA or sulfapyridine. As all three components are simultaneously present in the plasma of patients taking the drug, (at typical concentrations of 40-50μM), increasing concentrations (10μM, 20μM, 40μM, and 50μM) of a mixed solution of sulfasalazine, 5-ASA and sulfapyridine were also tested, in this physiological range (ie the 10μM solution was composed of 10μM each of sulfasalazine, 5-ASA, and
sulfapyridine; the 20µM solution contained 20µM each of sulfasalazine, 5-ASA, and sulfapyridine; and so on for the 40µM and 50µM solutions) (22). Aliquots of PRP incubated with 1% DMSO (vehicle control) and 20µM aspirin were used as negative and positive controls, respectively. The negative control is representative of the normal platelet aggregation response in the absence of medication, and the positive control of 20µM acetylsalicylic acid is representative of the physiological plasma concentration of aspirin present in patients taking low dose aspirin (75mg daily) for CVD prevention (38). The platelet count in each sample was assayed pre- and post-incubation using a standard Sysmex haematology cell counter. Platelet function was assessed using conventional light transmission aggregometry (LTA) on a 4-channel Chronolog aggregometer, and the platelet aggregation responses to 500µg/ml of arachidonic acid were tested, as described (39, 40). The positive and negative controls were used in every aggregometry run alongside 2 other samples. All concentrations of each drug were tested at least 3 times for every donor.

2.11 Statistics

Demographic data categorical variables were analysed using the Fisher’s Exact Test. Disease activity and serological data were analysed using the Wilcoxon matched pairs test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for per cent platelet aggregation were calculated with the nominal level of significance set at 5%, and multiple comparisons between different agonist concentrations were adjusted using the Bonferroni correction. Due to the relatively small sample size for the patient studies in Chapters 4 and 6, differences in pre and post-treatment platelet aggregation responses for each concentration of agonist were also tested using the non-parametric Wilcoxon matched pairs test. Sigmoidal dose-response curves of platelet function profiles were compared using the extra sum-of-squares F-test and EC50 values expressed as 95% confidence intervals. Statistical advice was obtained from the biostatistics consulting and support services within RCSI.
Chapter 3: The Influence of Disease Activity on Platelet Function in Patients with Inflammatory Arthritis
3.1 Background

Patients with inflammatory arthritis (IA) are at increased risk of adverse cardiovascular disease (CVD) (1). While traditional risk factors are known to play a role in the pathogenesis of cardiovascular events, recent work has highlighted the key role of inflammation in atherothrombosis (4) and as a risk factor for CVD in patients with rheumatoid arthritis (5).

Myocardial infarction results from a complex chain of events leading to the thrombotic occlusion of a coronary artery. Platelets are central to the development of these thrombotic events (4). It is well established that inflammation activates platelets (6) and that platelet-derived mediators in turn potentiate the inflammatory response (4). To date, platelet function in the IA population has not been well characterised.

3.2 Rationale

Previous studies have shown that patients with inflammatory rheumatic conditions exhibit increased in vitro platelet aggregation (7). However, these patients were not characterized in terms of disease activity. More recent studies have shown a strong correlation between elevated markers of disease activity (CRP, ESR) and markers of platelet activation (CD62P or p-selectin, CD63) in RA (8). However, the functional responsiveness or reactivity of these platelets was not assessed.

Platelet reactivity is a measure of how easily platelets will adhere to one another to form thrombus. Several lines of evidence suggest that platelet reactivity is useful in clinical practice to predict the risk of future adverse cardiovascular events (9-12). Therefore, we decided to examine platelet reactivity in the IA population and to assess the influence of disease activity on platelet function.

3.3 Patients and Methods

For a detailed description of patients and methods please see Chapter 2. In brief, patients attending the rheumatology outpatient clinic of MMUH were recruited. Only patients with an established diagnosis of IA and
aged between 18 and 70 years were considered for inclusion. Patients who did not have a clear diagnosis of IA were excluded. Patients with a prior history of ischaemic heart disease, diabetes mellitus, and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine, or dipyrimadole), or thromboembolic prophylaxis (heparin or warfarin) were excluded.

Patients with a serum creatinine > 140 mmol/l, a platelet count < 120,000/mm$^3$, or who were pregnant, or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

Ethical approval was obtained from the Mater Misericordiae Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients prior to phlebotomy.

3.3.1 Disease Activity

Several methods were used to accurately determine inflammatory disease activity. Tender joint count, swollen joint count, duration of early morning stiffness (EMS), visual analogue scale of disease activity (VASDA), and visual analogue scale of pain (VASPAIN) were recorded. Demographic data and traditional cardiovascular risk factors were also recorded. A detailed and comprehensive medication record was also obtained from each patient. Serum inflammatory markers (erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), and fibrinogen (Fib)) were obtained as outlined in Chapter 2. In addition, the internationally validated DAS-28 score (32) was calculated for those patients with a diagnosis of RA and PsA.

3.3.2 Platelet Function

Phlebotomy and platelet rich plasma (PRP) preparation were performed as outlined in Chapter 2.

To assess platelet function, we used a novel platelet function assay based on a modification of light transmission aggregometry, described in detail elsewhere (16, 35), (36). In brief, 180 μl of PRP was added to each
well of a 96-well plate containing the different agonists. Light absorbance was measured at standard times. To characterise platelet aggregation, increasing concentrations of the agonists were tested. Platelet aggregation measured as a percentage of absorbance from baseline, using a 572nm filter, was assayed at each time point. The agonists used were arachidonic acid, collagen, epinephrine, ADP and TRAP. The 96-well plate was then read using a Victor 3 Multilabel plate reader (Perkin Elmer, Wellesley, MA, USA). The time from blood draw until the end of the assay protocol was recorded.

The reproducibility of this novel platelet function assay has been established recently and is described in detail elsewhere (16). In brief, the between assay precision of each agonist is; 12% for arachidonic acid, 10% for collagen, 8% for ADP, 11% for epinephrine, and 11% for TRAP. A value of <12% for between assay precision is regarded as acceptable for assay validation (37). The within assay precision is <1% for all agonists, indicating excellent reproducibility (16).

### 3.3.3 Patient Groups

The entire cohort was divided into two separate groups based on disease activity. Those with clinically active disease were assigned to the Active group and those patients with well controlled disease were assigned to the Control group. This assignment was made independent of platelet function. Platelet assay data were recorded separate to the clinical data and the two were merged thereafter.

Maximal platelet aggregation was defined as no change in light absorbance with incremental concentration of agonist at two consecutive time points. Sigmoidal dose/response curves were then plotted for each agonist at all concentrations. We then compared platelet function in patients with active inflammatory disease (Active Group) to those in clinical remission (Control Group).

### 3.3.4 Statistics

Demographic data categorical variables were analysed using Fischers Exact Test. Disease activity data were analysed using the Wilcoxon
Signed Rank Test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for % platelet aggregation was then assessed by Repeated Measures Two-Way ANOVA.

The nominal level of significance was 5% and multiple comparisons between different agonist concentrations were adjusted using the Bonferroni correction.

Sigmoidal dose-response curves for the two groups were compared separately for each agonist using the Extra Sum-of-Squares F Test.

### 3.4 Results

107 patients were initially enrolled in the study. 11 patients were excluded for the following reasons: 4 from whom a sufficient blood sample for platelet analysis could not be obtained; 5 for failure to complete the assay within the timeframe; 2 where complete baseline serological data was missing.

Data from a total of 96 patients was analysed. The average time from blood draw to completion of the assay was 40 +/- 5 mins.

#### 3.4.1 Demographic Data:

The two groups (Active vs Control) were similar in terms of age, gender, diagnosis and traditional cardiovascular risk factors (Table 3.1). The majority of patients (65%) were diagnosed with RA. There were significant differences between the two groups in their use of medications. Anti-tumour necrosis factor (TNF) α therapy use was significantly higher in the Control group (p=0.0062), while non-steroidal anti-inflammatory (NSAID) use was significantly higher in the Active disease group (p=0.0017).
<table>
<thead>
<tr>
<th></th>
<th>ENTIRE COHORT N=96</th>
<th>ACTIVE GROUP N=38</th>
<th>CONTROL GROUP N=58</th>
<th>“p” value</th>
</tr>
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<td>AGE yrs</td>
<td>49 +/-10</td>
<td>50 +/-9</td>
<td>49 +/-12</td>
<td></td>
</tr>
<tr>
<td>MALE (%)</td>
<td>50 (52%)</td>
<td>19 (50%)</td>
<td>31 (53%)</td>
<td>0.6814</td>
</tr>
<tr>
<td>DIAGNOSIS (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>62 (65%)</td>
<td>21 (55%)</td>
<td>41 (71%)</td>
<td>0.134</td>
</tr>
<tr>
<td>PSA</td>
<td>16 (17%)</td>
<td>8 (21%)</td>
<td>8 (14%)</td>
<td>0.407</td>
</tr>
<tr>
<td>AS</td>
<td>7 (7%)</td>
<td>3 (8%)</td>
<td>4 (7%)</td>
<td>0.999</td>
</tr>
<tr>
<td>OTHER</td>
<td>11 (11%)</td>
<td>6 (16%)</td>
<td>5 (8%)</td>
<td>0.335</td>
</tr>
<tr>
<td>RISK FACTORS (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SMOKING</td>
<td>30 (31%)</td>
<td>13 (34%)</td>
<td>17 (29%)</td>
<td>0.656</td>
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<tr>
<td>DM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HTN</td>
<td>14 (15%)</td>
<td>6 (16%)</td>
<td>8 (14%)</td>
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</tr>
<tr>
<td>CHOL</td>
<td>12 (12%)</td>
<td>4 (11%)</td>
<td>8 (14%)</td>
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</tr>
<tr>
<td>FAMILY HX</td>
<td>18 (19%)</td>
<td>8 (21%)</td>
<td>10 (6%)</td>
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</tr>
<tr>
<td>MEDICATIONS (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAIDS</td>
<td>47 (49%)</td>
<td>27 (71%)</td>
<td>20 (34%)</td>
<td>0.001**</td>
</tr>
<tr>
<td>ANTI-TNFα</td>
<td>26 (27%)</td>
<td>4 (11%)</td>
<td>22 (38%)</td>
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</tr>
<tr>
<td>MTX</td>
<td>49 (51%)</td>
<td>16 (42%)</td>
<td>33 (57%)</td>
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</tr>
<tr>
<td>SZP</td>
<td>23 (24%)</td>
<td>10 (26%)</td>
<td>13 (22%)</td>
<td>0.807</td>
</tr>
<tr>
<td>HCL</td>
<td>15 (16%)</td>
<td>6 (16%)</td>
<td>9 (16%)</td>
<td>0.999</td>
</tr>
<tr>
<td>AZA</td>
<td>5 (5%)</td>
<td>1 (3%)</td>
<td>4 (7%)</td>
<td>0.641</td>
</tr>
<tr>
<td>STEROIDS</td>
<td>10 (11%)</td>
<td>4 (11%)</td>
<td>6 (10%)</td>
<td>0.999</td>
</tr>
<tr>
<td>MMP</td>
<td>1 (1%)</td>
<td>1 (3%)</td>
<td>0</td>
<td>0.396</td>
</tr>
<tr>
<td>HRT/OCP</td>
<td>7 (7%)</td>
<td>3 (8%)</td>
<td>4 (7%)</td>
<td>0.999</td>
</tr>
</tbody>
</table>

** significant difference between the groups (ACTIVE v CONTROL)

Key:
RA= rheumatoid arthritis, PSA=psoriatic arthritis, AS= ankylosing spondylitis, Other= reactive arthritis, juvenile idiopathic arthritis, and gout. DM=diabetes mellitus, HTN= hypertension, Chol= hypercholesterolaemia, Hx= history.
NSAIDS= non-steroidal anti-inflammatory drugs, ANTI-TNFα= anti-tumour necrosis α drugs, MTX= methotrexate, SZP= salazopyrin, HCL= hydroxychloroquine, AZA= azathioprine, MMP= mycophenolate mofetil, HRT= hormone replacement therapy, OCP= oral contraceptive pill.

3.4.2 Clinical Data:
Clinical variables for the Active vs Control disease groups are shown in Table 3.2. There were significant differences in all of these variables between the two groups. DAS-28 scores for those patients with RA are shown separately.

3.4.3 Platelet Data:
Sigmoidal dose-response curves of % platelet aggregation in response to increasing concentrations of agonist are shown (Figures 3.1 and 3.2). Each agonist is graphed separately and the platelet aggregation responses for the Active and Control disease groups are directly compared to each other.

Platelet % aggregation values were normally distributed for all concentrations of agonists. Analysis of variance (ANOVA) results for each agonist demonstrated a significant difference between the groups in response to ADP only (p=0.01). The values obtained for the other agonists were; p=0.25 for TRAP, p=0.42 for collagen, p=0.54 for arachidonic acid, p=0.88 for epinephrine. The individual concentrations of ADP responsible for the significantly increased response in the Active disease group are in the submaximal range (1.25μM, 2.5μM, 5μM), as demonstrated by Bonferroni post-test values (p<0.001).
<table>
<thead>
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<th>Table 3.2: Clinical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entire Cohort</strong></td>
</tr>
<tr>
<td>N=96</td>
</tr>
<tr>
<td>ACTIVE</td>
</tr>
<tr>
<td>N=38</td>
</tr>
<tr>
<td>CONTROL</td>
</tr>
<tr>
<td>N=58</td>
</tr>
<tr>
<td><strong>Mean +/- SEM</strong></td>
</tr>
<tr>
<td><strong>Mean +/- SEM</strong></td>
</tr>
<tr>
<td><strong>Wilcoxon Signed Rank test</strong></td>
</tr>
<tr>
<td><strong>p” value</strong></td>
</tr>
</tbody>
</table>

**SEROLOGICAL MEASURES**

| ESR (mm/hr)  | 31.74 +/- 4.33 | 14.04 +/- 1.82 | 0.0109** |
| CRP (mg/l)   | 23.40 +/- 5.57 | 7.09 +/- 2.074 | 0.0105** |
| Fibrinogen (g/l) | 3.99 +/- 0.21 | 3.01 +/- 0.10 | 0.0056** |

**PATIENT MEASURES**

| VASDA (0-100) | 63.29 +/- 3.36 | 15.02 +/- 2.21 | <0.0001** |
| VASPAIN (0-100) | 61.79 +/- 5.06 | 16.41 +/- 2.69 | <0.0001** |
| EMS (mins)    | 44.11 +/- 12.26 | 4.33 +/- 1.29 | 0.0008** |

**RA Patient Cohort**

| N=62          |
| ACTIVE        |
| N=21          |
| CONTROL       |
| N=41          |
| DAS-28 SCORE  | 4.87 +/- 0.29 | 2.45 +/- 0.12 | 0.0002** |

Key:

ESR= erythrocyte sedimentation rate, CRP= C-reactive protein, VASDA= visual analogue scale of disease activity, VASPAIN= visual analogue scale of pain, EMS= early morning stiffness. DAS-28 = 28 joint count disease activity score.

**significant difference between the groups (ACTIVE v CONTROL)**
Figure 3.1: Platelet aggregation is enhanced in the actively inflamed patients (Active group) in response to submaximal concentrations of ADP, compared to those patients whose disease is well controlled (Control group).

*** p<0.001

Log Dose/Response Curves for Active v Control groups in response to ADP (adenosine diphosphate).

Log of agonist concentration is plotted on the “x” Axis.

Platelet % aggregation is plotted on the “y” Axis.
Figure 3.2: There is no difference in platelet aggregation between the Active and Control groups in response to the other agonists. The log dose/response curves for both groups are similar for arachidonic acid, collagen, epinephrine, and TRAP.


Log of agonist concentration is plotted on the “x” Axis.

Platelet % aggregation is plotted on the “y” Axis.
3.5 Conclusion
The results of this investigation demonstrate a number of key findings. Firstly, the patients in this study with objective evidence of active IA demonstrate increased platelet reactivity specifically to ADP. Secondly, there was no significant difference in platelet response to any of the other agonists in terms of disease activity. Thirdly, when maximal concentrations of agonists are used in platelet aggregation, there is no difference seen between the two groups.

This unique platelet hyper-reactivity in response to ADP in patients with IA who are actively inflamed may explain some of the cardiovascular risk associated with IA and has implications for therapy. Currently, despite this elevated cardiovascular risk, there are no guidelines for anti-platelet therapy in patients with IA. Furthermore, it is not known if targeting the ADP pathway would offer any specific benefit in ameliorating this risk, and further work in this area is warranted. These results are discussed in detail in Chapter 7.
Chapter 4 Pro-inflammatory cytokines and platelet ADP reactivity
4.1 Background

While traditional risk factors are known to play a role in the pathogenesis of cardiovascular events, recent work has highlighted the key role of inflammation in atherothrombosis (4) and as a risk factor for CVD in patients with IA (5). Pro-inflammatory cytokines drive the disease process in IA (17, 18) and their pathogenic role in CVD is becoming increasingly recognized (41). It is well established that inflammation activates platelets (6) and that platelet-derived mediators in turn potentate the inflammatory response (4). Several lines of evidence suggest that increased platelet reactivity as assessed by the aggregation response to agonists such as arachidonic acid (AA), adenosine diphosphate (ADP), collagen, and epinephrine is associated with recurrent ischaemic events (15).

4.2 Rationale

The pro-inflammatory cytokine interleukin 6 (IL-6) plays a key pathogenic role in both RA and CVD (19), and previous evidence also demonstrates that IL-6 increases platelet reactivity, particularly in response to ADP (20). Given that we demonstrated increased platelet reactivity, specific to the ADP pathway, in patients with IA who have objective evidence of inflammation compared to RA patients in disease remission (29), we chose to investigate this further.

It is not known if treatment targeted against the IL-6 pathway alters platelet behaviour in patients with RA. Therefore, we investigated the impact of IL-6 receptor inhibition with tocilizumab (TCZ) therapy on platelet function in patients with refractory RA. Furthermore, we also examined the effect of a range of pro-inflammatory cytokines, including IL-6, on the platelet ADP pathway in samples from healthy volunteers.

4.3 Patients and Methods

This work was conducted alongside a PHASE IV study evaluating IL-6 receptor inhibition with TCZ in patients with RA who had failed treatment
with, or were intolerant of, conventional DMARDs (including methotrexate) and/or biologic therapy with anti-TNF alpha agents.

Ethical approval was obtained from the Mater Misericordiae University Hospital Ethics Committee (code number: 1/378/1086) and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients and healthy volunteers prior to phlebotomy.

For a detailed description of patients and methods please refer to Chapter 2.

In brief, patients with an established diagnosis of RA aged between 18 and 70 years who were attending the rheumatology outpatients clinic and who had previously failed treatment with, or were intolerant of, methotrexate and/or anti-TNFα therapy were considered for inclusion. Patients with a history of ischaemic heart disease, diabetes mellitus and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine or dipyrimadole) or thromboembolic prophylaxis (heparin or warfarin) were excluded.

Patients with a serum creatinine>140mmol/l, a platelet count <120,000/mm³, or who were pregnant or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

Patients who fulfilled these criteria and who were already enrolled in the aforementioned PHASE IV clinical trial evaluating tocilizumab therapy in RA were invited to participate in this study. Patients were evaluated pre and post- treatment on each of 2 separate occasions, at baseline and 24 weeks (ie 4 weeks after their 6th intravenous infusion of tocilizumab (8mg/kg) given at 4-weekly intervals).

4.3.1 Patient clinical data

Demographic data and cardiovascular risk factors were recorded. A detailed medication record was also obtained from each patient. Disease activity was assessed both clinically and serologically. Tender joint count, swollen joint count, duration of early morning stiffness, and visual
analogue scales of disease activity and pain were recorded. Standard inflammatory markers (ESR, CRP, and fibrinogen) were measured, and the internationally validated 28-joint count of disease activity (DAS-28) (32) was calculated for all patients. Routine serological data (full blood count, renal, liver and bone profiles) and fasting samples of traditional cardiovascular risk factors (blood glucose, lipids and insulin level) were also collected. Insulin resistance was calculated using the established HOMA-IR method (33).

4.3.2 Phlebotomy and platelet function

Pre and post-treatment blood samples for platelet function testing were obtained at baseline and 24 weeks. Platelet rich plasma (PRP) was prepared as described in Chapter 2. Using the multichannel pipette, the PRP was dispensed across wells in a 96-well plate (black isoplate with clear flat-bottomed wells, Perkin Elmer) containing incremental concentrations of the different agonists arachidonic acid, collagen (type 1 soluble calf skin), adenosine diphosphate (ADP), and epinephrine. Thrombin Receptor Activating Peptide (TRAP) was not utilised due to a change in batch production resulting in varying platelet responses.

Platelet function was assessed using a modification of light transmission aggregometry, described in detail elsewhere (16, 29, 35) and in Chapter 2. The time from blood draw until the end of the assay protocol was recorded.

Maximal platelet aggregation was defined as no change in light absorbance with incremental concentration of agonist at two consecutive time points. Sigmoidal log dose-response curves were plotted for each agonist at all concentrations. Pre and post-treatment platelet function profiles were then compared for all patients, with each patient acting as their own control.

4.3.3 Healthy Volunteers

These were all aged between 18 and 30 years, had no underlying medical conditions and were receiving no prescribed medications, over the counter drugs, or dietary supplements.
4.3.4 Phlebotomy and platelet preparation

Uncuffed fasting samples were obtained as per the patient protocol above and the initial 5ml were discarded. As baseline serological data was not required, only 40 ml of 3.2% citrated blood was collected from each volunteer (n=6). PRP was obtained as per the patient studies, following which samples were aliquoted and incubated for 15min at 37°C with either vehicle control or 10ng/ml of each of the following pro-inflammatory cytokines; TNF alpha, IL1 beta, IL 6, and IL17a. Following incubation, the PRP samples were assayed to determine their effects or otherwise on the platelet ADP pathway using a modification of the LTA protocol described above, whereby only increasing concentrations of ADP were tested.

4.3.5 Flow Cytometry

In addition, to investigate whether there was a direct effect of these pro-inflammatory cytokines on platelet activation, platelet surface P-Selectin (CD62P) expression in resting and ADP-stimulated PRP samples was measured by an established flow cytometry assay using the fluorescently labelled anti–CD62P antibody.

For a detailed description of this assay see Chapter 2. In brief, using a total reaction volume of 100 μl, 10 μl of each PRP sample was further incubated with 10 μl of 1μM ADP or 0.9% NaCl in the presence of 10 μl of FITC-labelled ant-CD62P or an appropriate FITC labelled isotype control (1.25 μg/ml). All incubations were performed at room temperature for 10 minutes. P-Selectin expression was calculated as the percentage of platelets positive for anti-CD62P binding relative to the isotype control.

4.3.6 Statistics

Demographic data categorical variables were analysed using the Fisher’s Exact Test. Disease activity and serological data were analysed using the Wilcoxon matched pairs test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for per cent platelet aggregation were calculated with the nominal level of significance set at 5%, and multiple comparisons between different agonist concentrations were adjusted.
using the Bonferroni correction. Due to the relatively small sample size, differences in pre and post-treatment platelet aggregation responses for each concentration of agonist were also tested using the non-parametric Wilcoxon matched pairs test. Sigmoidal dose-response curves of platelet function profiles were compared using the extra sum-of-squares F-test and EC50 values expressed as 95% confidence intervals.

4.4 Results

4.4.1 Healthy Volunteers

Data from a total of 6 subjects (3 male, 3 female) were analysed. Log dose-response curves of ADP-induced platelet aggregation following incubation with 10ng/ml each of the four cytokines (IL17a, TNFα, IL1β, and IL-6) versus control PRP are depicted in Figure 4.1. Increasing concentration of agonist caused increased platelet aggregation. IL-17a (EC50 2.97-6.5) vs PRP control (EC50 3.71-7.38) had no effect, p=0.36. There was a small difference with TNFα (EC50 1.65-3.34, p<0.03) and significantly larger effects with IL1β (EC50 0.54-1.92, p<0.001) and IL6 (0.42-1.67, p<0.001), respectively.

Results of the percentage of platelets positive for P-Selectin expression, (an established marker of platelet activation), in resting vs ADP stimulated PRP samples following cytokine incubation are shown in Figure 4.2. Following stimulation with 1μM ADP, compared to vehicle control, there was a significant increase in platelet activation for all of the cytokines tested (p<0.01). Furthermore, there was also a significant difference in P-selectin expression in the resting samples (ie not stimulated by the ADP agonist) pre-incubated with TNFa and IL6, compared to the control resting sample (p<0.05).
Figure 4.1. Effect of pro-inflammatory cytokines on ADP-induced platelet aggregation.

Log of agonist concentration is plotted on the “x” Axis.

Platelet % aggregation is plotted on the “y” Axis.
Figure 4.2. P-Selectin expression of resting vs ADP stimulated PRP samples following cytokine incubation, as measured by flow cytometry.
4.4.2 Patients

Eight patients who fulfilled the inclusion and exclusion criteria were enrolled in the study. Of these, two patients discontinued due to an adverse skin reaction and non-specific raised transaminases, respectively. Data from all of the remaining patients (n=6) were analysed. The average time from blood draw to completion of the platelet function assay was 40+-5 mins.

4.4.3 Clinical Data

All patients were middle-aged females with seropositive (RF and anti-CCP antibody) RA whose disease had been resistant to treatment with both methotrexate and anti-TNF alpha therapy. Pre and post-treatment disease activity data and plasma indices are shown in Table 4.1. There was a significant decrease in post-treatment mean disease activity for all of the indices measured (p<0.05). Mean platelet count also decreased significantly (Mean, 95% C.I. 408, 323-496 x 10^9/L at baseline and 277, 202-351 x 10^9/l post treatment, p<0.05) while there was a small but significant increase in mean LDL cholesterol (Mean, 95% C.I 2.9, 2.1-3.6 mmol/L at baseline and 3.5, 2.7-4.4 mmol/L post-treatment, p<0.05). Insulin resistance profiles were similar for both time points.

The direction and magnitude of change in ESR (Panel A), CRP (Panel B), DAS-28 (Panel C), and platelet count (Panel D) for each individual patient is depicted in Figure 4.3 and all demonstrate a significant decrease between baseline and post-treatment values (p<0.05)
Table 4.1 Clinical Data

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<th>Pre-Tx</th>
<th>Post-Tx</th>
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<tr>
<td>{mean, (95% CI)}</td>
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<td></td>
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</tr>
<tr>
<td>ESR</td>
<td>59 (19.4-99.2)</td>
<td>22 (9.78-34.29)</td>
<td>p&lt;0.05</td>
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<td>CRP</td>
<td>32 (3.3-67.9)</td>
<td>3.07 (0.31-5.8)</td>
<td>p&lt;0.05</td>
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<tr>
<td>Fibrinogen</td>
<td>4.5 (3.8-5.1)</td>
<td>2.5 (1.6-3.4)</td>
<td>p&lt;0.05</td>
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<tr>
<td>DAS 28</td>
<td>5.2 (4.6-5.8)</td>
<td>2.6 (1.7-3.4)</td>
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<tr>
<td><strong>Plasma Indices</strong></td>
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<tr>
<td>Platelet Count</td>
<td>410 (323-492)</td>
<td>276 (202-350)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>HOMA - IR</td>
<td>1.2 (0.5-1.9)</td>
<td>1.2 (0.42-1.96)</td>
<td>p=0.71</td>
</tr>
<tr>
<td>LDL</td>
<td>2.9 (2.1-3.6)</td>
<td>3.5 (2.7-4.4)</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>
Figure 4.3. Disease activity indices pre and post treatment with the IL 6 inhibitor, tocilizumab.
Figure 4.4. Log dose-response curves of platelet aggregation in response to increasing concentrations of various agonists (collagen, epinephrine, arachidonic acid, and ADP) in samples taken from RA patients (n=6), pre-and post treatment. Log of agonist concentration is plotted on the “x” Axis. Platelet % aggregation is plotted on the “y” Axis.
4.4.4 Platelet Function Data

Increasing concentrations of agonist caused increasing platelet aggregation. The response to each agonist was plotted as a sigmoidal log dose-response curve and the pre and post-treatment platelet reactivity profiles for each agonist were compared for all patients (Figure 4.4). There was a significant reduction in the post-treatment platelet aggregation responses to ADP (Panel D) for all concentrations of agonist tested (p<0.001). Similarly, platelet responses to the four highest concentrations of arachidonic acid (Panel C) were significantly decreased post-treatment (p<0.01). There was also a small but significant decrease in post treatment platelet reactivity in response to epinephrine (Panel B) but this effect was only observed at very low submaximal concentrations (0.0195 and 0.078 μM, p<0.05). Platelet responses to collagen (Panel A) were similar at both time points.

4.5 Conclusion

The results of this investigation demonstrate a number of key findings. First, this is a novel prospective demonstration that improved disease control with IL-6 receptor inhibition in patients with RA is accompanied by a significant change in platelet function. This is particularly apparent in the ADP pathway with a decrease in aggregation response to all concentrations tested, but also observed with low concentrations of epinephrine and high concentrations of arachidonic acid. Interestingly, there was no change in platelet responses to collagen, and the reasons for this are unclear. Secondly, pro-inflammatory cytokines known to be involved in the pathogenesis of RA enhance the platelet aggregation and/or activation responses to ADP, in samples from healthy volunteers at the concentrations tested. Finally, while the improved control of inflammation with tocilizumab had no impact on insulin sensitivity, there was a significant increase in levels of LDL cholesterol, a consistent finding in several recent studies involving a much greater number of patients (42). These findings are discussed in detail in Chapter 7.
Chapter 5 Investigation of the inhibitory effect of sulfasalazine on arachidonic acid mediated platelet aggregation.
5.1 Background

Sulfasalazine is an older generation anti-rheumatic drug first synthesized in the 1940’s as a combination of an antibiotic (sulfapyridine) and an NSAID (5-aminosalicylic acid) (21). Patients with rheumatoid arthritis (RA) who regularly take sulfsalazine have a significantly reduced risk of myocardial infarction (MI), independent of disease severity, (hazard ratio 0.82, p<0.05) (5). The mechanisms underlying this cardiovascular risk reduction with sulfasalazine therapy are not entirely understood. Sulfasalazine is a pro-drug composed of two moieties, sulfapyridine and 5-aminosalicylic acid (5-ASA), linked by an azo bond (21). Plasma concentrations of all three substances in RA patients receiving stable doses of sulfasalazine have recently been reported (22). Sulfasalazine inhibits platelet thromboxane generation which should theoretically alter platelet aggregation (23), but the exact effect(s) of sulfasalazine on platelet function is unclear.

5.2 Rationale

One of our earliest observations from the initial cohort was that patients receiving sulfsalazine therapy had a poor aggregation response to arachidonic acid. However, this finding was confounded by concomitant NSAID use, which as COX inhibitors, interfere with the arachidonic acid pathway (43, 44) thus severely limiting any interpretation of the results. Therefore we decided to assess the influence of sulfasalazine therapy on platelet function in patients with inflammatory arthritis (IA) by excluding all patients who had used an NSAID within the preceding 14 days.

Our hypothesis was that sulfasalazine would have an inhibitory effect on platelet function in patients with IA and that this would be less than the inhibition achieved by standard low-dose aspirin in patients with CVD. Somewhat surprisingly, patients taking sulfasalazine had equivalent inhibition of arachidonic acid (AA) induced platelet aggregation to patients...
taking standard doses of aspirin. Therefore, we also examined the effect(s) of sulfasalazine and its individual components (sulfapyridine and 5-aminosalicylic acid) on AA-induced platelet aggregation \textit{in vitro}, in blood samples obtained from healthy volunteers.

\textbf{5.3 Patients and Methods}

Ethical approval was obtained from the Mater Misericordiae University Hospital Ethics Committee (code number: 1/378/1086) and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients and healthy volunteers prior to phlebotomy.

For a detailed description of patients and methods please see Chapter 2.

In brief, patients with an established diagnosis of IA (RA, PsA, and AS) aged between 18 and 70 years and attending the rheumatology outpatients clinic were considered for inclusion. Patients with a history of ischaemic heart disease, diabetes mellitus and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine or dipyrimadole) or thromboembolic prophylaxis (heparin or warfarin) were excluded. As non-steroidal anti-inflammatory drugs (NSAIDs) are known to affect platelet function (43), patients who had taken an NSAID within the preceding 14 days were also excluded.

Patients with a serum creatinine>140mmol/l, a platelet count <120,000/mm\(^3\), or who were pregnant or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

Based on the previously published reliability and reproducibility characteristics of our platelet function assay, a 10\% difference in platelet aggregation response is regarded as significant (16, 45). To evaluate whether patients with IA taking sulfasalazine (Sulfasalazine group) had a different platelet function profile compared to patients in receipt of other anti-rheumatic medications (Other DMARDs group), and to achieve 80\% power with a nominal significance of 5\%, we required 10 patients in each
group. To ensure the analysis would be robust, statistically viable, and informative, we aimed to recruit at least 15 patients with IA in each group. Stable CVD patients (n=15) with no history of IA and who were receiving standard ant-platelet monotherapy with low-dose aspirin (75mg) were recruited as a positive controls (Aspirin group).

5.3.1 Clinical data

Demographic data and cardiovascular risk factors were recorded. A detailed medication record was also obtained from each patient. Disease activity was assessed both clinically and serologically as described in Chapter 2.

5.3.2 Phlebotomy and platelet preparation

Blood was drawn from all patients by the same phlebotomist as described in Chapter 2. In brief, all samples were obtained uncuffed, patients had rested for 30mins prior to phlebotomy and all samples were obtained between 10am and 12noon, approximately 2 hours after a light breakfast. Blood was sent for baseline serological data and inflammatory markers. Platelet-rich plasma (PRP) aspirated from the supernatant was prepared and dispensed across wells in a 96-well plate (black isoplate with clear flat-bottomed wells, Perkin Elmer) containing incremental concentrations of the five agonists arachidonic acid, collagen (type 1 soluble calf skin), adenosine diphosphate (ADP), epinephrine and thrombin receptor activating peptide (TRAP).

5.3.3 Platelet Function Assay

Platelet function was assessed using a modification of light transmission aggregometry, described elsewhere (16, 29, 35) and detailed in Chapter 2. In brief, PRP was added to each well of a 96-well plate containing the different agonists. Light absorbance was measured at standard times. To characterise platelet aggregation, increasing concentrations of the agonists were tested. Platelet aggregation measured as a percentage of
absorbance from baseline was standardised times. The time from blood draw until the end of the assay protocol was recorded.

Maximal platelet aggregation was defined as no change in light absorbance with incremental concentration of agonist at two consecutive time points. Sigmoidal log dose-response curves were plotted for each agonist at all concentrations. We then compared platelet function in IA patients taking sulfasalazine (2-3g daily) (Sulfasalazine group, n=15) to that of IA patients using alternative agents (anti-TNFα therapy, methotrexate, hydroxychloroquine, steroids), alone or in combination (Other DMARDs group, n=17). Stable CVD patients with no history of IA and receiving anti-platelet monotherapy with 75mg of aspirin were used as a positive control group (Aspirin group, n=15).

5.3.4 Platelet function with sulfasalazine and metabolites in vitro

To assess the specific effect of sulfasalazine and its individual components (5-ASA and sulfapyridine) on the platelet thromboxane pathway (with aspirin as a positive control), blood samples were obtained from healthy volunteers (n=18, 9 male and 9 female) who were not taking any medication. Sulfasalazine, 5-ASA, sulfapyridine, and acetylsalicylic acid (aspirin) were purchased from Sigma laboratories. All drugs were reconstituted with dimethyl sulfoxide (DMSO) and diluted to their respective concentrations with deionised water. The final concentration of DMSO in each solution tested was <1%. Phlebotomy and platelet preparation were carried out as described above and assayed using standard light transmission aggregometry as described in Chapter 2. In brief, the PRP obtained was divided into aliquots of 1ml and these were incubated (for 45mins at 37°C) with increasing concentrations (40µM, 50µM, and 100µM) of either sulfasalazine, 5-ASA or sulfapyridine. As all three components are simultaneously present in the plasma of patients taking the drug, (at typical concentrations of 40-50µM), increasing concentrations (10µM, 20µM, 40µM, and 50µM) of a mixed solution of sulfasalazine, 5-ASA and sulfapyridine were also tested, in this
physiological range (22). Aliquots of PRP incubated with 1% DMSO (vehicle control) and 20\( \mu \)M aspirin were used as negative and positive controls, respectively. The negative control is representative of the normal platelet aggregation response in the absence of medication, and the positive control of 20\( \mu \)M acetylsalicylic acid is representative of the physiological plasma concentration of aspirin present in patients taking low dose aspirin (75mg daily) for CVD prevention (38). The platelet count in each sample was assayed pre- and post-incubation using a standard Sysmex haematology cell counter. Platelet function was assessed using conventional light transmission aggregometry (LTA) on a 4-channel Chronolog aggregometer, and the platelet aggregation responses to 500\( \mu \)g/ml of arachidonic acid were tested, as described (39, 40). The positive and negative controls were used in every aggregometry run alongside 2 other samples. All concentrations of each drug were tested at least 3 times for every donor.

5.3.5 Statistics

Demographic data categorical variables were analysed using the Fisher’s Exact Test. Disease activity data were analysed using the Wilcoxon signed-rank test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for per cent platelet aggregation were calculated with the nominal level of significance set at 5%, and multiple comparisons between different agonist concentrations were adjusted using the Bonferroni correction. Sigmoidal dose-response curves for all groups were compared separately for each agonist using the extra sum-of-squares F-test.

As the maximal aggregation responses obtained from the series of in vitro LTA experiments were not all normally distributed, these results were analysed using the non-parametric Wilcoxon signed-rank test.
5.4 Results

5.4.1 Patient data

135 consecutive patients with an established diagnosis of IA were screened for inclusion. 32 patients with IA [RA (n=21), PsA (n=8), AS (n=3)] who fulfilled the inclusion and exclusion criteria were enrolled. The average time from blood draw to completion of the platelet function assay was 40+/-5 minutes.

5.4.2 Patient characteristics

Demographic data and disease activity indices are shown in Table 5.1. The two groups with IA (Sulfasalazine v Other DMARDs) were similar in terms of age, gender, diagnosis, and standard cardiovascular risk factors. There was no significant difference in disease activity, with both groups displaying some objective evidence of inflammation in the low to moderate disease activity range (Mean +/- sem; Sulfasalazine, 3.82 +/- 0.46, and Other DMARDs, 3.4 +/- 0.46, p=0.48), as defined by DAS-28 score (32).

The only significant differences between the two groups related to their use of medications (p<0.001). Patients in the Sulfasalazine group (n=15) were all receiving sulfasalazine 2-3g daily in divided doses (13 sulfasalazine alone and 2 with methotrexate). Patients in the Other DMARDs group (n=17) were using stable doses of alternative DMARDs (anti-TNFα therapy, methotrexate, hydroxychloroquine, steroids), alone or in combination.
Table 5.1: Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>IA COHORT N=32</th>
<th>Sulfasalazine N=15</th>
<th>Other DMARDs N=17</th>
<th>“p” value</th>
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<tbody>
<tr>
<td>AGE yrs</td>
<td>49+/-10</td>
<td>50+/-9</td>
<td>49+/-12</td>
<td>ns</td>
</tr>
<tr>
<td>MALE (%)</td>
<td>14 (44%)</td>
<td>6 (40%)</td>
<td>8 (47%)</td>
<td>ns</td>
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<tr>
<td>DIAGNOSIS (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>21 (66%)</td>
<td>9 (66%)</td>
<td>12 (71%)</td>
<td>ns</td>
</tr>
<tr>
<td>PsA</td>
<td>8 (25%)</td>
<td>3 (20%)</td>
<td>5 (29%)</td>
<td>ns</td>
</tr>
<tr>
<td>AS</td>
<td>3 (7%)</td>
<td>1 (7%)</td>
<td>2 (12%)</td>
<td>ns</td>
</tr>
<tr>
<td>RISK FACTORS (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMOKING</td>
<td>10 (31%)</td>
<td>4 (27%)</td>
<td>6 (35%)</td>
<td>ns</td>
</tr>
<tr>
<td>DM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>HTN</td>
<td>5 (16%)</td>
<td>2 (13%)</td>
<td>3 (17%)</td>
<td>ns</td>
</tr>
<tr>
<td>CHOL</td>
<td>4 (12%)</td>
<td>2 (13%)</td>
<td>2 (12%)</td>
<td>ns</td>
</tr>
<tr>
<td>FAMILY HX</td>
<td>4 (12%)</td>
<td>2 (13%)</td>
<td>2 (12%)</td>
<td>ns</td>
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<td>MEDICATIONS(%)</td>
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<td></td>
</tr>
<tr>
<td>SZP</td>
<td>15 (47%)</td>
<td>15 (100%)</td>
<td>0 (0%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>ANTI-TNFα</td>
<td>8 (25%)</td>
<td>0 (0%)</td>
<td>8 (47%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>MTX</td>
<td>11 (34%)</td>
<td>2 (13%)</td>
<td>9 (53%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>HCL</td>
<td>3 (9%)</td>
<td>0 (0%)</td>
<td>3 (17%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>STEROIDS</td>
<td>3 (9%)</td>
<td>0 (0%)</td>
<td>4 (17%)</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Disease Activity Data</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(mean +/- SEM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>30.13 +/- 4.91</td>
<td>28.89 +/- 6.18</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>19.78 +/- 6.87</td>
<td>16.71 +/- 7.67</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.61 +/- 0.3</td>
<td>3.51 +/- 0.28</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>VASDA (0-100)</td>
<td>41.07 +/- 7.75</td>
<td>29.44 +/- 7.65</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>VASPAIN (0-100)</td>
<td>36 +/- 8.8</td>
<td>33.39 +/- 7.88</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>EMS (mins)</td>
<td>32.50 +/- 14.41</td>
<td>28.33 +/- 19.95</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.41</td>
<td>19.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS-28 SCORE</td>
<td>3.82 +/- 0.46</td>
<td>3.4 +/- 0.46</td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

** significant difference between the groups
RA= rheumatoid arthritis, PSA=psoriatic arthritis, AS= ankylosing spondylitis, DM=diabetes mellitus, HTN= hypertension, Chol= hypercholesterolaemia, Hx= history.
ANTI-TNFα= anti- tumour necrosis α drugs, MTX= methotrexate, SZP= salazopyrin, HCL= hydroxychloroquine.

ESR= erythrocyte sedimentation rate, CRP= C-reactive protein, VASDA= visual analogue scale of disease activity, VASPAIN= visual analogue scale of pain, EMS= early morning stiffness. DAS-28 = 28 joint count disease activity score.
5.4.3 Platelet function data

Increasing concentrations of agonist caused increasing platelet aggregation. The response to each agonist was plotted as a sigmoidal log dose-response curve and the curves for each agonist compared in the three groups. The results of these platelet function assays in the three cohorts of patients receiving sulfasalazine (Sulfasalazine group, n=15), other disease-modifying agents (Other DMARDs group, n=17), and the stable CVD patients taking aspirin (Aspirin group, n=15) were compared.

Patients in the Sulfasalazine group had a significant reduction in platelet aggregation response to arachidonic acid (AA) compared to patients in the Other DMARDs group (p<0.001) (Figure 5.1A). The maximal aggregation responses to the maximum dose (500μg/ml) of arachidonic acid were (Mean, SD) Sulfasalazine group, (9+/−7%) v Other DMARDs group, (77+/−12%), respectively (p<0.001). This decreased response to arachidonic acid in the patients taking sulfasalazine was identical to that seen in patients taking aspirin (10+/−6%), (Figure 5.1B).

Log dose-response curves for collagen, epinephrine, TRAP, and ADP are shown in Figure 5.2. Platelet aggregation responses for all of these agonists were similar in the Sulfasalazine and Other DMARDs groups. Patients in the Aspirin group also had a similar response to collagen. There was a small but significantly increased platelet response to submaximal concentrations of epinephrine (0.00122 μM, 0.00488 μM, 0.0195 μM, and 0.078 μM) and TRAP (0.156 μM, 0.313 μM, and 0.625 μM) (p<0.05) in both groups of patients with IA (Sulfasalazine and Other DMARDs groups) compared to the Aspirin group, albeit in the lowest dose ranges of these agonists. Furthermore both groups of patients with IA also had a significantly increased response to the highest concentrations (2.5 μM, 5 μM, 10 μM, and 20 μM) of ADP (p<0.01), compared to the aspirin-treated patients.
Figure 5.1: Platelet aggregation in response to arachidonic acid is significantly inhibited in both the Sulfasalazine and Aspirin group compared to those on Other DMARDs.

(Panel A) Log dose-response curves of platelet aggregation in response to incremental concentrations of Arachidonic Acid. *** p<0.001. (Panel B) Mean maximal platelet aggregation in response to 500μg/ml of Arachidonic Acid. *** p<0.001.
The maximal platelet aggregation responses to collagen, epinephrine, TRAP, and ADP are shown in Figure 5.3. Mean maximal aggregation across all 3 groups was similar in response to the highest doses of collagen (190μg/ml), epinephrine (20μM), and TRAP (20μM) (Figure 5.3A). However, there was a significant increase in the mean maximal aggregation response to ADP (20μM) in both of the IA groups (Sulfasalazine group 1, 80+/- 5%; Other DMARDs group, 81+/- 6%) compared to that of the aspirin treated group (Aspirin group, 68+/- 6%), (p<0.01, Figure 5.3B).

Maximal aggregation responses are similar in all three groups for collagen, epinephrine, and TRAP (Panel A). Both groups of patients with IA have an enhanced maximal aggregation response to 20μM ADP compared to CVD patients taking aspirin (Panel B). p<0.01.

Mean platelet counts were similar in both groups of patients with IA (Sulfasalazine group (95% CI 280, 364 x 10^9/l), Other DMARDs (95% CI 232, 359 x 10^9/l)), and while the mean platelet count in the aspirin treated CVD patients (Aspirin group (95% CI 217,300)) was similar to that of the Other DMARDs group, it was significantly less than that of the Sulfasalazine group (p<0.05). However this difference was small and mean platelet counts in all three groups fell within the normal range (150, 450 x 10^9/l).
Figure 5.2: ADP induced platelet aggregation is significantly greater in both groups of IA patients compared to CVD patients taking aspirin.

Log dose-response curves of platelet aggregation in response to incremental concentrations of the agonists, collagen (Panel A), epinephrine (Panel B), TRAP (Panel C), and ADP (Panel D). Platelet responses are similar for both groups of patients with IA. Platelet aggregation in response to low concentrations of epinephrine (Panel B) and TRAP (Panel C) and to the highest concentrations of ADP (Panel D) are significantly greater in patients with IA. *p<0.05, **p<0.01.
Figure 5.3: Maximal aggregation responses reveal a uniquely enhanced ADP response in patients with IA compared to CVD patients taking aspirin.
5.4.4 Sulfasalazine and its metabolites *in vitro*

There were no significant differences between the pre- and post-incubation platelet counts for any of the samples. Sulfasalazine, 5-ASA, and sulfapyridine each inhibited arachidonic acid-induced platelet aggregation to some degree. The mean maximal aggregation responses to 500μg/ml of arachidonic acid (AA) was 86+/-7%. Platelet aggregation in response to AA in the presence of 50μM sulfapyridine was 71+/-6% (p<0.01), in the presence of 50μM 5-ASA was 47 +/-12% (p<0.001), and 32+/-9% in the presence of 50μM sulfasalazine (p<0.001) (Figure 5.4A). Increasing concentrations (10 μM each of sulfasalazine, 5-ASA, and sulfapyridine; 20 μM each of sulfasalazine, 5-ASA, and sulfapyridine; 40 μM each of sulfasalazine, 5-ASA, and sulfapyridine; and 50 μM each of sulfasalazine, 5-ASA, and sulfapyridine) of a combined solution of all three agents synergistically inhibited arachidonic acid-induced platelet aggregation in a dose-dependent manner (Figure 5.4B). At physiological plasma concentrations that are seen *in vivo* (40μM each of sulfasalazine, 5-ASA, and sulfapyridine) (22), this solution effectively abrogated the platelet response to 500μg/ml of arachidonic acid (p<0.001) and achieved a similar level of inhibition to a physiological concentration (20μM) of aspirin (38) (Figure 5.4B).
Figure 5.4: Platelet aggregation in response to Arachidonic Acid is inhibited by sulfapyridine, 5-ASA, and sulfasalazine (Panel A).

A mixed solution (containing equal parts sulfasalazine, 5-ASA, and sulfapyridine) dose-dependently inhibits platelet aggregation in response to Arachidonic Acid (Panel B). **p<0.01, ***p<0.001.

Platelet aggregation in response to 500μg/ml of arachidonic acid.
5.5 Conclusion

The results of this investigation demonstrate a number of novel findings. First, patients with IA receiving sulfasalazine therapy have a significantly inhibited platelet response to arachidonic acid (AA) compared to patients with IA taking other DMARD’s. This inhibition of AA-induced platelet aggregation in patients taking sulfasalazine is comparable to that of CVD patients taking aspirin. Secondly, both groups of IA patients demonstrate a significantly enhanced maximal platelet response to ADP compared to CVD patients taking aspirin. Finally, both sulfasalazine and its components reduce the platelet response to arachidonic acid *in vitro* with an effective abrogation of AA-induced platelet aggregation by a mixture of sulfasalazine and its components, at physiological concentrations. The relevance of these findings is discussed in detail in Chapter 7.
Chapter 6 The impact of anti-TNF therapy on platelet function, insulin metabolism, and lipid profile in patients with inflammatory arthritis
6.1 Background
Patients with rheumatoid arthritis (RA) die prematurely from cardiovascular disease (CVD) (46). There is an emerging body of evidence that patients with other forms of inflammatory arthritis (IA), such as psoriatic arthritis (PsA) and seronegative spondyloarthopathy (SpA), are at a similar increased CVD risk (46). Biologic therapy with anti-TNFα agents has revolutionised the treatment of IA (24) and data from the British Biologics Registry demonstrates a clear reduction in the incidence of MI in patients with RA who respond to anti-TNFα therapy (25).

6.2 Rationale
The mechanisms underlying the CVD risk reduction in RA patients receiving anti-TNFα drugs are unclear, with some studies indicating possible effects on lipid profile (26) and insulin metabolism (27, 28). The effects of anti-TNFα therapy on platelet function are unknown. Furthermore, having demonstrated increased platelet reactivity, specific to the ADP pathway, in patients with IA who have objective evidence of inflammation compared to IA patients in disease remission (29) we sought to examine, in a prospective manner, whether this phenomenon is reversible. Therefore we decided to investigate the impact of improved disease control with anti-TNFα therapy on platelet function, LDL cholesterol, and insulin resistance in patients with IA.

6.3 Patients and Methods
Ethical approval was obtained from the Mater Misericordiae University Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients prior to phlebotomy.

For a detailed description of patients and methods please see Chapter 2.

In brief, patients with an established diagnosis of IA aged between 18 and 70 years who were attending the rheumatology outpatients clinic and who
had previously failed treatment with, or were intolerant of, methotrexate and/or whose disease, in the opinion of their attending consultant rheumatologist, warranted treatment with an anti-TNFα agent were considered for inclusion. Exclusion criteria were applied as described previously.

Patients were evaluated pre and post-treatment on each of 2 separate occasions, at baseline and after 4 months of treatment with one of the following subcutaneously administered anti-TNFα agents; adalimumab, etanercept, or golimumab.

6.3.1 Patient clinical data
Demographic data and traditional cardiovascular risk factors (dyslipidaemia, hypertension, smoking status, and family history) were recorded. A detailed medication record was also obtained from each patient. Disease activity was assessed both clinically and serologically as described previously. Standard inflammatory markers (ESR, CRP, and fibrinogen) were measured, and the internationally validated 28-joint count of disease activity (DAS-28) (32) was calculated for all patients.

Patients were classified as Responders if they demonstrated a reduction of at least one disease activity category by DAS 28 criteria (32), or by a 40 percent improvement in VASDA for those patients with spondyloarthritis in which the DAS28 has not been validated (47). Routine serological data (full blood count, renal, liver and bone profiles) and fasting samples of traditional cardiovascular risk factors comprising blood glucose, insulin level, and lipid profile were also collected. Total cholesterol, HDL, triglycerides, and LDL levels were obtained as per standard laboratory assessment and the HDL/LDL ratio calculated for all subjects (34). Insulin resistance was measured using the established HOMA-IR method (33).

6.3.2 Phlebotomy and platelet preparation
Pre- and post-treatment blood samples for platelet function testing were obtained at baseline and after 4 months treatment. Blood was drawn from all patients by the same phlebotomist. All samples were obtained
uncuffed and following an overnight fast. Blood was collected through a 19-gauge Butterfly needle and the first 30 ml was sent for baseline serological data, inflammatory markers, and cardiovascular risk factors. A further 40ml was collected into a syringe containing 3.2% sodium citrate. Blood was then centrifuged for 10 min at 150g. Platelet-rich plasma (PRP) aspirated from the supernatant was placed in a reagent reservoir. Using a multichannel pipette, the PRP was dispensed across wells in a 96-well plate (black isoplate with clear flat-bottomed wells, Perkin Elmer) containing incremental concentrations of the different agonists arachidonic acid, collagen (type 1 soluble calf skin), adenosine diphosphate (ADP), and epinephrine.

6.3.3 Platelet function assay
Platelet function was assessed using a modification of light transmission aggregometry, described in detail elsewhere (16, 29, 35) and in Chapter 2. In brief, 180 μl of PRP was added to each well of a 96-well plate containing the different agonists. Light absorbance was measured at standard times. To characterise platelet aggregation, increasing concentrations of the agonists were tested. The time from blood draw until the end of the assay protocol was recorded. Maximal platelet aggregation was defined as no change in light absorbance with incremental concentration of agonist at two consecutive time points. Sigmoidal log dose-response curves were plotted for each agonist at all concentrations. Pre and post-treatment platelet function profiles were then compared for all patients, with each patient acting as their own control.

6.3.4 Statistics
Demographic data categorical variables were analysed using the Fisher’s Exact Test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for per cent platelet aggregation were calculated with the nominal level of significance set at 5%, and multiple comparisons between different agonist concentrations were adjusted using the
Bonferroni correction. Due to the relatively small sample size, differences in pre and post-treatment disease activity scores and serological indices were analysed using the non-parametric Wilcoxon signed rank test or the Mann-Whitney test, where appropriate. Sigmoidal dose-response curves of platelet function profiles were compared using the extra sum-of-squares F-test and EC50 values expressed as 95% confidence intervals.

6.4 Results

A total of 30 patients were initially enrolled in the study. However, due to a variety of factors (five patients discontinued, serological markers were unavailable for another, and one subject was lost to follow up) complete data from a total of 23 patients were analysed. Of those who discontinued, two had recurrent upper respiratory tract infections, two had persistent skin site reactions and the final one elected to stop her medication. Baseline demographic data, rheumatological diagnoses, traditional cardiovascular risk factors, and medication use are summarised in Table 6.1.

Based on the pre-set criteria of a reduction by at least one DAS 28 disease activity category (n=20) or a 40% improvement in VASDA (n=3), where applicable, n=18 achieved a discernible clinical response and thus were deemed “Responders” while n=5 were classified as “Non-responders”. There were no differences in clinical response rates among the different anti-TNF agents used. All patient characteristics including underlying diagnosis, anti-body positivity, CVD risk factors, and combination DMARD use were similar among both groups.
Table 6.1: Demographics

<table>
<thead>
<tr>
<th></th>
<th>Entire Cohort (N=23)</th>
<th>Responders (N=18)</th>
<th>Non-responders (N=5)</th>
<th>‘p’ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female Gender</td>
<td>15</td>
<td>12</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Disease (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>69</td>
<td>72</td>
<td>60</td>
<td>0.62</td>
</tr>
<tr>
<td>RF pos</td>
<td>52</td>
<td>55</td>
<td>40</td>
<td>0.64</td>
</tr>
<tr>
<td>CCP pos</td>
<td>48</td>
<td>50</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>RF/CCP dual pos</td>
<td>43</td>
<td>50</td>
<td>20</td>
<td>0.33</td>
</tr>
<tr>
<td>PSA</td>
<td>17</td>
<td>17</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>AS</td>
<td>9</td>
<td>11</td>
<td>20</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>CVD Risk Factor (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>22</td>
<td>22</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Hypertension</td>
<td>22</td>
<td>17</td>
<td>40</td>
<td>0.29</td>
</tr>
<tr>
<td>Family Hx</td>
<td>35</td>
<td>33</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Medication use (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etanercept</td>
<td>65</td>
<td>67</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>30</td>
<td>28</td>
<td>40</td>
<td>0.62</td>
</tr>
<tr>
<td>Golimumab</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Combination DMARDs (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>65</td>
<td>67</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>Plaquenil</td>
<td>9</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>9</td>
<td>11</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Steroid</td>
<td>9</td>
<td>6</td>
<td>20</td>
<td>0.39</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>26</td>
<td>17</td>
<td>60</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Disease activity, serological indices, and lipid profiles are summarised in Table 6.2. Across the entire cohort there were significant differences in mean values of DAS-28 score (5.2 v 3.4, p<0.001), sedimentation rate (29mm/h v 18mm/h, p<0.02), C-reactive protein (27mg/dl v 8mg/dl, p<0.02), plasma fibrinogen (4.1g/l vs 3.1g/l, p<0.01), VASDA (59.36mm v 32.83mm, p<0.01), platelet count (315 x 10^9 v 258 x 10^9, p<0.001) and the HOMA-IR ratio (2.6 v 1.9, p<0.001), pre- and post treatment, respectively, while lipid profiles were similar. Among non-responders alone, there were no differences in any of these variables whereas responders demonstrated significant post-treatment reductions in markers of inflammation (ESR, C-reactive protein, and fibrinogen), disease activity indices (DAS-28 and VASDA) and platelet count, with no change in lipid profile (HDL, LDL, HDL/LDL ratio, TG, and Total Cholesterol). There were significant differences between responders and non-responders in baseline values of DAS-28 score (5.6 v 3.8, p<0.01), C-reactive protein (34.0 mg/dl v 3.0 mg/dl, p<0.01), LDL cholesterol (2.9 mmol/l v 3.7 mmol/l, p<0.05), and HDL/LDL ratio (0.50 v 0.35, p<0.05), while values for the remaining variables were similar. Post-treatment, in addition to a persistent difference between responders and non-responders in relation to LDL (2.9 mmol/l v v 4.2 mmol/l, p<0.05), total cholesterol (4.7 mmol/l v 6.3 mmol/l, p<0.05) and triglyceride levels (0.9 mmol/l v 2.3 mmol/l, p<0.05) were also higher in non-responders. Also, while post-treatment blood markers of inflammation (ESR, CRP, and fibrinogen) and DAS-28 scores were similar, responders had significantly lower VASDA indices compared to non-responders (27 mm v 54 mm, p<0.05).
<table>
<thead>
<tr>
<th>Clinical Parameter (95% CI)</th>
<th>Entire Cohort</th>
<th>Responders</th>
<th>Non-responders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 months</td>
<td>Baseline</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>29(16,42)</td>
<td>18(9,27)</td>
<td>0.01</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>27(4,50)</td>
<td>8(2,14)</td>
<td>0.02</td>
</tr>
<tr>
<td>Fib (g/L)</td>
<td>4.1(3,4,4.6)</td>
<td>3.1(2,6,3.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Platelet count (x10⁹)</td>
<td>315(265,364)</td>
<td>258(229,288)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>4.9(4,4,5.3)</td>
<td>5.1(4,5,5.6)</td>
<td>0.27</td>
</tr>
<tr>
<td>TG</td>
<td>1.2(0.8,1.5)</td>
<td>1.2(0.8,1.6)</td>
<td>0.60</td>
</tr>
<tr>
<td>LDL</td>
<td>3.1(2,7,3,4)</td>
<td>3.2(2,7,3,6)</td>
<td>0.64</td>
</tr>
<tr>
<td>HDL</td>
<td>1.3(1,2,1,4)</td>
<td>1(1,3,1,6)</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL/ LDL Ratio</td>
<td>0.47(0,39,0,55)</td>
<td>0.59(0,42,0,62)</td>
<td>0.35</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.6(2,2,3)</td>
<td>1.9(1,6,2,2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DAS-28 score</td>
<td>5.2(4,6,5,8)</td>
<td>3.4(2,9,3,8)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>VASDA</td>
<td>59(48,71)</td>
<td>33(22,43)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Significant differences Responders v Non-Responders at Baseline * p<0.05, ** p<0.01

Φ Significant differences Responders v Non-Responders at 4 months Φ p<0.05
Platelet function data are described in Table 6.3. There was no difference between pre and post-treatment platelet responses to arachidonic acid, collagen, epinephrine and TRAP across the entire cohort, see Figure 6.1. However, as illustrated in Figure 6.2, post-treatment platelet reactivity to ADP was significantly reduced following treatment with anti-TNF agents (EC50 1.18 vs 1.31, P<0.05). Interestingly, among Non-responders, platelet responses to ADP were almost identical (EC50 1.18 vs 1.17, p=0.89). Therefore, the significant reduction in the platelet ADP aggregation profile across the entire cohort is wholly accounted for by the markedly reduced post-treatment values among Responders alone (EC50 1.97 vs 1.17, p<0.001), see Figure 6.3. Platelet reactivity profiles for all agonists remained largely unchanged.
Table 6.3: Platelet Function Data

| Agonist Concentration (EC50) | Entire Cohort | | | Responders | | | Non-responders | | |
|-----------------------------|---------------|| | | | | | | | |
|                             | Baseline  | 4 months | ‘p’ value | Baseline | 4 months | ‘p’ value | Baseline | 4 months | ‘p’ value |
| AA µg/ml                    | 381       | 376       | 0.84       | 372       | 357       | 0.44       | 379       | 358       | 0.47       |
| Collagen µg/ml              | 90        | 102       | 0.54       | 97        | 94        | 0.92       | 90        | 98        | 0.93       |
| ADP µM                      | 1.18      | 1.31      | 0.03*      | 1.17      | 1.97      | <0.001**   | 1.18      | 1.17      | 0.89       |
| Epinephrine µM              | 3.28      | 3.49      | 0.94       | 3.37      | 3.45      | 0.99       | 3.21      | 3.54      | 0.88       |
| TRAP µM                     | 1.39      | 1.36      | 0.89       | 1.31      | 1.25      | 0.88       | 1.32      | 1.28      | 0.94       |

* Significant Difference between the groups * p<0.05, ** p<0.01
Figure 6.1 Pre and post-treatment platelet aggregation responses (clockwise from top left) to Arachidonic Acid (AA), Collagen, TRAP, and epinephrine are similar across the Entire Cohort.
Figure 6.2 Platelet reactivity to ADP is decreased following treatment with Anti-TNF therapy, p<0.05.
**Figure 6.3.** There is a significant reduction in ADP induced platelet aggregation among Responders to Anti-TNF therapy only, $p<0.001$. 
6.5 Conclusion
The results of this investigation reveal a number of key findings. First, these data demonstrate a significant decrease in platelet reactivity, that is particular to the ADP pathway, in patients with IA who respond to anti-TNFα therapy. Second, there is a clear association between improved disease control with anti-TNFα inhibition and reduced insulin resistance in these patients. Finally, while pre and post treatment lipid levels were largely unchanged across the entire cohort, there are clear differences in lipid profiles between responders and non-responders, both before and after treatment with anti-TNFα agents. These findings are discussed in detail in Chapter 7.
Chapter 7 Discussion
The findings from each of the four individual studies reported in Chapters 3 through 6 above are discussed separately below and are followed by concluding remarks.

7.1 Inflammation and platelet function in patients with IA.

The results of this investigation demonstrate a number of key findings. Firstly, the patients in this study with objective evidence of active IA demonstrate increased platelet reactivity specifically to ADP. Secondly, there was no significant difference in platelet response to any of the other agonists in terms of disease activity. Thirdly, when maximal concentrations of agonists are used in platelet aggregation, there is no difference seen between the two groups. Epidemiological evidence has clearly shown that IA is an independent risk factor for CVD (3) and that this risk is greatest in those with chronic active disease (5). Several lines of evidence support the use of platelet reactivity in predicting the risk of future cardiovascular events (9-12). Thus the unique platelet hyper-reactivity in response to ADP in patients with active IA may explain some of this risk, and has implications for therapy.

Platelets express at least two ADP receptors, P2Y1 and P2Y12. Both are G-protein coupled surface receptors, P2Y1 coupled to G\textsubscript{q} and P2Y12 to G\textsubscript{i}. P2Y1 is a weak agonist of platelet aggregation, whereas the P2Y12 receptor is the major receptor involved in amplifying and sustaining platelet aggregation in response to ADP (4). The therapeutic importance of the ADP-P2Y12 receptor interaction is evident from the reduction in adverse cardiovascular events when this receptor is inhibited with drugs such as ticlopidine and clopidogrel (48).

The mechanisms underlying the enhanced platelet response to ADP in active IA are currently unexplained. Studies have shown an increased platelet responsiveness to ADP in insulin resistant states (49). Furthermore, insulin resistant patients with elevated fibrinogen levels treated with the ADP-P2Y12 antagonist clopidogrel, exhibit less inhibition
of platelet reactivity in response to ADP, than those with normal plasma fibrinogen (50). Of interest in our study, plasma fibrinogen was the most consistently elevated serological marker of active disease. Several studies have demonstrated increased insulin resistance in patients with IA (51), and this is associated with active inflammation (27, 52). Insulin resistance has been shown to ameliorate in response to treatment with disease modifying anti-rheumatic drugs (DMARDs), in particular anti-TNF\(\alpha\) agents (28). The use of anti-TNF\(\alpha\) therapy was significantly higher in our controlled disease cohort and this group did not show the same response to ADP compared to those with active IA. Moreover, insulin resistance is associated with abnormal platelet function (53). Insulin at physiological concentrations in normal individuals has an anti-aggregating effect on platelets. This response is perturbed in insulin resistant subjects (54). Thus a link between active inflammatory disease, insulin resistance, fibrinogen, and platelet ADP hyper-responsiveness seems possible. We did not evaluate insulin resistance prospectively in our patient study, hence the rationale for its inclusion in the analysis reported in Chapter 6.

Platelet activation is associated with systemic inflammation and platelet-derived mediators of inflammation potentiate the inflammatory response (4). CD40 ligand is stored in intracellular granules of resting platelets and rapidly presents on the platelet surface after platelet activation (55). This ligand is a trimeric transmembrane protein in the TNF family. On the platelet surface, CD40 ligand is cleaved to its active soluble fragment. This active soluble fragment inhibits endothelial ADP-ase, thus increasing the bioavailability of ADP, and also induces endothelial cells to produce reactive oxygen species to generate further mediators of inflammation (56). Clopidogrel significantly inhibits ADP-induced CD40 ligand expression (55). Furthermore, TNF\(\alpha\) has been shown to directly upregulate CD40 ligand on the platelet surface in patients with heart failure (57). Patients with active IA have markedly elevated TNF\(\alpha\) levels. It is possible that actively inflamed IA patients also upregulate CD40 ligand expression and consequently have a physiological bias towards increased ADP bioavailability.
TNFα is also implicated in the development of insulin resistance in RA and TNFα blockade reduces insulin resistance in this cohort (28). We have already referred to the association between increased plasma fibrinogen and platelet ADP hypersensitivity in the insulin resistant state (50) and the fact that fibrinogen levels were consistently elevated in our active disease cohort. Activated platelets bind fibrinogen following a conformational change in the GPIIbIIIa receptor (4). Intracellular platelet fibrinogen stores are continuously replenished from the platelet external environment. Thus patients with IA who have chronic active inflammation and insulin resistance may exhibit enhanced fibrinogen binding in the setting of a hyper-reactive platelet ADP receptor-effector system.

7.2 Pro-inflammatory cytokines and the platelet ADP pathway

The results of this investigation demonstrate a number of key findings. First, this is a novel prospective demonstration that improved disease control with IL-6 receptor inhibition in patients with previously refractory RA is accompanied by a significant change in platelet function. This is particularly apparent in the ADP pathway with a decrease in aggregation response to all concentrations tested, but also observed with low concentrations of epinephrine and high concentrations of arachidonic acid. Interestingly, there was no change in platelet responses to collagen. Secondly, pro-inflammatory cytokines known to be involved in the pathogenesis of RA enhance the platelet aggregation and/or activation responses to ADP, in samples from healthy volunteers at the concentrations tested. Finally, while the improved control of inflammation with tocilizumab had no impact on insulin sensitivity, there was a significant increase in levels of LDL cholesterol, a consistent finding in several recent studies involving a much greater number of patients (42). The reasons for these changes in platelet function in patients with RA following control of inflammation are unclear. While the phenomenon of “reactive thrombocytosis” during acute flares in RA has long been recognised as cytokine driven (18, 58), the reduction in platelet count following treatment (as occurred in all of our patients) does not explain changes in platelet function (59). Indeed, previous work has
demonstrated that artificially controlling for platelet count actually inhibits platelet aggregation measurements, especially in response to ADP (60).

Prior studies have indicated significant differences in thromboxane/prostacyclin balance in response to treatment in patients with RA (61) and, as this is the primary regulator of platelet arachidonic acid metabolism, this is a possible explanation for the observed decreased response to the higher concentrations of this agonist. Similarly, others have found clear differences in neuroendocrine adrenergic acute stress responses in patients with RA coupled with IL-6 levels (62). Conversely, inhibition of IL-6 could theoretically lead to lower levels of endogenous epinephrine thereby explaining the decreased platelet responses to low submaximal concentrations in the treated patients that are overcome with the addition of higher concentrations of exogenous epinephrine used in our assay.

Given the recent compelling evidence that platelet microparticles, generated in a collagen-dependent manner, drive joint inflammation mediated by the platelet GPVI receptor (63), we had expected to observe some difference in platelet collagen responses with improved control of inflammation. This did not occur. However, being the most thrombogenic component of the subendothelium and with other receptors such as the integrin α2β1 intricately involved in the platelet response to collagen (64), it is plausible that the inflammatory potential of platelet microparticle generation by collagen is entirely independent of its platelet aggregating properties.

The very clear differences in platelet responses to ADP warrant special consideration. Numerous studies have shown a clear association between elevated markers of inflammation (eg CRP and fibrinogen) and a poor response to ADP-blockade with clopidogrel in patients with CVD, and that a poor response to ADP inhibition manifested by “high on treatment platelet reactivity” predicts future adverse cardiovascular events (65, 66). Furthermore, an imbalance between pro and anti-inflammatory cytokines is also associated with sub-optimal ADP antagonism in CVD patients presenting with acute coronary syndromes.
Moreover, several pro-inflammatory cytokines known to be elevated in patients with active RA have been strongly implicated in driving atherosclerotic disease (68). Previous work has highlighted the both the pathogenic role of IL-6-GP130 interaction in CVD (19) and the pro-thrombotic effects of IL-6 on platelet function (20). Furthermore, following stimulation by a variety of agonists, release of intra-platelet stores of IL-6 receptor and subsequent binding of the IL-6/IL6 receptor complex to the platelet GP 130 receptor to initiate a positive feedback loop has been clearly demonstrated (69). Thus in the context of platelet activation by ADP, the most important agonist in maintaining and propagating the platelet aggregation response (4), this may help to explain the potentiating effect of IL-6 on platelet function (69).

Therefore, it is not altogether surprising that treatment with an IL-6 inhibitor such as tocilizumab could have a dampening influence on platelet responsiveness. As platelets do not possess the standard IL-6 receptor on their extracellular membrane (69), this is less likely due to a direct anti-platelet effect of tocilizumab and more probably a consequence of an altered inflammatory milieu and decreased ancillary binding of the IL-6/receptor complex with platelet GP 130.

In addition to IL 6, the results of the in vitro experiments on the samples from healthy volunteers corroborate previous findings regarding the enhancement of platelet function by TNF alpha (57) and, more recently, IL1beta (70) but are discordant with a recent study involving IL 17a and ADP-induced platelet aggregation (71). However, the latter investigation used both murine and human platelet preparations and a complex analysis of second messenger phosphorylation signalling. Nonetheless, our work did demonstrate an ability of IL-17a to increase platelet activation, as confirmed by P selectin expression on flow cytometry, in conjunction with the other cytokines.

An increase in LDL cholesterol following treatment with tocilizumab has been well reported and substantiated by a recent Cochrane systematic
review (42). However, the significance of this in patients with RA remains undetermined given the known “lipid paradox” in this disease population, whereby untreated RA patients with lower levels of LDL cholesterol have an increased risk of CVD (72). Therefore, the attainment of good disease control may lead to a normalisation of previously suppressed LDL levels, and this might explain the increase in LDL observed in our patients, as has been reported elsewhere (72).

Notwithstanding the clear findings, our study has a number of limitations. These patients were using other disease-modifying drugs that may confound interpretation of the data. However, patients were recruited prospectively, each acting as their own control on pre/post treatment testing, and thus reflect real life clinical practice. Moreover, to the best of our knowledge there is no known anti-platelet effect of the other agents these patients were taking. Regarding the possible mechanisms outlined above, we did not measure serum thromboxane, urinary catecholamine metabolites, soluble GPVI levels, or plasma cytokines. Similarly we did not investigate platelet GP130 interactions with the IL6/receptor complex. Future work in this area should incorporate such assays. Furthermore, the concentrations of cytokines used in the in vitro experiments are in the nanogram range, and are therefore of questionable physiological relevance. However, concentrations of this level have been reported in patients with active RA (17), and clear differences in both aggregation and activation are demonstrated. Finally, a major limitation of this work is that we did not perform any experiments to directly investigate whether the observed augmentation of the platelet ADP response in vitro by these cytokines could be blocked by specific antagonism. Furthermore, as we used platelet rich plasma (PRP) it is theoretically possible that the observed effects could have been due to cytokine stimulation of any remaining lymphocytes or other white blood cells leading to the release of platelet activating factors. Therefore we would recommend using washed platelets and incubating samples with known cytokine antagonists as part of any future investigation of this phenomenon.
**7.3 Inhibition of platelet function by Sulfasalazine**

The results of this study demonstrate a number of novel findings. First, patients with IA receiving sulfasalazine therapy have a significantly inhibited platelet response to arachidonic acid (AA) compared to patients with IA taking other DMARD’s. This inhibition of AA-induced platelet aggregation in patients taking sulfasalazine is comparable to that of CVD patients taking aspirin. Secondly, both groups of IA patients demonstrate a significantly enhanced maximal platelet response to ADP compared to CVD patients taking aspirin. Finally, both sulfasalazine and its components reduce the platelet response to arachidonic acid *in vitro* with an effective abrogation of AA-induced platelet aggregation by a mixture of sulfasalazine and its components, at physiological concentrations.

Patients with rheumatoid arthritis (RA) die prematurely from cardiovascular disease (CVD) (73). In patients with CVD, anti-platelet therapy is effective in reducing mortality (74). Aspirin is used in secondary prevention of future adverse cardiovascular events in patients with established CVD and has an integral role in the primary prevention of CVD in patients at high risk of same (75). Despite the well-known risk of cardiovascular death there are no recognized guidelines on the use of anti-platelet therapy for primary prevention of CVD in patients with IA (46).

The underlying mechanisms by which sulfasalazine lowers CVD risk in patients with IA are currently unknown. However, since we observed that platelets from patients receiving sulfasalazine in our initial investigation appeared to respond poorly to arachidonic acid (albeit confounded by possible concomitant NSAID use), and it has been shown that sulfasalazine inhibits platelet thromboxane production (23), we hypothesised that sulfasalazine may inhibit platelet function. Our results show that platelet aggregation in response to AA is inhibited in patients...
taking sulfasalazine, and is comparable to the inhibition seen in patients with CVD taking aspirin. While we had expected some inhibition of platelet function we were surprised that there was nearly complete inhibition of AA-induced aggregation. Therefore, we characterised the contribution of the individual components of the drug (5-ASA and sulfapyridine) and when combined in the form of their parent compound, sulfasalazine. Thus, while sulfasalazine and its individual components are generally poorly absorbed, our study demonstrates a synergistic inhibition of platelet reactivity in response to arachidonic acid at physiological concentrations of sulfasalazine, 5-ASA, and sulfapyridine (22).

The enhanced platelet reactivity response to ADP in both groups of patients with IA compared to the aspirin-treated group is noteworthy. This could be due to the weak effect of aspirin on platelet aggregation induced by other agonists, as evidenced by the reduced platelet responses to very low doses of epinephrine and TRAP observed in the aspirin-treated patients in this study. However, this inhibitory effect is usually overcome at higher concentrations of agonist (as was the case with both epinephrine and TRAP in these patients) and previous work has demonstrated that the weak inhibitory effect of aspirin on the ADP pathway is nullified at concentrations of ADP above 10μM (76). Differences in platelet count would also not account for the differences seen in the aggregation measurements (59). The clear increase in platelet response to 20 μM ADP in the patients with IA may also be partly as a result of their underlying inflammatory burden, as both groups display objective evidence of ongoing inflammation. Numerous studies have shown a clear association between markers of inflammation and a poor response to ADP-blockade with clopidogrel in patients with CVD and that a poor response to ADP inhibition predicts future adverse cardiovascular events (65, 66). Furthermore, we have already demonstrated an enhanced platelet response to ADP in patients with IA who have objective evidence of inflammation compared to IA patients in disease remission (29). As several lines of evidence support the use of
platelet reactivity in predicting the risk of future adverse cardiovascular events (9, 11), the unique platelet hyper-reactivity in response to ADP (but not epinephrine, collagen, or TRAP) in patients with IA is certainly worthy of future investigation.

This study has a number of limitations. The use of other disease-modifying drugs was not identical between the two IA groups and could potentially confound interpretation of the data. However, patients were recruited consecutively and, in fact, reflect real life clinical practice. Moreover, to the best of our knowledge there is no known anti-platelet effect of the other drugs these patients were taking.

7.4 The impact of anti-TNF therapy on platelet function, insulin metabolism, and lipid profile.

The results of this study reveal a number of key findings. First, these data demonstrate a significant decrease in platelet reactivity, that is particular to the ADP pathway, in patients with IA who respond to anti-TNFα therapy. Second, there is a clear association between improved disease control with anti-TNFα inhibition and reduced insulin resistance in these patients. Finally, while pre and post treatment lipid levels were largely unchanged across the entire cohort, there are clear differences in lipid profiles between responders and non-responders, both before and after treatment with anti-TNFα agents.

Given the greatly increased risk of premature CVD in patients with IA (5) these findings are certainly of interest. While the exact mechanisms underlying the elevated CVD risk in patients with IA are poorly understood, it has become clear that although traditional risk factors remain important, this risk is strongly associated with inflammation (77). Furthermore, recent work has highlighted the key role of inflammation in the pathogenesis of CVD in the general population (41, 78), and trials are currently underway targeting inflammation as a secondary prevention
strategy in patients with known CVD but without a history of a recognised inflammatory disorder (79).

Heightened platelet reactivity (ie an increased tendency of platelets to aggregate) predisposes CVD patients to an increased risk of thrombosis (9) and emerging evidence has established a clear link between levels of inflammation and increased platelet reactivity in patients with established CVD who subsequently experience a recurrent MI (65). While multiple pathways and effector-receptor systems are involved in platelet aggregation, the ADP pathway plays the major role in the amplification and propagation of the aggregation response to form thrombus (4). As previously discussed, platelets express at least 2 ADP receptors, P2Y1 and P2Y12. Both are G-protein coupled surface receptors, P2Y1 coupled to G\(_D\)q and P2Y12 to G\(_D\)i. P2Y1 is a weak agonist of platelet aggregation, whereas the P2Y12 receptor is the major receptor involved in amplifying and sustaining platelet aggregation in response to a variety of agonists (4). Agents that specifically target the P2Y12 receptor (eg clopidogrel) have revolutionised the secondary prevention of CVD, particularly following coronary stent insertion (4). Furthermore, data from the CAPRIE trial demonstrates the superiority of clopidogrel over aspirin in the primary prevention of CVD in certain high-risk groups (eg Type 2 diabetics) while dual anti-platelet therapy with both agents dramatically improves outcomes in the secondary prevention of recurrent events, notwithstanding the associated bleeding risk (80).

Patients with IA share a similar cardiovascular risk profile to those with Type 2 diabetes (81), yet continue to remain unprotected in terms of anti-platelet therapy. We have already shown that patients with active IA have an enhanced platelet response to ADP compared to those in a state of disease remission (29). This finding has since been corroborated by other investigators in a similar study of inflammatory disease activity and platelet reactivity in patients with PsA (82). However, both of these studies were cross-sectional in nature with only one set of measurements. To the best of our knowledge this current work is the first to prospectively demonstrate that improved disease control with anti-
TNFα therapy is associated with a decrease in platelet reactivity to ADP. While this was associated with a reduction in absolute platelet count, this does not explain any observed changes in platelet responsiveness (59) and previous work has demonstrated grossly inaccurate results when native platelet count is manipulated, particularly in response to ADP (60).

Several studies have demonstrated increased insulin resistance (IR) in patients with IA (51), especially in active disease (27), and IR is an independent risk factor for adverse CVD events in this patient population (51). Therefore the clear improvement in insulin sensitivity in patients who respond to anti-TNFα agents is an important finding that is supported by previous work (28). Furthermore, IR is also associated with abnormal platelet function (53). Insulin at physiological concentrations in normal individuals has an anti-aggregating effect on platelets but this response is perturbed in insulin resistant subjects (54). Moreover, several studies have demonstrated increased platelet responsiveness to ADP in insulin resistant states (49), and recent evidence has shown that insulin resistant patients with elevated fibrinogen levels have less inhibition of platelet reactivity by the P2Y12/ADP receptor antagonist, clopidogrel ((50).

Interestingly, while post-treatment plasma fibrinogen levels were similar across all subjects, anti-TNFα responders in our cohort demonstrated a significant reduction in plasma fibrinogen when compared to pre-treatment levels. Unlike the other acute phase reactants measured (ESR, CRP), fibrinogen has a clear functional role in thrombus formation and this link between active inflammation, increased IR, elevated fibrinogen and ADP hyper-responsiveness may significantly contribute to the greatly increased CVD risk in patients with IA. Conversely, controlling inflammation per se should decrease this risk. Taken together, this prospective demonstration that reducing inflammation with anti-TNFα therapy in our patient cohort leads to altered platelet function and improved insulin metabolism may partially explain the empirical evidence that the greatest CVD risk reduction in patients with IA is observed in those who respond to anti-TNFα agents (25).

Perhaps the most interesting finding in this study is the absence of any significant changes to the lipid profile following treatment with anti-TNFα
therapy, across the entire cohort. There is a well described "lipid paradox" in patients with IA, in that those patients with the lowest LDL typically have the worst CVD outcomes, a finding that is thought to be due the fact that these patients have the highest levels of inflammatory disease activity (72). Therefore, the attainment of good disease control should, theoretically at least, lead to an increase in previously suppressed LDL levels. As discussed above, this phenomenon has certainly been observed following control of inflammation in RA with the anti-IL 6 agent tocilizumab, and substantiated by a recent Cochrane systematic review (42). However, previous work has also failed to demonstrate a change in lipid profile following anti-TNFα treatment despite significantly decreased levels of inflammation, in patients who had active disease at baseline (26). Furthermore, a recent investigation involving the specific effects of anti-TNFα therapy on lipid metabolism confirms that patients with IA who the highest levels of disease activity demonstrate an anti-atherogenic lipid profile (ie high HDL/LDL ratio) and that this may possibly be used to predict clinical response to these agents (83). Interestingly, all of the anti-TNFα responders in our cohort had HDL/LDL ratio greater than 0.4, a previously defined cut-off value associated with better outcomes in patients with CVD (34), while all the non-responders demonstrated the opposite. However, the clinical usefulness and physiological relevance of these findings in patients with IA remains uncertain.

Notwithstanding the clear findings, our study has a number of limitations. These patients were using other disease-modifying drugs that may confound interpretation of the data. However, patients were recruited prospectively, each acting as their own control on pre/post treatment testing, and thus reflect real life clinical practice. While none of the patients were using any specific targeted anti-platelet therapy there is a well recognized anti-platelet effect with NSAID use (84), but this effect is confined to the arachidonic acid pathway (85). Furthermore, as discussed above, we have also demonstrated an inhibitory effect on arachidonic acid induced platelet aggregation by sulfasalazine and its metabolites (86). However there were no differences between the groups in their use
of these agents, and to the best of our knowledge there are no known anti-platelet effects with any of the other medications these patients were receiving. Finally, while the study was adequately powered to detect differences in platelet function, larger numbers may be required to substantiate the observed improvements in insulin metabolism and the clinical relevance of HDL/LDL ratio in predicting response to anti-TNF\(\alpha\) treatment.

**7.5 Conclusion**

It is well known that patients with IA have a markedly elevated risk of adverse cardiovascular events, and that this risk is greatest in those with poor disease control. The results of our initial investigation reveal a unique enhanced response of platelets to ADP in patients with active disease. It is not known if specific anti-platelet therapy targeting the ADP receptor in patients with IA can reduce cardiovascular risk in this patient cohort, and further studies in this area are warranted.

Furthermore, our observation that improved disease control with IL6 receptor inhibition decreases platelet reactivity in patients with RA, and, limitations aside, our novel prospective demonstration that a good clinical response to anti-TNF\(\alpha\) therapy is accompanied by decreased platelet reactivity to ADP, improved insulin sensitivity, and preservation of a favourable anti-atherogenic lipid profile may offer unique mechanistic insights as to how controlling inflammation by targeted therapy reduces adverse cardiovascular events in this high-risk cohort.

Finally, we reveal that sulfasalazine, although poorly absorbed, has an anti-platelet effect that is comparable to that of aspirin. Since there are currently no anti-platelet guidelines for primary prevention of CVD in patients with IA, our results suggest a new therapeutic indication for sulfasalazine in this patient population.
REFERENCES


APPENDICES
Appendix 1
List of relevant publications

Peer Reviewed Publications


Published Abstracts


PMID: 18841585 [PubMed - indexed for MEDLINE]


Paul A. MacMullan 1 Ann M. Madigan 2 Paola Bagaglia 2 Laura J. Durcan 2 Dermot Kenny 3 Geraldine M. McCarthy 2The Impact of Anti-Tumour Necrosis Factor Alpha Therapy on Platelet Function, Lipid Profile, and
Insulin Metabolism in Patients with Inflammatory Arthritis: A Prospective Cohort Study Rheumatology (2015) 54 (suppl 1): i87-i88
doi:10.1093/rheumatology/kev088.096

Paul A. MacMullan1,2, Anne M. Madigan1, Laura J. Durcan3, Karl Egan2, Paola M. Bagaglia1, Dermot Kenny4 and Geraldine M. McCarthy1,
1Rheumatology, Mater Misericordiae University Hospital, Dublin 7, Ireland, 2Cardiovascular Biology Laboratory, RCSI, Dublin, Ireland, 3Rheumatology, Mater Misericordiae University Hospital, Dublin 7, Ireland, 4Molecular and Cellular Therapeutics, RCSI, Dublin 2, Ireland

Anti-Tumour Necrosis Factor Alpha Therapy Reduces Platelet Reactivity and Is Associated With Improved Insulin Sensitivity In Patients With Inflammatory Arthritis ABSTRACT NUMBER: 1470 Meeting:
Appendix 2

RHEUMATOLOGY

Concise report

Platelet hyper-reactivity in active inflammatory arthritis is unique to the adenosine diphosphate pathway: a novel finding and potential therapeutic target

Paul A. Mac Mullan¹, Aaron J. Peace², Anne M. Madigan¹, Anthony F. Tedesco², Dermot Kenny² and Geraldine M. McCarthy³

Abstract

Objective. To assess the influence of disease activity on platelet function in patients with inflammatory arthritis (IA).

Methods. Ninety-six patients with an established diagnosis of IA (RA, PsA, seronegative SpA) were recruited. Patients with a history of cardiovascular disease (CVD), diabetes mellitus or receiving anti-platelet therapy were excluded. Demographic data, traditional CVD risk factors and medication use were recorded. Patients were characterized as active disease (n = 38) or control disease (n = 58) groups, respectively, based on internationally validated measures of disease activity (comprising serological markers [ESR, CRP, fibrinogen], patient measures [visual analogue scale of disease activity], evaluator global assessment and the 28-joint disease activity score). Platelet function was assessed using a novel assay of platelet reactivity. Platelet aggregation to multiple concentrations of arachidonic acid, collagen, epo-epo, thrombin receptor activating peptide and adenosine diphosphate (ADP) were measured simultaneously using a modification of light transmission aggregometry.

Results. The two groups (active vs control) were similar in terms of demographics and CVD risk factors. Anti-TNF-α therapy use was higher in the control group (P = 0.004), whereas NSAID use was higher in the active group (P < 0.001). There was a significant difference between the two groups in platelet response to ADP (P < 0.001). Platelet aggregation, in response to submaximal concentrations of ADP, was increased in the active disease group compared with the control group. There was no difference in platelet reactivity between the groups in response to any of the other agonists.

Conclusion. Patients with active IA demonstrate enhanced platelet reactivity, unique to the ADP pathway. This potential pro-thrombotic bias may contribute to their increased cardiovascular risk.

Key words: Platelet reactivity, Inflammatory arthritis, Adenosine diphosphate.

Introduction

Following a diagnosis of inflammatory arthritis (IA) (RA, PsA, AS and seronegative SpA), the 10-year risk of myocardial infarction is more than twice that of the general population and is equivalent to that of patients with Type 2 diabetes mellitus. While traditional risk factors are known to play a role in the pathogenesis of cardiovascular events, recent work has highlighted the key role of inflammation in atherothrombosis and as a risk factor for cardiovascular disease (CVD) in patients with RA [2]. Myocardial infarction results from a complex chain of events leading to the thrombotic occlusion of a coronary artery. Platelets are central to the development of these thrombotic events [2]. It is well established that inflammation activates platelets [4] and that platelet-
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Introduction

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derived mediators in turn potentiate the inflammatory response [2]. To date, platelet function in the IA population has not been well characterized. Previous studies have shown that patients with inflammatory rheumatic conditions exhibit increased in vitro platelet aggregation [5]. However, these patients were not characterized in terms of disease activity. More recent studies have shown a strong correlation between elevated markers of disease activity (CRP and ESR) and markers of platelet activation (CD62P, CD63) in RA [6]. However, the functional responsiveness or reactivity of these platelets was not assessed.

Platelet reactivity is a measure of how easily platelets will adhere to one another to form thrombus. Several lines of evidence suggest that platelet reactivity is useful in clinical practice to predict the risk of future adverse cardiovascular events [7, 8]. Therefore, we decided to examine platelet reactivity in the IA population and assess the influence of disease activity on platelet function. Standard platelet function assays using light transmission aggregometry typically assess the platelet response to single agonists at maximal concentrations and have limited clinical applicability [9]. Platelet aggregation in vivo is complex and involves multiple agonists, receptor–effector mechanisms and signaling pathways [2]. We developed an assay that measures the response of platelets to multiple agonists at multiple concentrations, simultaneously [10]. We used this novel assay of platelet reactivity and compared the ex vivo platelet function of patients with active inflammatory disease to that of patients with no clinical evidence of inflammation.

**Patients and methods**

Ethical approval was obtained from the Mater Misericordiae Hospital Ethics Committee and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients prior to phlebotomy.

**Patients**

Patients attending the rheumatology outpatient clinic were recruited. Only patients with an established diagnosis of IA (RA, PsA, AS, seronegative SpA, gout and Still’s disease) and those aged between 18 and 70 years were considered for inclusion. Patients with a prior history of ischaemic heart disease, diabetes mellitus and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine or dipyridamole) or thromboembolic prophylaxis (heparin or warfarin) were excluded.

Patients with a serum creatinine >140 mmol/l, a platelet count <120 000/mm², or who were pregnant or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

**Disease activity**

Several methods were used to accurately determine the disease activity. An initial assessment based on history and clinical examination was performed on all patients by the same clinician. All patients were then examined by a clinical nurse specialist. Tender joint count, swollen joint count, duration of early morning stiffness, visual analogue scale of disease activity and visual analogue scale of pain were recorded. Demographic data and traditional cardiovascular risk factors were also recorded.

A detailed and comprehensive medication record was also obtained from each patient. Serum inflammatory markers (ESR, CRP and fibrinogen (Fg)) were obtained as outlined below. In addition, the internationally validated 28-joint disease activity score (DAS-28) [11] was calculated for those patients with a diagnosis of RA.

**Phlebotomy and platelet preparation**

For detailed methodology regarding blood sampling and platelet preparation see supplementary data available at *Rheumatology* online.

**Platelet function assay**

To assess the platelet function, we used a novel platelet function assay based on a modification of light transmission aggregometry, described in detail elsewhere [10] (see supplementary data available at *Rheumatology* Online).

**Patient groups**

The entire cohort was divided into two separate groups based on disease activity. Those with clinically active disease were assigned to the active group, whereas those patients with well-controlled disease were assigned to the control group. This assignment was made independent of platelet function. Platelet assay data were recorded and both the platelet assay data and clinical data were merged thereafter.

**Statistical analysis**

Demographic data categorical variables were analysed using the Fisher’s Exact Test. Disease activity data were analysed using the Wilcoxon signed-rank test. Platelet function data continuous variables were tested for normality using the D’Agostino–Pearson omnibus test. Analysis of variance (ANOVA) for per cent platelet aggregation was then assessed by repeated measures two-way ANOVA. The nominal level of significance was 5% and multiple comparisons between different agonist concentrations were adjusted using the Bonferroni correction. Sigmoidal dose–response curves for the two groups were compared separately for each agonist using the extra sum-of-squares F-test.

**Results**

One hundred and seven patients were initially enrolled in the study. Eleven patients were excluded for the following reasons: four from whom a sufficient blood sample for platelet analysis could not be obtained; five for failure to complete the assay within the timeframe and two where complete baseline serological data were missing. Data from a total of 96 patients were analysed. The average time from drawing the blood to completion of the assay was 40±5 min.
Patient characteristics

The two groups (active vs control) were similar in terms of age, gender, diagnosis and traditional cardiovascular risk factors (Table 1). The majority of patients (65%) were diagnosed with RA. There were significant differences between the two groups in their use of medications. Anti-TNF-α therapy use was significantly higher in the control group \((P = 0.0062)\), whereas NSAID use was significantly higher in the active disease group \((P = 0.0017)\).

Disease activity data for the active vs control disease groups are also shown in Table 1. There are significant differences in all of these variables between the two groups. DAS-28 scores for those patients with RA are shown separately.

Coagulation profiles, prothrombin and partial thromboplastin times were similar in both groups, and all were within the normal range.

Mean platelet count was higher in the active group \((95\%\ CI 303, 391 \times 10^9/l)\) than the control group \((95\%\ CI 266, 315 \times 10^9/l)\). However, both fall within the normal range \((150, 450 \times 10^9/l)\).

**Platelet function data**

Sigmoidal dose–response curves of per cent platelet aggregation in response to increasing concentrations of agonist for the active and control disease groups were directly compared with each other.

**Table 1 Patient characteristics**

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Entire cohort, n = 96</th>
<th>Active group, n = 38</th>
<th>Control group, n = 58</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (s.d.), years</td>
<td>49 (10)</td>
<td>50 (9)</td>
<td>49 (12)</td>
<td>0.6814</td>
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<tr>
<td>Male, n (%)</td>
<td>50 (52)</td>
<td>19 (50)</td>
<td>31 (53)</td>
<td>0.062</td>
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<tr>
<td>Diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>62 (65)</td>
<td>21 (55)</td>
<td>41 (71)</td>
<td>0.134</td>
</tr>
<tr>
<td>PSA</td>
<td>16 (17)</td>
<td>8 (21)</td>
<td>8 (14)</td>
<td>0.407</td>
</tr>
<tr>
<td>AS</td>
<td>7 (7)</td>
<td>3 (8)</td>
<td>4 (7)</td>
<td>0.999</td>
</tr>
<tr>
<td>Other</td>
<td>11 (11)</td>
<td>6 (16)</td>
<td>5 (8)</td>
<td>0.335</td>
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<tr>
<td>Risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>30 (31)</td>
<td>13 (34)</td>
<td>17 (29)</td>
<td>0.656</td>
</tr>
<tr>
<td>DM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>HTN</td>
<td>14 (15)</td>
<td>6 (16)</td>
<td>8 (14)</td>
<td>0.776</td>
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<tr>
<td>Chol</td>
<td>12 (12)</td>
<td>4 (11)</td>
<td>8 (14)</td>
<td>0.758</td>
</tr>
<tr>
<td>Family Hx</td>
<td>18 (19)</td>
<td>8 (21)</td>
<td>10 (6)</td>
<td>0.789</td>
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<td>Medications, n (%)</td>
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<td></td>
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<tr>
<td>NSAIDS</td>
<td>47 (49)</td>
<td>27 (71)</td>
<td>20 (34)</td>
<td>0.001**</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>26 (27)</td>
<td>4 (11)</td>
<td>22 (38)</td>
<td>0.004**</td>
</tr>
<tr>
<td>MTX</td>
<td>49 (51)</td>
<td>16 (42)</td>
<td>33 (57)</td>
<td>0.211</td>
</tr>
<tr>
<td>SZP</td>
<td>23 (24)</td>
<td>10 (26)</td>
<td>13 (22)</td>
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<td>HCQ</td>
<td>15 (16)</td>
<td>6 (16)</td>
<td>9 (16)</td>
<td>0.999</td>
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<tr>
<td>AZA</td>
<td>5 (5)</td>
<td>1 (3)</td>
<td>4 (7)</td>
<td>0.641</td>
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<tr>
<td>Steroids</td>
<td>10 (11)</td>
<td>4 (11)</td>
<td>6 (10)</td>
<td>0.999</td>
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<tr>
<td>MMP</td>
<td>1 (1)</td>
<td>1 (3)</td>
<td>0</td>
<td>0.396</td>
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<tr>
<td>HRT/OCP</td>
<td>7 (7)</td>
<td>3 (8)</td>
<td>4 (7)</td>
<td>0.999</td>
</tr>
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</table>

**Serological markers**

<table>
<thead>
<tr>
<th>Entire cohort, n = 96</th>
<th>Active group, n = 38</th>
<th>Control group, n = 58</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR, mm/h</td>
<td>31.74 (4.33)</td>
<td>14.04 (1.82)</td>
<td>0.0109**</td>
</tr>
<tr>
<td>CRP, mg/l</td>
<td>23.40 (5.57)</td>
<td>7.09 (2.07)</td>
<td>0.0105**</td>
</tr>
<tr>
<td>Fib, g/l</td>
<td>3.99 (0.21)</td>
<td>3.01 (0.10)</td>
<td>0.0056**</td>
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</table>

**Patient measures**

<table>
<thead>
<tr>
<th>Entire cohort, n = 96</th>
<th>Active group, n = 38</th>
<th>Control group, n = 58</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VASDA (0–100)</td>
<td>63.29 (3.36)</td>
<td>15.02 (2.21)</td>
<td>0.0001**</td>
</tr>
<tr>
<td>VASPAIN (0–100)</td>
<td>61.79 (5.06)</td>
<td>16.41 (2.69)</td>
<td>0.0001**</td>
</tr>
<tr>
<td>EMS, min</td>
<td>44.11 (12.26)</td>
<td>4.33 (1.29)</td>
<td>0.0008**</td>
</tr>
<tr>
<td>RA patients only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA, n = 62</td>
<td>Active group, n = 21</td>
<td>Control group, n = 41</td>
<td></td>
</tr>
<tr>
<td>DAS-28 score, mean (s.e.m.)</td>
<td>4.87 (0.29)</td>
<td>2.45 (0.12)</td>
<td>0.0002**</td>
</tr>
</tbody>
</table>

**Significant difference between the groups (active vs control). Other: ReA, juvenile idiopathic arthritis and gout; DM: diabetes mellitus; EMS: early morning stiffness; HTN: hypertension; Chol: hypercholesterolaemia; Hx: history; SZP: salazopyrin; VASDA: visual analogue scale of disease activity; VASPAIN: visual analogue scale of pain.
There is a significant difference between the two groups in response to adenosine diphosphate (ADP) only ($P < 0.001$). The active disease group exhibits increased platelet reactivity at submaximal concentrations (Fig. 1).

The dose–response curves for arachidonic acid, epinephrine, collagen and thrombin receptor activating peptide (TRAP) are similar for both groups (see supplementary figure 1 available as supplementary data at Rheumatology Online).

Per cent platelet aggregation values were normally distributed for all concentrations of agonists. ANOVA results for each agonist demonstrated a significant difference between the groups in response to ADP only ($P = 0.01$). The values obtained for the other agonists were: $P = 0.25$ for TRAP, $P = 0.42$ for collagen, $P = 0.54$ for arachidonic acid and $P = 0.88$ for epinephrine. The individual concentrations of ADP responsible for the significantly increased response in the active disease group are in the submaximal range (1.25, 2.5 and 5 $\mu$m), as demonstrated by the Bonferroni post-test values ($P < 0.001$).

We then exclusively examined those patients with a diagnosis of RA. A unique enhanced response to ADP was also seen at the same submaximal concentrations in those patients with active disease only (data not shown).

**Discussion**

The results of this investigation demonstrate a number of key findings. First, the patients in this study, with objective evidence of active IA, demonstrate increased platelet reactivity specifically to ADP. Secondly, there was no significant difference in platelet response to any of the other agonists in terms of disease activity. Thirdly, when maximal concentrations of agonists are used in platelet aggregation, there is no difference seen between the two groups. Epidemiological evidence has clearly shown that IA is an independent risk factor for CVD [1] and that this risk is greatest in those with chronic active disease [3]. Several lines of evidence support the use of platelet reactivity in predicting the risk of future cardiovascular events [7, 8]. Thus, the unique platelet hyper-reactivity in response to ADP in patients with active IA may explain some of this risk, and has implications for therapy.

Platelets express at least two ADP receptors: P2Y1 and P2Y12. Both are G-protein-coupled surface receptors. P2Y1 is a weak agonist of platelet aggregation, whereas the P2Y12 receptor is the major receptor involved in amplifying and sustaining platelet aggregation in response to ADP [2]. The therapeutic importance of the ADP–P2Y12 receptor interaction is evident from the reduction in adverse cardiovascular events when this receptor is inhibited with drugs such as ticlopidine and clopidogrel [12].

The mechanisms underlying the enhanced platelet response to ADP in active IA are currently unexplained. Although mean platelet count was higher in the active group, it was within the normal range, and previous work has demonstrated that this does not account for differences in platelet aggregation measurements [13]. Increased receptor expression could account for this difference. However, whereas the platelet ADP–P2Y12 receptor has been cloned for some time, monoclonal antibodies to this receptor that are suitable for surface receptor quantification by flow cytometry are currently not commercially available.

Studies have shown an increased platelet responsiveness to ADP in insulin-resistant states [14]. Furthermore, insulin-resistant patients with elevated Fib levels treated with the ADP–P2Y12 antagonist clopidogrel, exhibit less inhibition of platelet reactivity in response to ADP than those with normal plasma Fib [15]. Interestingly, in our study, plasma Fib was the most consistently elevated serological marker of active disease. Several studies have demonstrated increased insulin resistance in patients with IA, and this is associated with active inflammation [16]. Insulin resistance has been shown to ameliorate in response to treatment with DMARDs, in particular anti-TNF-α agents [17]. The use of anti-TNF-α therapy was significantly higher in our controlled disease cohort and this group did not show the same response to ADP as compared with those with active IA. Moreover, insulin resistance is associated with abnormal platelet function [18]. Thus, a link among active inflammatory disease, insulin resistance, Fib and platelet ADP hyper-responsiveness seems possible. We did not evaluate insulin resistance prospectively in our patient study.

Platelet activation is associated with systemic inflammation and platelet-derived mediators of inflammation potentiating the inflammatory response [2]. The pro-inflammatory cytokine CD40 ligand is one such mediator, which is stored intracellularly and rapidly presents on the platelet surface after platelet activation [19]. This ligand is a trimeric transmembrane protein in the TNF family and when cleaved to its active soluble fragment it inhibits endothelial ecto-ADPase and induces the endothelium.

**Fig. 1** Log dose–response curves for active vs control groups in response to ADP. Log of agonist concentration is plotted on the x-axis, whereas platelet per cent aggregation is plotted on the y-axis. Platelet aggregation is enhanced in the active group in response to submaximal concentrations of ADP. ***$P < 0.001$.**
to generate further pro-inflammatory mediators [19]. Interestingly, clopidogrel significantly inhibits ADP-induced CD40 ligand expression [19]. Furthermore, TNF-α has been shown to directly up-regulate CD40 ligand on the platelet surface in patients with heart failure [20]. Patients with active IA have markedly elevated TNF-α levels. It is possible that actively inflamed IA patients also up-regulate CD40 ligand expression and consequently have a physiological bias towards increased ADP bioavailability. We have already referred to the association between increased plasma Fib and platelet ADP hypersensitivity in the insulin-resistant state [15] and the fact that Fib levels were consistently elevated in our active disease cohort. Activated platelets bind Fib following a conformational change in the GPIIbIIIa receptor [2]. Intracellular platelet Fib stores are continuously replenished from the platelet external environment. Thus, patients with IA who have chronic active inflammation and insulin resistance may exhibit enhanced Fib binding in the setting of a hyper-reactive platelet ADP receptor-effector system.

In conclusion, it is well known that patients with IA have a markedly elevated risk of adverse cardiovascular events, and that this risk is greatest in those with poor disease control. The results of the present investigation demonstrate a unique enhanced response of platelets to ADP in patients with active disease. It is not known if specific anti-platelet therapy targeting the ADP receptor in patients with IA can reduce the cardiovascular risk in this patient cohort, and further studies in this area are warranted.

Rheumatology key messages

- Patients with active IA demonstrate enhanced platelet function, specific to the ADP pathway.
- The increased platelet response to ADP may represent a pro-thrombotic bias in patients with inflammation.
- Trials of targeted anti-platelet therapy should be considered in this population.

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Regarding the novel platelet reactivity assay used in this study, a patent has been filed entitled ‘A Method of Generating a Platelet Reactivity Profile for an Individual’ with a priority date of 6 October 2006 and an International Application Number of PCT/IE2007/000096. Prof. Dermot Kenny and Dr Aaron Peace are named on the patent application. The patent is owned by the Royal College of Surgeons in Ireland. The College (RCSI) holds the rights to the patent. As the College is the sole owner, there should not be any conflict of interest for Prof. Kenny or Dr Peace.

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Supplementary data

Supplementary data are available at Rheumatology Online.

References


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Sulfasalazine and its metabolites inhibit platelet function in patients with inflammatory arthritis

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Abstract The purpose of this study is to assess the effect of sulfasalazine and its metabolites on platelet function in patients with inflammatory arthritis (IA). One hundred thirty-five consecutive patients with an established diagnosis of IA were screened. Those with a history of cardiovascular disease (CVD), taking anti-platelet agents or non-steroidal anti-inflammatory drugs (NSAIDs) were excluded. A total of 32 patients were investigated, 15 taking sulfasalazine and 17 taking other disease-modifying anti-rheumatic drugs (DMARDs) and no sulfasalazine. These two cohorts were compared to 15 patients with stable CVD on long-term aspirin. The effect of sulfasalazine and its metabolites on arachidonic acid (AA)-induced platelet aggregation was also tested in vitro in samples from healthy donors (n=18). Demographics, CVD risk factors and disease activity indices were similar in the sulfasalazine and other DMARD groups. AA-induced platelet aggregation was significantly inhibited in the sulfasalazine group (9±7 %) and comparable to that in the aspirin group (10±6 %). In contrast, there was no effect on AA-induced platelet aggregation in the other DMARDs group (77±12 %) (p<0.001). Furthermore, sulfasalazine therapy had no effect on platelet aggregation in response to multiple other agonists. Sulfasalazine and its metabolites (5-aminosalicylic acid and sulfapyridine) exerted an additive and dose-dependent inhibitory effect on AA-induced platelet aggregation in vitro (p<0.001). The inhibition of AA-induced platelet aggregation by sulfasalazine is comparable to that achieved by aspirin and is dependent on both sulfasalazine and its metabolites. This represents a potential mechanism that may contribute to the known cardioprotective effect of sulfasalazine in patients with IA.

Keywords Cardiovascular disease · DMARDs · Inflammation · Platelet function · Sulfasalazine

Introduction

Sulfasalazine is an older generation anti-rheumatic drug first synthesised in the 1940s as a combination of an antibiotic (sulfapyridine) and an NSAID (5-aminosalicylic acid) [1]. Patients with rheumatoid arthritis (RA) who regularly take sulfasalazine have a significantly reduced risk of myocardial infarction (MI), independent of disease severity (hazard ratio 0.82, p<0.05) [2]. The mechanisms underlying this cardiovascular risk reduction with sulfasalazine therapy are not

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entirely understood. Sulfasalazine is a pro-drug composed of two moieties, sulfapyridine and 5-aminosalicylic acid (5-ASA), linked by an azo bond [1]. Approximately 10–15% of the parent compound is absorbed from the small intestine, with 10% of the 5-aminosalicylate moiety and 40–60% of the sulfapyridine component absorbed from the large intestine following cleavage of the azo bond by colonic bacteria [3]. Plasma concentrations of all three substances in RA patients receiving stable doses of sulfasalazine have recently been reported [3]. Sulfasalazine inhibits platelet thromboxane generation [4], but the effect of sulfasalazine on platelet function is not known. Therefore, we decided to assess the influence of sulfasalazine therapy on platelet function in patients with inflammatory arthritis (IA).

Standard platelet function assays using light transmission aggregometry typically assess the platelet response to single agonists at maximal concentrations and have limited clinical applicability [5]. Platelet aggregation in vivo is complex and involves multiple agonists, receptor-effector mechanisms and signaling pathways [6]. In patients with cardiovascular disease (CVD) taking dual anti-platelet therapy with both aspirin and clopidogrel, we have recently shown that dual anti-platelet therapy unmasks a unique platelet reactivity profile using an assay that assesses platelet function in response to multiple concentrations of multiple agonists simultaneously [7]. Our hypothesis was that sulfasalazine would have an inhibitory effect on platelet function in patients with IA, and that this would be less than the inhibition achieved by standard low-dose aspirin in patients with CVD. Somewhat surprisingly, patients taking sulfasalazine had equivalent inhibition of arachidonic acid (AA)-induced platelet aggregation in vitro in blood samples obtained from healthy volunteers. We demonstrate a dose-dependent inhibition of the platelet arachidonic acid pathway by the parent compound (sulfasalazine) and its metabolites (sulfapyridine and 5-aminosalicylic acid) at drug concentrations equivalent to those present physiologically in patients receiving sulfasalazine therapy.

**Methods**

Ethical approval was obtained from the Mater Misericordiae University Hospital Ethics Committee, and the study complied with the Declaration of Helsinki. Informed consent was obtained from all patients and healthy volunteers prior to phlebotomy.

**Patients**

Patients with an established diagnosis of IA (RA, PsA and AS) aged between 18 and 70 years and attending the rheumatology outpatient clinic were considered for inclusion. Patients with a history of ischaemic heart disease, diabetes mellitus and those receiving anti-platelet therapy (aspirin, clopidogrel, ticlopidine or dipyridamole) or thromboembolic prophylaxis (heparin or warfarin) were excluded. As non-steroidal anti-inflammatory drugs (NSAIDs) are known to affect platelet function [8], patients who had taken an NSAID within the preceding 14 days were also excluded.

Patients with a serum creatinine >140 mmol/l, a platelet count <120,000/mm³ or who were pregnant or had hepatic dysfunction (defined by hepatic enzymes more than twice the upper limit of normal) were also excluded.

Based on the previously published reliability and reproducibility characteristics of our platelet function assay, a 10% difference in platelet aggregation response is regarded as significant [7, 9]. To evaluate whether patients with IA taking sulfasalazine (sulfasalazine group) had a different platelet function profile compared to patients in receipt of other anti-rheumatic medications (other disease-modifying anti-rheumatic drugs (DMARDs) group) and to achieve 80% power with a nominal significance of 5%, we required 10 patients in each group. To ensure the analysis would be robust, statistically viable and informative, we aimed to recruit at least 15 patients with IA in each group. Stable CVD patients (n=15) with no history of IA and who were receiving standard anti-platelet monotherapy with low-dose aspirin (75 mg) were recruited as positive controls (aspirin group).

**Clinical data**

Demographic data and cardiovascular risk factors were recorded. A detailed medication record was also obtained from each patient. Disease activity was assessed both clinically and serologically. Tender joint count, swollen joint count, duration of early morning stiffness and visual analogue scales of disease activity and pain were recorded. Standard inflammatory markers (erythrocyte sedimentation rate (ESR), CRP and fibrinogen) were measured, and the internationally validated 28-joint count of disease activity (DAS-28) [10] was calculated for all patients.

**Phlebotomy and platelet preparation**

Blood was drawn from all patients by the same phlebotomist. All samples were obtained uncuffed. Blood was collected through a 19-gauge butterfly needle and the first 20 ml was sent for baseline serological data and inflammatory markers.
A further 40 ml was collected into a syringe containing 3.2 % sodium citrate. Blood was then centrifuged for 10 min at 150 g. Platelet-rich plasma (PRP) aspirated from the supernatant was placed in a reagent reservoir. Using a multichannel pipette, the PRP was dispensed across wells in a 96-well plate (black isoplate with clear flat-bottomed wells, Perkin Elmer) containing different concentrations of the five agonists arachidonic acid, collagen (type 1 soluble calf skin), adenosine diphosphate (ADP), epinephrine and thrombin receptor-activating peptide (TRAP).

**Platelet function assay**

To assess platelet function, we used a novel platelet function assay based on a modification of light transmission aggregometry described in detail elsewhere [7, 11]. In brief, 180 µl of PRP was added to each well of a 96-well plate containing the different agonists. Light absorbance was measured at standard times. To characterise platelet aggregation, increasing concentrations of the agonists were tested. Platelet aggregation measured as a percentage of absorbance from baseline, using a 572-nm filter, was assayed at 0, 3, 6, 9, 12, 15 and 18 min. Between each of the standardised times, the plate was rotated at 1,000 rpm through a 0.1-mm orbit. The concentrations of the agonists used were 500, 375, 188, 83.8, 46.9, 23.4, 11.8 and 5.86 µg/ml for arachidonic acid; 190, 143, 71.3, 35.6, 17.8, 8.9, 4.45 and 2.23 µg/ml for collagen; 20, 10, 5, 2.5, 1.25, 0.625, 0.313 and 0.156 µM for ADP and TRAP; and 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.0395, 0.0195, 0.00488 and 0.00122 µM for epinephrine. The agonist volumes used were 50 µl of arachidonic acid, 50 µl of collagen, 40 µl of ADP, 40 µl of epinephrine and 40 µl of TRAP. The 96-well plate was then read using a Victor 3 Multilabel plate reader (Perkin Elmer, Wellesley, MA, USA). The time from blood draw until the end of the assay protocol was recorded.

Maximal platelet aggregation was defined as no change in light absorbance with incremental concentration of agonist at two consecutive time points. Sigmoidal dose/response curves were then plotted for each agonist at all concentrations. We then compared platelet function in IA patients taking sulfasalazine (2–3 g daily) (sulfasalazine group, n=15) to that of IA patients using alternative agents (anti-TNF-α therapy, methotrexate, hydroxychloroquine and steroids), alone or in combination (other DMARDs group, n=17). Stable CVD patients with no history of IA and receiving anti-platelet monotherapy with 75 mg of aspirin were used as a positive control group (aspirin group, n=15).

The reproducibility of this novel platelet function assay has been established recently and is described in detail elsewhere [7]. In brief, the between-assay precision of each agonist is 12 % for arachidonic acid, 10 % for collagen, 8 % for ADP, 11 % for epinephrine and 11 % for TRAP. A value of <12 % for between-assay precision is regarded as acceptable for assay validation [12]. The within-assay precision is <1 % for all agonists, indicating excellent reproducibility [7].

**Platelet function with sulfasalazine and metabolites in vitro**

In essence, the effects on platelet function of physiological plasma concentrations of sulfasalazine and its components, present in patients who are receiving stable doses of sulfasalazine (2 g daily) [3], were directly compared to the effect on platelet function of the physiological plasma concentration of aspirin present in patients taking low-dose aspirin (75 mg daily) for CVD prevention [13]. Platelet function was assessed using conventional light transmission aggregometry (LTA) on a 4-channel Chrono-log aggregometer, and the platelet aggregation responses to 500 µg/ml of arachidonic acid were tested, as described [14, 15].

To assess the specific effect of sulfasalazine and its individual components (5-ASA and sulfapyridine) on platelet aggregation (with aspirin as a positive control), blood samples were obtained from healthy volunteers (n=18, 9 males and 9 females) who were not taking any medication. Sulfasalazine, 5-ASA, sulfapyridine and acetylsalicylic acid (aspirin) were purchased from Sigma laboratories. All drugs were reconstituted with dimethyl sulfoxide (DMSO) and diluted to their respective concentrations with deionised water. The final concentration of DMSO in each solution tested was <1 %. Phlebotomy and platelet preparation were carried out as described above. The PRP obtained was divided into aliquots of 1 ml and these were incubated (for 45 min at 37 °C) with increasing concentrations (40, 50 and 100 µM) of either sulfasalazine, 5-ASA or sulfapyridine. As all three components are simultaneously present in the plasma of patients taking the drug (at typical concentrations of 40–50 µM), increasing concentrations (10, 20, 40 and 50 µM) of a mixed solution of sulfasalazine, 5-ASA and sulfapyridine were also tested in this physiological range (i.e. the 10 µM solution was composed of 10 µM each of sulfasalazine, 5-ASA and sulfapyridine; the 20 µM solution contained 20 µM each of sulfasalazine, 5-ASA and sulfapyridine; and so on for the 40 and 50 µM solutions) [3]. Aliquots of PRP incubated with 1 % DMSO (vehicle control) and 20 µM aspirin were used as negative and positive controls, respectively. The negative control is representative of the normal platelet aggregation response in the absence of medication, and the positive control of 20 µM acetylsalicylic acid is representative of the physiological plasma concentration of aspirin present in patients.
taking low-dose aspirin (75 mg daily) for CVD prevention [13]. The platelet count in each sample was assayed pre- and post-incubation using a standard Sysmex haematology cell counter. Platelet function was assessed using conventional light transmission aggregometry (LTA) on a 4-channel Chronolog aggregometer, and the platelet aggregation responses to 500 μg/ml of arachidonic acid were tested, as described [14, 15]. The positive and negative controls were used in every aggregometry run alongside two other samples. All concentrations of each drug were tested at least three times for every donor.

Statistics

Patient studies

Demographic data categorical variables were analysed using the Fisher’s exact test. Disease activity data were analysed using the Wilcoxon signed-rank test. Platelet function data continuous variables were tested for normality using the D’Agostino-Pearson omnibus test. Analysis of variance (ANOVA) for percent platelet aggregation was calculated with the nominal level of significance set at 5 %, and multiple comparisons between different agonist concentrations were adjusted using the Bonferroni correction. Sigmoidal dose-response curves for all groups were compared separately for each agonist using the extra sum-of-squares F test.

Healthy volunteers

As the maximal aggregation responses obtained from the series of in vitro LTA experiments were not all normally distributed, these results were analysed using the non-parametric Wilcoxon signed-rank test.

Results

Patient data

One hundred thirty-five consecutive patients with an established diagnosis of IA were screened for inclusion. Thirty-two patients with IA [RA (n=21), PsA (n=8), AS (n=3)] who fulfilled the inclusion and exclusion criteria were enrolled. The average time from blood draw to completion of the platelet function assay was 40±5 min.

Patient characteristics

Demographic data and disease activity indices are shown in Table 1. The two groups with IA (sulphasalazine vs other DMARDs) were similar in terms of age, gender, diagnosis and standard cardiovascular risk factors. There was no significant difference in disease activity, with both groups displaying some objective evidence of inflammation in the low to moderate disease activity range (mean±SEM; sulphasalazine, 3.82±0.46; and other DMARDs, 3.4±0.46, p=0.48), as defined by DAS-28 (ESR) score [10].

The only significant differences between the two groups were related to their use of medications (p<0.001). Patients in the sulphasalazine group (n=15) were all receiving sulphasalazine 2–3 g daily in divided doses (13 sulphasalazine alone and 2 with methotrexate). Patients in the other DMARDs group (n=17) were using stable doses of alternative DMARDS (anti-TNF-α therapy, methotrexate, hydroxychloroquine and steroids), alone or in combination.

Platelet function data

Increasing concentrations of agonist caused increasing platelet aggregation. The response to each agonist was plotted as a sigmoidal log dose-response curve and the curves for each agonist compared in the three groups. The results of these platelet function assays in the three cohorts of patients receiving sulphasalazine (sulphasalazine group, n=15), other disease-modifying agents (other DMARDs group, n=17) and the stable CVD patients taking aspirin (aspirin group, n=15) were compared.

Patients in the sulphasalazine group had a significant reduction in platelet aggregation response to arachidonic acid (AA) compared to patients in the other DMARDs group (p<0.001) (Fig. 1a). The maximal aggregation responses to the maximum dose (500 μg/ml) of arachidonic acid were (mean, SD) sulphasalazine group (9±7 %) vs other DMARDs group (77±12 %) respectively (p<0.001). This decreased response to arachidonic acid in the patients taking sulphasalazine was identical to that seen in patients taking aspirin (10±6 %) (Fig. 1b).

Log dose-response curves for collagen, epinephrine, TRAP and ADP are shown in Fig. 2. Platelet aggregation responses for all of these agonists were similar in the sulphasalazine and other DMARD groups. Patients in the aspirin group also had a similar response to collagen. There was a small but significantly decreased platelet response to submaximal concentrations of epinephrine (0.00122, 0.00488, 0.0195 and 0.078 μM) and TRAP (0.156, 0.313 and 0.625 μM) (p<0.05) in the aspirin group compared to both groups of patients with IA (sulphasalazine and other DMARD groups), albeit in the lowest dose ranges of these agonists. Furthermore, the aspirin-treated patients also had a significantly decreased response to the four highest concentrations (2.5, 5, 10 and 20 μM) of ADP (p<0.01) compared to both groups of patients with IA.

Mean platelet counts were similar in both groups of patients with IA (sulphasalazine group (95 % CI 280, 364×10^9/l)
and other DMARDs (95 % CI 232, 359 × 10⁹/l), and while the mean platelet count in the aspirin-treated CVD patients {aspirin group (95 % CI 217,300)} was similar to that of the other DMARDs group, it was significantly less than that of the sulfasalazine group (p<0.05). However, this difference was small, and mean platelet counts in all three groups fell within the normal range (150, 450 × 10⁹/l).

Sulfasalazine and its metabolites in vitro

There were no significant differences between the pre- and post-incubation platelet counts for any of the samples. Sulfasalazine, 5-ASA and sulfapyridine each inhibited arachidonic acid-induced platelet aggregation to some degree. The mean maximal aggregation response to 500 μg/ml of arachidonic acid (AA) was 86±7 %. Platelet aggregation in response to AA in the presence of 50 μM sulfapyridine was 71±6 % (p<0.01) in the presence of 50 μM 5-ASA was 47±12 % (p<0.001) and 32±9 % in the presence of 50 μM sulfasalazine (p<0.001) (Fig. 3a). Increasing concentrations (10 μM each of sulfasalazine, 5-ASA and sulfapyridine; 20 μM each of sulfasalazine, 5-ASA and sulfapyridine; 40 μM each of sulfasalazine, 5-ASA and sulfapyridine; and 50 μM each of sulfasalazine, 5-ASA and sulfapyridine) of a combined solution of all three agents inhibited arachidonic acid-induced platelet aggregation in a dose-dependent manner (Fig. 3b). At physiological plasma concentrations that are seen in vivo (40 μM each of sulfasalazine, 5-ASA and sulfapyridine) [3], this solution effectively abrogated the platelet response to 500 μg/ml of arachidonic acid (p<0.001) and achieved a similar level of inhibition to a physiological concentration (20 μM) of aspirin [13] (Fig. 3b).

Discussion

The results of this investigation demonstrate a number of novel findings. First, patients with IA receiving sulfasalazine therapy have a significantly inhibited platelet response to arachidonic acid (AA) compared to patients with IA taking other DMARDs. This inhibition of AA-induced platelet aggregation in patients taking sulfasalazine is comparable to that of CVD patients taking aspirin. Secondly, sulfasalazine
therapy has no effect on platelet aggregation in response to multiple other agonists. Thirdly, both groups of IA patients demonstrate a significantly enhanced platelet response to sub-maximal concentrations of epinephrine and TRAP and to the four highest concentrations of ADP compared to CVD patients taking aspirin. Finally, both sulfasalazine and its components reduce the platelet response to arachidonic acid in vitro with an effective abrogation of AA-induced platelet aggregation by a mixture of sulfasalazine and its components at physiological concentrations observed in patients receiving stable doses of sulfasalazine reported elsewhere [3].

Patients with rheumatoid arthritis (RA) die prematurely from cardiovascular disease (CVD) [16]. Their incident risk of CVD is at least as great as that of patients with type 2 diabetes mellitus [17] and patients with RA experience higher mortality following a first acute cardiovascular event [18]. There is an emerging body of evidence that patients with other forms of inflammatory arthritis (IA), in particular psoriatic arthritis (PsA) and ankylosing spondylitis (AS), are at a similarly increased risk of CVD [19]. Myocardial infarction results from a complex chain of events leading to the thrombotic occlusion of a coronary artery. Platelets are central to the development of these thrombotic events [6]. It is well established that inflammation activates platelets [20] and that platelet-derived mediators in turn potentiate the inflammatory response [6]. Furthermore, elevated markers of platelet activation such as P-selectin (CD62P) and CD63 are associated with disease activity in RA [21], but the significance of this in terms of platelet function has not been well characterised. In
patients with CVD, anti-platelet therapy is effective in reducing mortality [22]. Aspirin is used in secondary prevention of future adverse cardiovascular events in patients with established CVD and has an integral role in the primary prevention of CVD in patients at high risk [23]. Despite the well-known risk of cardiovascular death, there are no recognized guidelines on the use of anti-platelet therapy for primary prevention of CVD in patients with IA [19].

The underlying mechanisms by which sulfasalazine lowers CVD risk in patients with IA are currently unknown. This may be partly achieved through improved control of inflammation, as other more potent DMARDs such as methotrexate and anti-TNF-α agents have been shown to decrease CVD risk in patients with IA, with the greatest reduction in the incidence of myocardial infarction observed in those patients who respond to anti-TNF-α therapy [24]. However, since it has been shown that sulfasalazine inhibits platelet thromboxane production [4], we hypothesised that sulfasalazine may also inhibit platelet function. Our results show that platelet aggregation in response to AA is inhibited in patients taking sulfasalazine and is comparable to the inhibition seen in patients with CVD taking aspirin. While we had expected some inhibition of platelet function, we were surprised that there was nearly complete inhibition of AA-induced aggregation. Therefore, we characterised the contribution of the individual components of the drug (5-ASA and sulfapyridine) and when combined in the form of their parent compound, sulfasalazine. Thus, while sulfasalazine and its individual components are generally poorly absorbed, our study demonstrates an additive inhibition of platelet reactivity in response to arachidonic acid at physiological concentrations of sulfasalazine, 5-ASA and sulfapyridine in a dose-dependent manner [3]. Correlating plasma levels of these three compounds with platelet aggregation data would further enhance the mechanistic understanding of the minimum concentrations required to achieve the inhibitory effects observed in our patients with IA receiving sulfasalazine therapy.

The enhanced platelet reactivity response to the highest concentrations of ADP in both groups of patients with IA compared to the aspirin-treated group is noteworthy, and although it may be due to factors unrelated to IA, it is certainly worthy of consideration. Differences in platelet count do not account for the differences seen in the aggregation measurements [25]. The differences in aggregation responses could be due to the weak effect of aspirin on platelet aggregation induced by other agonists, as evidenced by the reduced platelet responses to very low doses of epinephrine and TRAP observed in the aspirin-treated patients in this study. However, this inhibitory effect is usually overcome at higher concentrations of agonist (as was the case with both epinephrine and TRAP in these patients), and previous work has demonstrated that the weak inhibitory effect of aspirin on the ADP pathway is nullified at concentrations of 10 μM ADP and above [26]. Thus, while the differences in platelet response to the 2.5 and 5 μM ADP may well be explained by an inhibitory effect of aspirin therapy on ADP-induced platelet aggregation, the clear increase in platelet response to the highest concentrations of ADP (10 and 20 μM) in the patients with IA may also be partly a result of their underlying inflammatory burden, but the lack of unexposed controls make the differences observed difficult to interpret. Numerous studies have shown a clear association between markers of inflammation and a poor response to ADP blockade with clopidogrel in patients with CVD and that a poor response to ADP inhibition predicts future adverse cardiovascular events [27, 28]. Furthermore, we have recently demonstrated an enhanced platelet response to ADP in patients with IA who have objective evidence of inflammation compared to IA patients in disease remission [29]. As several lines of evidence support the use of platelet reactivity in predicting the risk of future adverse cardiovascular events [30, 31], the unique platelet hyper-reactivity in response to the highest concentrations of ADP (but not epinephrine, collagen or TRAP) in patients with IA is certainly worthy of further investigation. Such work would require matched controls unexposed to any
real or potential anti-platelet medications and serial measurements of platelet function.

This study has a number of limitations. While the stable cardiovascular patients receiving aspirin represented a positive control group, the lack of unexposed controls without any history of inflammatory arthritis or cardiovascular disease weakens the external validity. Also, the use of other disease-modifying drugs was not identical between the two IA groups and could potentially confound interpretation of the data. However, patients were recruited consecutively and, in fact, reflect real-life clinical practice. Hydroxychloroquine has been reported as inhibiting platelet activation induced by anti-phospholipid antibodies [32], but to the best of our knowledge, there is no known anti-platelet effect of any of the other drugs these patients were taking. Furthermore, while our study demonstrates similar pharmacological efficacy of sulfasalazine therapy to aspirin on arachidonic acid-mediated platelet aggregation, we did not prospectively measure serum thromboxane production and future studies should incorporate such assays. Finally, the fact that sulfasalazine blocks the platelet response to exogenous arachidonic acid does not necessarily imply that it has the same anti-thrombotic effects nor the same clinical efficacy as aspirin in the prevention of adverse cardiovascular events in patients with inflammatory arthritis. To truly answer that question, a large prospective study with hard clinical end points and long-term longitudinal follow-up would be required.

Conclusion

In summary, this investigation demonstrates that sulfasalazine, although poorly absorbed, has an anti-platelet effect on the arachidonic acid pathway that is comparable to that of aspirin. Furthermore, this inhibitory effect is dependent on both sulfasalazine and its metabolites. Finally, this represents a potential mechanism that may contribute to the known cardioprotective effect of sulfasalazine in patients with IA, who are at significantly increased risk of adverse cardiovascular events and remain unprotected in terms of anti-platelet therapy.

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Authors’ contributions Paul MacMullan was involved in all aspects of this research, including study design, data collection, analyses and manuscript preparation. Anne Madigan’s main contribution centred around patient recruitment, disease activity assessment, data collection and write-up. Nevin Paul performed the experiments for the in vitro work and subsequent data collection and analysis. Aaron Peace was involved in study design, adaptation of the appropriate platelet function testing procedures, data analysis and write-up. Ahmed Alagha helped to design the in vitro experiments, prepared the agents to be tested and collected data. Kevin Nolan oversaw the in vitro work and contributed to the write-up. As the senior authors, both Geraldine McCarthy and Dermot Kenny were involved in all aspects of this work, including study design, data analyses and preparation of final submission.

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As one of the two senior authors, Professor Geraldine McCarthy is a world-renowned rheumatologist with an international research reputation. She has published widely in many different areas and is a sought-after speaker with a particular expertise in gout.

Prof. Dermot Kenny is a cardiologist and clinician scientist based at the RCSI in Dublin. He is an international expert and authority on platelet function and is a member of the International Society for Thrombosis and Haemostasis (ISTH) committee for recommendations and guidelines surrounding platelet function testing.

Conflict of interest All authors have completed the Unified Competing Interest form and declare no financial relationships with any organisations that might have an interest in the submitted work and no other relationships or activities that could appear to have influenced the submitted work.

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References

Appendix 4

Summary of Protocol and Procedure for Microtitre Platelet Test

1) Set-up machinery:
   - Switch on the plate-reader (Wallac), computer and set it to 37 degrees.
   - perform any calibrations (eg pH meter)

2) Prepare 50mls JNL:
   - Make up JNL solution using:
     o 5mls JNL-A
     o 5mls JNL-B
     o 5mls JNL-D
     o Only 0.5mls JNL-E
     o 25mls deionised water
   - Allow it to warm to room temp then pH it to 7.35
   - Add deionised water to make up to a final volume of 50mls.

3) Prepare syringe for collecting blood:
   - Draw up sodium citrate into a syringe (one tenth of the final volume of blood to be collected).
   - Remove any air bubbles from the syringe.
   - Get the syringe up to 37 degrees, or at least room temperature.

4) Ensure you have fresh stock of the agonists:
   - Arachidonic Acid [AA]: add 0.5mls deionised water to vial.
   - Collagen [Coll]: add 0.5mls deionised water to vial (room temp).
   - Adenosine Di-Phosphate [ADP]: add 0.5mls deionised water to vial.
   - Epinephrine [Epi]: add 0.5mls deionised water to vial and then do a 1:4 dilution.
   - Thrombin Receptor Activating Peptide [TRAP]: add 0.490μL deionised water to the frozen stock.

5) Prepare the 96-well plates:
   - Add JNL to wells as shown below (in μL)

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- **Add AA to column 1:**
  - Reverse pipette 20μL of (thawed) AA into well H1.
  - Reverse pipette 30μL of AA into well G1.
  - Do serial dilutions for rest of column 1 (Beware bubbles).

- **Add Coll to column 2:**
  - This is done exactly as for AA above.
  - Reverse pipette 20μL of Collagen into well H2.
  - Reverse pipette 30μL of Collagen into well G2.
  - Do serial dilutions for rest of column 2 (Beware bubbles. Collagen is particularly bad for this!).

- **Add ADP to column 3:**
  - This is similar to AA above except only 20μL is added to well G3.
  - Reverse pipette 20μL of ADP into well H3.
  - Reverse pipette 20μL of ADP into well G3.
  - Do serial dilutions for rest of column 3.

- **Add Epi to column 4:**
  - This is done similarly except that most volumes are 10μL.
  - Reverse pipette 20μL of Epi into well H4.
  - Reverse pipette 10μL of Epi into well G4.
  - Do serial dilutions for rest of column 4.
  - Extra step: withdraw and discard 10μL from wells G4 to A4.

- **Add TRAP to column 5:**
  - This will be identical to ADP.
  - Reverse pipette 20μL of (thawed) TRAP into well H5.
  - Reverse pipette 20μL of TRAP into well G5.
  - Do serial dilutions for rest of column 5.

- The addition of (raw, unmixed) agonists is summarised below:

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6) Collecting a blood sample
   - **Slowly** draw the blood sample to the exact amount required.
Blood should flow slowly and freely to avoid sheer stresses on the platelets.
Leave the blood to rest 15 to 30 minutes before centrifugation.

7) Centrifuge the blood sample in 5ml aliquots at 150g for 10 minutes

8) Collect Platelet-Rich Plasma (PRP) and prepare PPP.

9) Put PPP and PRP into plate and obtain measurements

**Detailed Protocol and Procedure for Microtitre Platelet Test**

1) Set-up machinery:
- First switch on the Wallac (large green switch at back).
- Then switch on computer (always do this after switching on the Wallac or else the computer can fail to detect the Wallac machine).
- Using the computer to operate the Wallac, prepare the Wallac temperature:
  - Start the Wallac Manager Program as found on the desktop. This will take a bit of time to initiate itself and you will hear the Wallac make its initiation noises.
  - Click on the “Temperature” tab.
  - set the heating plate to “ON”
  - set the temperature to 37.0 degrees
  - click on the “Apply” button
  - After a minute, confirm this has worked by looking at the temperature graph. It will display the current Wallac temperature (blue line) and the temperature it is aiming for (horizontal dotted line). The blue line grows slowly and will be seen to be rising towards the dotted horizontal line as the heating plate warms up.
- perform any calibrations (eg pH meter)

2) Prepare 50mls JNL:
- JNL is an artificial solution of salts, dextrose and pH buffers, used to match the physiological environment for platelets.
- Remove JNL ingredients (JNL A, B, D and E and the ACD) from fridge and check them for cloudiness, impurities and expiry date (each lasts 4 weeks if stored in fridge). Keep them refrigerated at all times.
- Obtain a clean dry 50ml container, add:
  - 5mls JNL-A using a sterile pipette.
  - 5mls JNL-B
  - 5mls JNL-D
  - But only 0.5mls JNL-E
- Using a fresh sterile pipette each time.
- Add deionised water to achieve a volume of 40mls approx.
- Allow the solution to get to warm to room temperature as this will affect the pH measurements in the next step.
- The solution made up so far will have a pH of about 8.8 (approximately). Using the pH meter to monitor your progress, carefully add ACD solution until the pH just reaches 7.35. Use a small 3ml plastic “transfer pipette”. Note 3.5mls should get you very close, then add ACD slowly, drop by drop. Stir the solution whilst doing this to ensure homogeneity.
- Add deionised water to make up to a final volume of 50mls.
- Unstable pH may indicate bacterial contamination of JNL ingredients.
- Use it that day only: once made up, it only lasts for a day.

3) Prepare syringe for collecting blood:
- Obtain a 50ml syringe.
- Remove the sodium citrate solution from the fridge.
- Draw up the correct dose of sodium citrate into the syringe, where the amount of sodium citrate will be one tenth of the final volume of blood. Therefore:
  - For a female patient, we need 30mls blood, so draw up 3mls Sodium Citrate.
  - For a male patient, we need 40mls blood, so draw up 4mls Sodium Citrate.
- Put the sodium citrate solution back in the fridge.
- Remove any air bubbles from the syringe so that the correct amount of blood will be drawn when the blood is collected.
- Try to get the syringe up to 37 degrees, or at least room temperature, so as not to shock the platelets into aggregation during blood collection.

4) Make up fresh stock of the agonists:
This doesn't need to be done every day, only when you run out of agonist (Arachidonic Acid [AA], Collagen [Coll], Adenosine Di-Phosphate [ADP], Epinephrine [Epi] and Thrombin-Related Activating Peptide [TRAP]).
- Remove the vial with its powdered agonist from the fridge. The exception is TRAP, which is in concentrated liquid form and is kept frozen.
- Check the agonist ampoules to ensure it's not out of date
- Make up new ampoules using deionised water:
  - For AA, Coll and ADP:
    - Open the glass ampoule (the metal capping first and then the grey soft rubber bung), taking care to avoid losing any of the fine powder within.
    - Measure 0.5mls deionised water accurately.
    - Add the water to the ampoule and replace the rubber bung.
    - Shake the ampoule so that all the powder is fully dissolved (including any stuck to the rubber bung),
leaving only a clear colourless fluid (Collagen is slightly cloudy).

- Label it with the date it was re-constituted.
- For Coll and ADP: store in the fridge.
- AA needs to be stored frozen unless used that day: divide the re-constituted AA into 54μL aliquots in 0.5ml Eppendorf “safe-lock” tubes, so that they can be frozen and individually used when required. One vial of freshly made AA will make 9 eppendorfs. Store these in the freezer in an upright position.
  - Note the product information for AA states that it lasts 24hrs under refrigeration and much less at room temperature. Keep it cold.
- For Epi:
  - Follow the steps above for ADP to make up an ampoule of full-strength Epi, which is 1mM.
  - Dilute this down to the assay-strength of 200μM in a plastic 1.5ml “safe-lock” tube. This can be done by transferring 200μL of the Epi into the safe-lock tube and adding 800μL of deionised water (ie a 1:4 dilution).
  - Label and date.
  - Store in the fridge.
- For TRAP
  - Take an ampoule of frozen full-strength TRAP from the freezer (10mM in 10μL).
  - Add 490μL deionised water (making a total of 500μL at 200μM strength), which is our final desired strength.
  - It’s easiest to keep the ADP, Col and Epi together in a small container in the fridge so that they don’t get mixed up or lost amongst the un-prepared ampoules.
  - Keep TRAP and AA frozen when not in use.

5) Prepare the 96-well plates:
- The plates used to test platelet samples have 96 polystyrene wells (12 columns of 8). Of these, the test uses 48 wells (6 columns, each with 8 rows) per patient.
- Each well, once fully prepared, will have various concentrations of reagents. But the amount of reagents in each well will be 20μL. If you end up with wells which look very different, you have made a mistake and need to start again.
- Each new plate needs to have the reagents accurately measured and inserted carefully in their appropriate wells.
- When adding liquid to a well at any point, always touch the pipette tip to the bottom edge of the well and inject slowly.
- Avoid any air bubbles whenever possible.
- Avoid contact with the sides of the wells.
- Avoid spraying liquid above the wells, as droplets can contaminate nearby wells.
- As you work, try to hold the plate at an angle so any fluid will sit at the bottom edge of a well, allowing you to see the pipette tip easily.

- Add JNL to appropriate wells. This is described for columns 1-6 (columns 7-12 will correspond respectively).
  - pour the JNL into a clean dry dispensing trough
  - Dispense JNL using "reverse-pipetting": over-fill the pipettes by fully depressing the pipette plunger to the 2nd stop prior to immersing the tips in the JNL. This draws up more than the required amount into each pipette. Then dispense the JNL into the wells by pressing the plunger but only to the 1st stop, without over-pressing it. Keep your thumb down at the 1st stop as you withdraw from the wells. Then get more JNL for the next set of wells.
  - Using the multi-channel pipette:
    - Add 10μL of JNL to rows A-G of column 4 (leave the H-well empty).
    - Add 20μL of JNL to rows A-G of columns 3, 4, 5 and 6. Note at this stage column 4 will now have 30μL of JNL.
    - Add 20μL of JNL to rows A-F of columns 1 and 2. (leave the G and H wells empty)
    - Add 20μL JNL to the well H6.
    - Add 10μL to wells G1 and G2
  - Return the remaining JNL to its beaker and cover and store away in the fridge.

- You can follow your own method if you prefer, but the final volumes of JNL in the plate layout should be:

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- Add AA to column 1:
  - Reverse pipette 20μL of (thawed) AA into well H1.
  - Reverse pipette 30μL of AA into well G1.
  - G1 already had 10μL of JNL, so it will now have 40μL of fluid. Mix these thoroughly by it drawing up and down fluid. Mix these thoroughly by it drawing up and down
with a 20μL pipette. This must be done using normal pipetting, not reverse pipetting. Do this about 10 times to ensure adequate mixing of the AA and the JNL, but do it carefully and avoid air bubbles.

- Remove 20μL from G1 and transfer it to F1. Again, use normal pipetting and draw the fluid in and out 10 times to ensure mixing.
- Remove 20μL from F1 and transfer it to E1, then mix as above.
- Repeat this 20μL transfer along the rest of column 1, mixing well each time and avoiding air bubbles.
- Discard the final 20μL drawn from well A1.
- Each well in column 1 (including H1) should now have 20μL of AA at various dilutions. Visually, they should all be similar in terms of the quantity of fluid present and the appearance; the fluid should coat the well’s plastic base, giving it an oily appearance.

- Add Coll to column 2:
  This is done exactly as for AA above. Beware bubbles as Collagen is quite viscous.

  - Remove 20μL from G2 and transfer it to F2. Again, draw the fluid in and out 10 times to ensure mixing.
  - Remove 20μL from F2 and transfer it to E2, then mix as above.
  - Repeat this 20μL transfer along the rest of column 2, mixing well each time and avoiding air bubbles.
  - Discard the final 20μL drawn from well A2.
  - Each well in column 2 should now have 20μL of Coll at various dilutions. Visually, they should all be similar in terms of the quantity of fluid present and the appearance.

- Add ADP to column 3:
  This is done exactly as for AA above except only 20μL of ADP is added to well G3.

  - Remove 20μL from G3 and transfer it to F3.
  - Remove 20μL from F3 and transfer it to E3, then mix as above.
  - Repeat this 20μL transfer along the rest of column 3, mixing well each time and avoiding air bubbles.
  - Discard the final 20μL drawn from well A3.
  - Each well in column 3 should now have 20μL of ADP at various dilutions. Visually, they should all be similar in terms of the quantity of fluid present and the appearance.
a 20μL pipette. Do this about 10 times to ensure adequate mixing.

- **Add Epi to column 4:**

  This is done similarly to the others except that
  (a) only 10μL of Epi is added to well G4,
  (b) only 10μL of liquid is transferred between the wells, and
  (c) once mixed, remove 10μL from wells G4 to A4 so they all end up with 20μL of liquid. The differences mean we get quarterly dilutions.

  - Reverse pipette 20μL of Epi into well H4.
  - Reverse pipette 10μL of Epi into well G4.
  - G4 already had 30μL of JNL, so it will now have 40μL of fluid. Mix these thoroughly by drawing up and dispensing with a 10μL pipette. Do this about 15 times to ensure adequate mixing.
  - Remove 10μL from G4 and transfer it to F4. Again, draw the fluid in and out 15 times to ensure mixing.
  - Repeat this 10μL transfer along the rest of column 4, mixing well each time and avoiding air bubbles.
  - Discard the final 10μL drawn from well A4.
  - Well G4 to A4 will now each contain 10μL of liquid. This needs to be reduced down to 20μL. Withdraw and discard a further 10μL from these wells so they all end up with a 20μL volume (No need to touch well H4 since it will already have the correct 20μL volume)
  - All the wells in column 4 should now have 20μL of Epi at various dilutions. Visually, they should all have a similar appearance in terms of the quantity of fluid.

- **Add TRAP to column 5:**

  This will be identical to ADP.

  - Reverse pipette 20μL of (thawed) TRAP into well H5.
  - Reverse pipette 20μL of TRAP into well G5.
  - G5 already had 20μL of JNL, so it will now have 40μL of fluid. Mix these thoroughly by drawing up and dispensing with a 20μL pipette. Do this about 10 times to ensure adequate mixing.
  - Remove 20μL from G5 and transfer it to F5. Again, draw the fluid in and out 10 times to ensure mixing.
  - Repeat this 20μL transfer along the rest of column 5, mixing well each time and avoiding air bubbles.
Discard the final 20μL drawn from well A5.
Each well in column 5 should now have 20μL of TRAP at various dilutions. Visually, they should all be similar in terms of the quantity of fluid present and the appearance.

- Column 6 will have just the JNL to act as a control. These will all already have 20μL of fluid. Thus all the wells (in columns 1 to 6) should now all have 20μL of fluid. They should all have a very similar appearance in terms of volume and as a clear colourless ball of fluid at the base of the well (except for the oily-looking AA of column 1).

- The addition of (raw, unmixed) agonists is summarised below:

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To do two patients on a single plate (ie to do a “double plate”), repeat the above JNL and agonist preparations for columns 7 to 12.

6) Collecting a blood sample

- prepare the necessary equipment:
  o a warmed syringe with the correct amount of sodium citrate, with no air bubbles
  o tourniquet
  o 19G butterfly needle
  o 5ml syringe
  o alcohol swab
  o cotton ball
  o sticking plaster
- attach the 5ml syringe to the butterfly
- place the tourniquet on the upper arm and clean the sample site with alcohol
- insert the needle until a flashback of blood is seen
- remove the tourniquet
- slowly draw 5mls of blood and discard
- remove this syringe and attach the 50ml syringe
- **Slowly** draw the blood sample to the exact amount required (total 30mls for women, 40mls for men).
- Blood should flow slowly and freely to avoid sheer stresses on the platelets, as this can activate aggregation
- Remove the needle, apply cotton wool and then a plaster.
- Gentle rock the syringe back and forth about 5 times to ensure adequate mixing of the citrate and the blood.
- Record the date and time that the blood is taken.
- Leave the blood to rest 15 to 30 minutes before centrifugation.

7) Centrifuge the blood sample
- Transfer the blood in 5ml aliquots into polyethylene test-tubes (6 tubes used for women, 8 tubes for men). Do this by tilting the test-tubes and allowing the blood to slowly run from the top of the test-tube down along the side of the tube into its base.
- Each tube should have the same amount of blood in them. This is essential to ensure the centrifuge is balanced and spins without agitation. Use plastic pipettes to correct any discrepancies in volume.
- Screw on the caps of the tubes and transfer them into the centrifuge.
- Place the test-tubes in a balanced fashion inside the centrifuge.
- Close the lid and set it to spin at 150g for 10 minutes
- Press the start button firmly.
- Check that the centrifuge starts by looking at the display
- Once finished, slowly and carefully remove the test-tubes and sit them vertically into a holding rack.

8) Collecting Platelet-Rich Plasma (PRP)
- Using a plastic pipette, remove the PRP from each test-tube, avoiding any red blood cells and the thin layer of white blood cells.
- Hold the test-tube vertically and try to keep the pipette tip just below the surface of the PRP to avoid collecting other blood cells.
- If you get too close to the other blood cells, you can see the different type of fluid being drawn into the pipette. Stop and quickly squeeze out a few drops so that the contaminating cells are removed.
- Add the PRP to a clean polyethylene test-tube by running the PRP along the side of the tube.

9) Obtain measurements
- Measure the amount of PRP obtained. 8.5 – 9.0mls is the bare minimum required for full testing. Aim for more than 12mls.
- Transfer 1ml of PRP into an Eppendorf and spin at high speed to remove any platelets. This will give you 1ml of Platelet-Poor Plasma (PPP). If you have less than 10mls PRP, you must prepare the PPP in a different way (Re-spin the whole blood tubes at 1500g for 5 minutes).
- Add 180 µL PPP to well H6, G6, F6 and E6 using reverse pipetting. (Normal pipetting will always cause air bubbles).
- Run the Wallac set-up wizard to set it to the correct program. Choose the columns to be “measured” and set the rest to “empty”.
- Enter in the sample details, such as the identity the sample, etc.
- Gently pour the PRP into a clean dry trough.
- At this stage, you should be ready to go. **CHECK that all is OK before proceeding** (ie Wallac is set up with temp at 37 degrees and program details entered and ready to go).
- Using reverse pipetting, add 180 μL PRP to the remaining wells, one column at a time. Do this by holding the pipettes almost horizontally across the tops of the well and allow the PRP to run from the *top* of each well, down the wall of each well, into the base where it will mix with the agonists.
- Do this fairly quickly as aggregation begins immediately once PRP has been added, but be careful and accurate.
- Quickly place the plate in the Wallac and click on start button.
- Once completed (25 minutes), save the data to the appropriate file.

10) Prepare any other samples for other tests:
- E.g. Store any remaining PRP in freezer.