Platelet dependent thrombosis, blood thrombogenicity and response to antiplatelet therapy in health, ageing and type 2 diabetes mellitus

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Abstract

Elderly and type 2 diabetes mellitus (T2DM) patients with stable coronary artery disease (CAD) have increased risk of atherothrombotic events despite recommended secondary prevention therapy. High platelet reactivity drives this risk. Novel approaches to antiplatelet therapy are needed.

Objectives:

To:

- determine the effect of age and T2DM on blood thrombogenicity and response to dual antiplatelet therapy in stable CAD
- assess the effect of changes in platelet count on thrombus quantity and quality with Rafigrelide

Methods:

Study 1: Patients with stable CAD, 4 groups: age<75 non-DM, age≥75 T2DM, age≥75 non-DM and age<75 T2DM studied at baseline and one week after clopidogrel. I performed Badimon chamber study, thromboelastography, VerifyNow® and Multiplate® aggregometry, coagulation and inflammatory biomarkers and scanning electron microscopy.

Study 2: Twelve volunteers took Rafigrelide (novel platelet lowering agent) singly and then with aspirin. I performed Badimon chamber study and thromboelastography at pre-defined intervals.

Results:

Study 1: At baseline and after clopidogrel therapy, there was no difference in thrombus area between the four groups. Serum TNFα levels were higher in elderly T2DM patients. Other coagulation and inflammatory markers were similar between the groups. Clopidogrel reduced thrombus area, lowered platelet content of thrombus and increased fibrin diameter and density in all four groups. Elderly and T2DM patients demonstrated high platelet reactivity and hyporesponsiveness to clopidogrel. Significant reduction in thrombus area was demonstrated both in good- and hypo-responders to clopidogrel. Point of care tests and thrombus area showed no correlation. Post chamber blood confirmed release of P selectin, CD40 ligand and PAI-1 from activated platelets.

Study 2: Rafigrelide reduced platelet count and thrombus area, delayed initiation of clot formation and reduced over all clot strength. Platelet count positively correlated with thrombus area.

Conclusion:

Elderly and T2DM patients had similar over all blood thrombogenicity but higher platelet reactivity when compared to young and non-diabetic patients. Addition of clopidogrel reduced thrombus area with ultrastructural changes in fibrin favouring fibrinolysis. Reduction in platelet count with Rafigrelide reduced thrombus formation and lowered viscoelastic strength. Dual antiplatelet therapy and novel therapeutic strategies may reduce future thrombotic risk in these high risk populations.

Dedications

I dedicate this work to my revered parents, dear wife, beloved sons Guru and Shiva, esteemed professors, colleagues and all the patients who volunteered for my study.

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The list would be incomplete without me mentioning all the patient volunteers who lent me their support and made this research study possible. I hope this study helps in some way to improve patient care in the field of the elderly, diabetes and cardiovascular medicine.

Declaration

I declare that this thesis submitted in complete fulfilment of the requirements for the award of the degree of Doctor of Philosophy to Newcastle University is a product of my original work and not submitted elsewhere for a degree or diploma. The study was conducted by me in The Newcastle upon Tyne Hospitals NHS Foundation trust and Institute of Cellular Medicine, Newcastle University. The research detailed within the thesis was performed by me between the years 2010 – 2014 and was supervised by Professor Sally M Marshall and Professor Azfar G Zaman. I was responsible for organisation of the clinical studies, recruitment of volunteers, conduction of the studies, collection and analysis of the samples, data management, data analysis and research governance. I have correctly acknowledged the specific contributions by others in the relevant sections of the thesis.

For one of the groups in the study (Patients <75 years with type 2 diabetes mellitus and stable coronary artery disease), I used the thrombus images from a previous study done by our research group and I recounted the thrombus area. I have also used data of three different point of care platelet function tests (thromboelastography, VerifyNow® and Multiplate®) for the same group in my study. That study was performed by Dr. Girish Viswanathan and I obtained his permission for using the data. Professors Sally M Marshall and Azfar G Zaman also supervised the previous study and I also had their permission. I sincerely thank Dr. G Viswanathan for permitting me to use some of his data for comparison in my study.

Chapter 1 Introduction 1

1.1 Epidemiology and Pathogenesis of Coronary Artery Disease (CAD) 1

1.1.1 Epidemiology of Cardiovascular Disease 1

1.1.2 Diabetes and Cardiovascular disease 2

1.1.3 Cardiovascular mortality and morbidity in type 2 diabetes mellitus (T2DM) 3

1.1.4 Risk factors for coronary artery disease in T2DM 4

1.1.5 Ageing and Cardiovascular disease 5

1.1.6 Cardiovascular disease mortality and morbidity in elderly 6

1.1.7 Factors contributing to increased risk of CAD in elderly 8

1.1.8 Ageing and bleeding 9

1.1.9 Ageing and Diabetes Mellitus 9

1.2 Pathophysiology of coronary atherosclerosis 10

1.2.1 Coronary artery anatomy 11

1.2.2 Laminar flow and protection against atherosclerosis 12

1.2.3 Initiation of plaque formation – endothelial dysfunction 12

1.2.4 Fatty streak 12

1.2.5 Progression and evolution of atheromatous lesion 15

1.2.6 Plaque vulnerability and rupture 18

1.3 Blood Haemostasis and thrombus formation 22

1.3.1 Mechanisms of thrombosis 22

1.3.2 Platelets in thrombogenesis 26

1.3.3 Platelet-neutrophil interaction 30

1.3.4 Platelet procoagulant activity 31

1.3.5 Coagulation physiology and factors 32

1.3.6 Regulation of coagulation 35

1.4 Blood thrombogenicity in Diabetes and Ageing 38

1.4.1 Platelets in Diabetes - “Angry Diabetic” platelets and Ageing platelets 39

1.4.2 Microparticles in Diabetes and Ageing 40

1.4.3 Platelet leukocyte aggregates (PLA) in Diabetes and Ageing 42

1.4.4 Coagulation in Diabetes and Ageing 42

1.4.5 Fibrinolysis in Diabetes and Ageing 44

1.4.6 Inflammation and thrombosis in Diabetes and Ageing 46
1.4.7 Markers of inflammation 49

1.5 Antiplatelet therapy in patients with CAD 50
1.5.1 Structure of aspirin 52
1.5.2 Pharmacokinetics of aspirin 52
1.5.3 Mechanism of action of aspirin 52
1.5.4 Dosage of aspirin for coronary artery disease 53
1.5.5 Side effects of aspirin 53
1.5.6 Aspirin resistance 54
1.5.7 Aspirin and T2DM 55
1.5.8 Aspirin and acute coronary syndrome 56
1.5.9 Aspirin and elderly population 57
1.5.10 Structure and metabolism of clopidogrel 57
1.5.11 Clinical studies of clopidogrel in coronary artery disease 60
1.5.12 Dosage of clopidogrel 62
1.5.13 Side effects of clopidogrel 62
1.5.14 Variability of clopidogrel response 63
1.5.15 Pharmacogenomics of clopidogrel 65
1.5.16 Clopidogrel and T2DM 67
1.5.17 Antiplatelet therapy in the elderly 68
1.5.18 Dose modification of antiplatelet agents 68
1.5.19 Dual antiplatelet therapy in stable coronary artery disease 69
1.5.20 Introduction to Rafigrelide – a novel platelet lowering agent 71

Chapter 2 Hypothesis and Objectives 73

2.1 Study 1: Elderly stable CAD study 73
2.1.1 Hypothesis 73
2.1.2 Primary Objectives 73
2.1.3 Secondary Objectives 73

2.2 Study 2: Rafigrelide (new platelet lowering agent) study – Phase 1 study 74
2.2.1 Hypothesis 74
2.2.2 Primary Objectives 74

2.2.3 Secondary Objectives 74

Chapter 3 Methods 75

3.1 General methods 75

3.2 Study 1: Elderly stable CAD study design 75

3.2.1 Patient selection 76

3.2.2 Screening and recruitment 77

3.2.3 Study specific procedures 79

3.2.4 Investigational Medicinal Product 80

3.2.5 Pharmacovigilance and data monitoring 81

3.2.6 Statistical methods 81

3.3 Study 2: Rafigrelide study design 85

3.3.1 Patient selection 86

3.3.2 Screening and recruitment 89

3.3.3 Study specific procedures: 93

3.3.4 Investigational Medicinal Product(s) 97

3.3.5 Pharmacovigilance and data monitoring 98

3.3.6 Statistical methods 99

3.4 Special methods 101

3.4.1 Badimon perfusion chamber – platelet dependent thrombus formation 101

3.4.2 Scanning electron microscopy (SEM) – Ultrastructural assessment of thrombus 111

3.4.3 Thromboelastography® and Platelet Mapping™ - assessment of viscoelasticity 122

3.4.4 VerifyNow® optical aggregometry – Measurement of platelet aggregation 135

3.4.5 Multiplate® impedance aggregometry – Measurement of platelet aggregation 139

3.4.6 Coagulation and Inflammatory biomarker assay – serum and plasma samples 145

Chapter 4 Results and discussion 151

4.1 Platelet dependant thrombus formation – Elderly stable CAD study 151

4.1.1 Results 151

4.1.2 Discussion 171

4.2 Thromboelastography® (TEG) and Platelet Mapping™ - Elderly stable CAD study 177

4.2.1 Results – Viscoelastic properties of thrombus 177

4.2.2 Discussion - viscoelastic properties of thrombus 206

4.3 VerifyNow® (Optical aggregometry) - Elderly stable CAD study 211

4.3.1 Results – Platelet reactivity indices 211

4.3.2 Discussion – platelet reactivity indices 242

4.4 Multiplate® (Impedance aggregometry) - Elderly stable CAD study 251

4.4.1 Results – platelet reactivity indices 251

4.4.2 Discussion – platelet reactivity indices 289

4.5 Coagulation biomarkers - Elderly stable CAD study 295

4.5.1 Results 295

4.5.2 Discussion 304

4.6 Inflammatory biomarkers - Elderly stable CAD study 311

4.6.1 Results 311

4.6.2 Discussion 319

4.7 Scanning electron microscopy (SEM) – Elderly stable CAD study 323

4.7.1 Results - Ultrastructural analysis of thrombus 323

4.7.2 Discussion - Ultrastructural analysis of thrombus 339

4.8 Rafigrelide study – Platelet dependant thrombosis and viscoelastic properties 343

4.8.1 Results 343

4.8.2 Discussion 364

Chapter 5 Conclusion 367

5.1 General discussion 367

5.2 Limitations 372

5.3 Future directions 374

List of Figures

Figure 1.1 Progression of coronary atherosclerosis 14
Figure 1.2 Schematic of the evolution of the atherosclerotic plaque. 20
Figure 1.3 Interplay between adaptive and innate immunity during atherogenesis. 21
Figure 1.4 Mechanism of thrombus formation. 25
Figure 1.5 Platelet components implicated in coagulation and atherosclerotic process. 29
Figure 1.6 The coagulation cascade. 36
Figure 1.7 Schematic diagram of the three pairs of polypeptide chains of fibrinogen. 36
Figure 1.8 Schematic diagram of fibrin polymerization. 37
Figure 1.9 Fibrinolytic system and its regulation. 45
Figure 1.10 Schematic representation of mechanisms of action of antiplatelet agents. 51
Figure 1.11 Structure of aspirin 51
Figure 1.12 Structure and metabolism of clopidogrel 59
Figure 1.13 Proposed Mechanisms Leading to Variability in Individual Responsiveness to Clopidogrel 64
Figure 1.14 Pharmacogenomics of clopidogrel. 66
Figure 3.1 Participant Pathway for Elderly stable CAD study 83
Figure 3.2 Elderly stable CAD study – Flow chart 84
Figure 3.3 Overview of study design 88
Figure 3.4 Rafigrelide study – Flow chart 100
Figure 3.5 Set up of the Badimon chamber 106
Figure 3.6 Electron microscopy appearance of platelets 116
Figure 3.7 Electron microscopic appearance of fibrin fibres 117
Figure 3.8 Image acquisition in SEM – Major Grid 121
Figure 3.9 Thromboelastography 125
Figure 3.10 Normal TEG® tracing as displayed digitally. 126
Figure 3.11 TEG® Platelet Mapping™ tracings 126
Figure 3.12 V curve parameters of TEG® 130
Figure 3.13 VerifyNow® assay 138
Figure 3.14 Multiplate® assay: Based on impedance aggregometry 141
Figure 3.15 Platelet function tests performed by Multiplate® aggregometry 142
Figure 3.16 MSD Electrochemiluminescence technology 149
Figure 4.1 High shear thrombus area in elderly stable CAD study. 159
Figure 4.2 Correlation of platelet count with high shear (A) and low shear thrombus (B) 160
Figure 4.3 High shear thrombus area (age<75 and age≥75) 165
Figure 4.4 High shear thrombus area (T2DM vs. Non-DM) 170
Figure 4.5 Changes in TEG® parameters after clopidogrel therapy 189
Figure 4.6 Changes in Platelet Mapping™ after clopidogrel therapy 190

Figure 4.7 Correlation between TEG®-Platelet mapping™ and thrombus area 191
Figure 4.8 Changes in Platelet Mapping™ (Age<75 vs. Age≥75) 196
Figure 4.9 Changes in Platelet Mapping™ (T2DM vs. Non-DM) 203
Figure 4.10 Changes in VerifyNow® indices 216
Figure 4.11 Percentage of good and hyporesponders to clopidogrel 217
Figure 4.12 Changes in VerifyNow® indices (Age<75 vs. Age≥75) 228
Figure 4.13 Changes in VerifyNow® indices (T2DM vs. Non-DM) 237
Figure 4.14 Changes in Multiplate® indices 265
Figure 4.15 Correlation: ADP AU with thrombus area, PRUz, MA and MA-ADP (baseline) 266
Figure 4.16 Correlation: ADP AU with thrombus, PRUz, MA & MA-ADP (after clopidogrel) 267
Figure 4.17 Correlation: ASP AU with ARU, MA and MA-AA 268
Figure 4.18 P Selectin, CD40 ligand & PAI-1 levels – pre- vs. post-chamber at baseline 302
Figure 4.19 P Selectin, CD40 ligand & PAI-1 levels – pre- vs. post-chamber after clopidogrel 303
Figure 4.20 Change in venous TNFα levels with clopidogrel therapy 318
Figure 4.21 Platelet and fibrin content of thrombus - scanning electron microscopy 334
Figure 4.22 Ultrastructure of platelet at baseline and one week after clopidogrel therapy 334
Figure 4.23 Fibrin ultrastructure at baseline and one week after clopidogrel therapy 335
Figure 4.24 Change in platelet content of thrombus, fibrin diameter and fibrin fibre density 336
Figure 4.25 Changes to number of hubs and spokes per square micron of thrombus 337
Figure 4.26 Correlation: SEM measured parameters and thrombus area 338
Figure 4.27 Effect of rafigrelide on platelet count and MPV - treatment period 1. Panel A and B 351
Figure 4.28 Effect of rafigrelide on high and low shear thrombus - treatment period 1. Panel A & B 352
Figure 4.29 Effect of rafigrelide + aspirin on platelet count & MPV - treatment period 2. Panel A & B . 353
Figure 4.30 Effect of rafigrelide on high and low shear thrombus - treatment period 2. Panel A and B 354
Figure 4.31 Correlation between thrombus area and platelet count 355
Figure 4.32 Effect of rafigrelide alone on TEG® parameters in panel A & B 358
Figure 4.33 Effect of rafigrelide alone on TEG®-PM™ indices in panel A & B 359
Figure 4.34 Effect of rafigrelide and aspirin on TEG® parameters in panel A & B 360
Figure 4.35 Effect of rafigrelide and aspirin on TEG®-PM™ indices in panel A & B 361
Figure 4.36 Reduction in thrombus area with rafigrelide (Period 1, Panel A). 362

List of Tables

Table 3.1 Stepwise protocol for modified Mason trichrome staining. 108
Table 3.2 Thromboelastography parameters 128
Table 3.3 TEG® step by step protocol 134
Table 4.1 Baseline characteristics for Elderly stable CAD study. 155
Table 4.2 Mean thrombus area - Elderly stable CAD study. 157
Table 4.3 Comparison of mean thrombus area. 158
Table 4.4 Baseline characteristics (age<75 years vs. age≥75 years) 163
Table 4.5 Mean thrombus area (age<75 years vs. age≥75 years) 164
Table 4.6 Comparison of thrombus area (age<75 years vs. age≥75 years) 164
Table 4.7 Baseline characteristics (T2DM vs. Non-DM) 168
Table 4.8 Mean thrombus area. (T2DM vs. Non-DM) 169
Table 4.9 Comparison of mean thrombus area (T2DM vs. Non-DM) 169
Table 4.10 Baseline characteristics of participants who underwent TEG® and platelet mapping™ 181
Table 4.11 Standard TEG® parameters, Platelet Mapping™ and V-curve data 184
Table 4.12 Thrombus area in patients who underwent TEG® measurements 185
Table 4.13 Comparison of TEG® parameters, Platelet Mapping™ and V-curve data 188
Table 4.14 Baseline characteristics - TEG® substudy (Age<75 vs. Age≥75) 194
Table 4.15 Changes in TEG®, Platelet Mapping™ and V-curve data (Age<75 vs. Age≥75) 195
Table 4.16 Comparison of TEG®, Platelet Mapping™ and V-curve data (age<75 vs. age≥75) 198
Table 4.17 Baseline characteristics - TEG® substudy (T2DM vs. Non-DM) 201
Table 4.18 Changes in TEG®, Platelet Mapping™ and V-curve data (T2DM vs. Non-DM) 202
Table 4.19 Comparison of TEG®, Platelet Mapping™ and V-curve data (T2DM vs. Non-DM) 205
Table 4.20 VerifyNow ® indices in Elderly stable CAD study 213
Table 4.21 Comparison of VerifyNow® platelet reactivity indices 214
Table 4.22 Changes in thrombus area and VerifyNow® indices (good responders) 218
Table 4.23 Comparison of thrombus area and VerifyNow® indices (good responders) 219
Table 4.24 Changes in thrombus area and VerifyNow® indices (hyporesponders) 220
Table 4.25 Comparison of thrombus area and VerifyNow® indices (hyporesponders) 221
Table 4.26 Correlation: VerifyNow® and thrombus area, TEG®, Platelet Mapping™, V-curve data 222
Table 4.27 Correlation: VerifyNow® and thrombus area, TEG®, Platelet Mapping™, V-curve data 223
Table 4.28 Changes in VerifyNow® indices (Age<75 vs. Age≥75) 226
Table 4.29 Comparison of thrombus area and VerifyNow® indices (Age<75 vs. Age≥75) 227
Table 4.30 Changes in thrombus area and VerifyNow® - good responders (Age<75 vs. Age≥75) 229
Table 4.31 Comparison of thrombus area and VerifyNow® - good responders (Age<75 vs. Age≥75) 230
Table 4.32 Thrombus area and VerifyNow® indices - hyporesponders (Age<75 vs. Age≥75) 231
Table 4.33 Thrombus area and VerifyNow® indices - hyporesponders (Age<75 vs. Age≥75) 232
Table 4.34 Changes in VerifyNow® indices (T2DM vs. Non-DM) 235
Table 4.35 Comparison of thrombus area and VerifyNow® (T2DM vs. Non-DM) 236

Table 4.36 Thrombus area and VerifyNow® indices - good responders (T2DM vs. Non-DM)..... 238
Table 4.37 Thrombus area and VerifyNow® indices - good responders (T2DM vs. Non-DM)..... 239
Table 4.38 VerifyNow® indices - hyporesponders (T2DM vs. Non-DM)..... 240
Table 4.39 Thrombus area and VerifyNow® - hyporesponders (T2DM vs. Non-DM) 241
Table 4.40 Baseline characteristics - Multiplate® sub study. 256
Table 4.41 Thrombus area and platelet reactivity indices - Multiplate® substudy. 257
Table 4.42 Comparison of Multiplate® indices 258
Table 4.43 Thrombus area and Multiplate® indices - good responders 259
Table 4.44 Thrombus area and Multiplate® indices - good responders 260
Table 4.45 Comparison of change in Multiplate® indices - good responders 261
Table 4.46 Thrombus area and Multiplate® indices - hyporesponders 262
Table 4.47 Thrombus area and Multiplate® indices - hyporesponders 263
Table 4.48 Comparison of change in thrombus area and Multiplate® indices - hyporesponders 264
Table 4.49 Baseline characteristics – Multiplate® sub study (Age<75 vs. Age≥75) 272
Table 4.50 Thrombus area and Multiplate® indices (Age<75 vs. Age≥75) 273
Table 4.51 Comparison of thrombus area and Multiplate® indices (Age<75 vs. Age≥75) 274
Table 4.52 Thrombus area and Multiplate® indices - good responders (Age<75 vs. Age≥75) 275
Table 4.53 Comparison of thrombus area and Multiplate® - good responders (Age<75 vs. Age≥75) 276
Table 4.54 Thrombus area and Multiplate® indices - hyporesponders (Age<75 vs. Age≥75) 277
Table 4.55 Multiplate® indices - hyporesponders (Age<75 vs. Age≥75) 278
Table 4.56 Baseline characteristics – Multiplate® sub study (T2DM vs. Non-DM) 282
Table 4.57 Thrombus area and Multiplate® indices (T2DM vs. Non DM) 283
Table 4.58 Comparison of Multiplate® indices (T2DM vs. Non-DM) 284
Table 4.59 Thrombus area and Multiplate® indices - good responders (T2DM vs. Non-DM) 285
Table 4.60 Comparison of Multiplate® indices - good responders (T2DM vs. Non-DM) 286
Table 4.61 Thrombus area and Multiplate® indices - hyporesponders (T2DM vs. Non-DM) 287
Table 4.62 Comparison of Multiplate® indices - hyporesponders (T2DM vs. Non-DM) 288
Table 4.63 Coagulation biomarkers – Elderly stable CAD study 297
Table 4.64 Change in coagulation markers pre and post chamber 298
Table 4.65 Comparison of coagulation biomarkers at baseline 299
Table 4.66 Comparison of coagulation biomarkers 300
Table 4.67 Change in coagulation biomarkers (delta) 301
Table 4.68 Inflammation biomarkers – Elderly stable CAD study; 312
Table 4.69 Change in inflammation markers pre and post chamber 313
Table 4.70 Comparison of inflammation biomarkers at baseline 314
Table 4.71 Comparison of inflammation biomarkers one week after clopidogrel therapy 315
Table 4.72 Change in inflammatory biomarkers (delta) 316
Table 4.73 Correlation between inflammatory biomarkers and thrombus area 317
Table 4.74 Baseline characteristics – SEM substudy 328

Table 4.75 Thrombus area and fibrin ultrastructure parameters 329
Table 4.76 Comparison of fibrin and platelet parameters at baseline 330
Table 4.77 Comparison of fibrin and platelet parameters one week after clopidogrel therapy 331
Table 4.78 TEG® and Platelet Mapping™ - SEM substudy 332
Table 4.79 VerifyNow® and Multiplate® indices - SEM substudy 333
Table 4.80 Baseline characteristics of healthy volunteers in Rafigrelide study 347
Table 4.81 Effect of Rafigrelide on platelet count and MPV - treatment period 1 and 2 348
Table 4.82 Effect of Rafigrelide on thrombus area - treatment period 1 and 2 in panel A 349
Table 4.83 Effect of Rafigrelide on thrombus area - treatment period 1 and 2 in panel B 350
Table 4.84 Changes to TEG-PM parameters - Panel A, treatment period 1 & 2 356
Table 4.85 Changes to TEG-PM parameters - Panel B, treatment period 1 & 2 357
Table 4.86 Pharmacokinetics of rafigrelide 363

Abbreviations

AA	Arachidonic acid
ACCORD	Action of Control Cardiovascular Risk in Diabetes
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AE	Adverse event
AGE	Advanced glycation end-products
AHA	American Heart Association
ANOVA	Analysis of variance
AOI	Area of interest
ARU	Aspirin Reaction Units
ATC	Antithrombotic Trialists' Collaboration
AU	Aggregation units
AUC	Area under the curve
BMI	Body mass index
BSE	Back-scattered electrons
CABG	Coronary artery bypass graft
CAD	Coronary Artery Disease
CAPRIE	Clopidogrel versus Aspirin in Patients' at risk of Ischaemic Events
CHARISMA	Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management and Avoidance
CI	Clot index

CKD	Chronic kidney disease
CL	Clot lysis
COMMIT	ClOpidogrel and Metoprolol in Myocardial Infarction Trial
COV	Co-efficient of variation
COX	Cyclo-oxygenase
CRF	Case report form
CRP	C-reactive protein
DALY	Disability-adjusted life years
DAPT	Dual antiplatelet therapy
DCCT	Diabetes Control and Complications Trial
DES	Drug eluting stents
DM	Diabetes mellitus
ECG	Electrocardiogram
EDTA	Ethylene diamine tetra acetic acid
EGF	Endothelial growth factor
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMA	European Medicines Agency
EQC	Electronic quality control
ESC	European Society of Cardiology
EU	European Union
FDA	Food and Drug Administration
GRACE	Global Registry of Acute Coronary Events

HCV	Hepatitis C virus
HDL	High-density lipoproteins
HEENT	Head, Eyes, Ears, Nose and Throat
HIV	Human immunodeficiency virus
HMWK	High molecular weight kininogen
HPR	High on-treatment platelet reactivity
IEC	Independent Ethics Committee
IMP	Investigational medicinal product
IQR	Interquartile range
IRAS	Insulin Resistance Atherosclerosis Study
IRB	Institutional Review Board
LDL	Low-density lipoproteins
LTA	Light transmission aggregometry
MA	Maximum amplitude
MEA	Multiple electrode aggregometry
MHC	Major histocompatibility complex
MHRA	Medicines and Health Regulatory Authority
MI	Myocardial infarction
MINAP	Myocardial Ischemia National Audit Project
MMP	Monocyte-derived microparticles
MPV	Mean platelet volume
MRL	Maximum rate of lysis
MRTG	Maximum rate of thrombus generation

MSD	Meso scale discovery
NCEP	National Cholesterol Education Program
NSAID	Non-steroidal anti-inflammatory drugs
NSTEMI	Non-ST elevation myocardial infarction
PAD	Peripheral arterial disease
PAR	Protease-activated receptors
PBS	Phosphate buffer solution
PCI	Percutaneous coronary intervention
PCT	Primary care trust
PDAY	Pathobiological Determinants of Atherosclerosis in Youth
PDGF	Platelet-derived growth factor
PDT	Platelet dependent thrombus
PGF	Placental growth factor
PIT	Pathological intimal thickening
PLA	Platelet leukocyte aggregates
PLATO	PLATelet inhibition and patient Outcomes
PM	Platelet mapping
PMA	Platelet-monocyte aggregates
PPA	Platelet procoagulant activity
PRP	Platelet-rich plasma
PRUb	P2Y12 reactive units - baseline
PRUz	P2Y12 reactive units - activated
PSGL	P-selectin glycoprotein ligand

PVD	Peripheral vascular disease
QALY	Quality adjusted life years
QC	Quality control
RANKL	Receptor activation of NF-kB ligand
RBC	Red blood cells
REACH	Reduction of Atherothrombosis for Continued Health
REC	Research ethics committee
SAE	Serious adverse event
SCAAR	Swedish Coronary Angiography Angioplasty Registry
SE	Secondary electrons
SEM	Scanning electron microscopy
SMC	Smooth muscle cells
ST	Stent thrombosis
STEMI	ST elevation myocardial infarction
SUSAR	Suspected Unexpected Serious Adverse Reaction
SWAP	SWitching Anti Platelet
TAFI	Thrombin-activated fibrinolysis inhibitor
TC	Total cholesterol
TEG	Thromboelastography
TEG-PM	Thromboelastography – Platelet Mapping
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TG	Thrombus generated

TIMI	Thrombolysis In Myocardial Infarction
TMRL	Time to maximum rate of clot lysis
TMRTG	Time to maximum rate of thrombus generation
TRAP	Thrombin receptor activating peptide
TRITON-TIMI	Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction
TTA	Total thrombus area
TTP	Thrombotic thrombocytopenic purpura
UA	Unstable angina
UKPDS	United Kingdom Prospective Diabetes Study
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WBC	White blood cell
WHO	World Health Organisation
WHR	Waist hip ratio

Chapter 1 Introduction

1.1 Epidemiology and Pathogenesis of Coronary Artery Disease (CAD)

1.1.1 Epidemiology of Cardiovascular Disease

Over the last decade, cardiovascular disease (CVD) has become the single largest cause of death worldwide. In 2004, CVD caused an estimated 17 million deaths and led to 151 million disability-adjusted life years (DALYs) lost – about 30% of all deaths and 14% of all DALYs that year ('World Health Organization: The Global Burden of Disease: 2004 Update.,' 2008). During the last century, similar to the high-income countries, an alarming increase in the rates of CVD has been observed in low- and middle-income countries and this change is accelerating. In approximately 38 % of individuals in high-income countries, death is due to CVD.

In high-income countries, despite the overall increase in CVD burden, the age adjusted death rates from CVD are declining, predominantly due to large stroke rate reductions. Almost 80% of deaths occur in those older than 60 years compared with 42% in low- and middle-income countries (Lopez *et al.*, 2006).

In Europe, CVD is the main cause of death: accounting for over 4.30 million deaths each year. Nearly half (48%) of all deaths are from CVD (54% of deaths in women and 43% of deaths in men). The main forms of CVD are coronary artery disease (CAD) and stroke. CAD by itself is the single most common cause of death in Europe: accounting for 1.92 million deaths each year. 20% of deaths before the age of 75 in men and 19% of deaths before the age of 75 in women are from CAD (Nichols *et al.*, 2012). In 2001, 75% of global deaths and 82% of total DALYs lost because of CAD occurred in low- and middle-income countries (Lopez *et al.*, 2006). Between 1990 and 2020, CAD deaths alone are anticipated to increase by 120% for women and 137% for men in developing countries (Leeder *et al.*, 2004).

1.1.2 Diabetes and Cardiovascular disease

Diabetes mellitus (DM) can be described as a metabolic disorder of multiple aetiologies characterised by chronic hyperglycaemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects of insulin secretion, insulin action, or a combination of both (DeFronzo, 2004). Clinical features include polydipsia, polyuria and weight loss.

The World Health Organisation biochemical diagnostic criteria for diabetes are:

i. Fasting plasma glucose ≥ 7.0 mmol/L and

ii. Plasma glucose two hours after ingestion of 75 grams of oral glucose load ≥ 11.1 mmol/L

In the presence of clinical features, one of the above is sufficient to make a diagnosis of DM (www.who.int/diabetes/publications/diagnosis_diabetes2006/en/, accessed on 07th July 2015)

iii. An HbA1c of 48mmol/mol (6.5%) is recommended as the cut off point for diagnosing diabetes. A value of less than 48mmol/mol (6.5%) does not exclude diabetes diagnosed using glucose tests.

(www.who.int/diabetes/publications/diagnosis_diabetes2011/en/, accessed on 07th July 2015)

The world prevalence of diabetes among adults (aged 20-79 years) was approximately 6.4%, affecting 285 million adults in 2010 and is predicted to rise to 7.7%, affecting 439 million adults by 2030 (Shaw *et al.*, 2010). Between 2010 and 2030, there will be a 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries. Type 2 diabetes mellitus (T2DM) accounts for ninety percent of patients with diabetes (www.who.int/mediacentre/factsheets, accessed on 07th July 2015). Insulin resistance usually precedes the onset of T2DM and is commonly accompanied by other related metabolic abnormalities such as hyperglycaemia, dyslipidaemia, hypertension, and pro-thrombotic factors, all of which contribute to the increased cardiovascular risk. This condition is called the metabolic syndrome (Hopkins *et al.*, 1996; Gray *et al.*, 1998). It is estimated that there will be 4 million people in the UK with diabetes by 2025 and the majority will have type 2 diabetes mellitus (T2DM). *(www.diabetes.org*.uk, *Accessed 03rd Jan 2011)*

1.1.3 Cardiovascular mortality and morbidity in type 2 diabetes mellitus (T2DM)

Cardiovascular Disease (CVD), particularly coronary artery disease (CAD) resulting from accelerated atherosclerosis, is the leading cause of morbidity and mortality in patients with T2DM accounting for 80% of all causes of death (Fox *et al.*, 2007). A large body of epidemiological and pathological data documents that diabetes is an important independent risk factor for CVD in both men and women (McGill and McMahan, 1998; Wilson *et al.*, 1998). Patients with T2DM have a fivefold risk of developing CVD compared to non-diabetics (Preis *et al.*, 2009).To make matters worse, when patients with diabetes develop clinical CVD, they have a poorer prognosis than the CVD patients without diabetes (Smith *et al.*, 1984; Singer *et al.*, 1989; Stone *et al.*, 1989). Cardiovascular mortality in patients with DM without a history of prior MI is comparable to mortality in non-diabetic subjects with previous MI (Haffner *et al.*, 1998). Hence, diabetes has been classified as a coronary “risk equivalent” ('Third report of the National Cholestrol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholestrol in Adults (Adult Treatment Panel III) final report ', 2002). In general, women have lower prevalence of cardiovascular disease and age specific cardiovascular mortality. However, pre-menopausal women with diabetes seem to not only lose most of their inherent protection against developing CVD but also have a poorer prognosis for cardiovascular illness (Brezinka and Padmos, 1994). Their mortality rates are 50% higher than in men (Mulnier *et al.*, 2006; Kautzky-Willer *et al.*, 2010).

Despite a significant overall reduction in cardiovascular mortality over the last two decades due to improved aggressive secondary prevention strategies, the relative risk of cardiovascular mortality in patients with diabetes remains around 2.5, unchanged over the last fifty years (Hanefeld *et al.*, 1996; Fox *et al.*, 2007). Cardiovascular morbidity is difficult to estimate in patients with diabetes. However, cardiovascular disease is responsible for significant number of hospitalisations, prolonged in-hospital admissions and loss of quality adjusted life years (QALY) in patients with diabetes (Sampson *et al.*, 2006).

Coronary Artery Disease (CAD) accounts for the majority of cardiovascular events in patients with T2DM. CAD can present as acute coronary syndrome (unstable angina, non ST elevation and ST elevation acute myocardial infarction) and chronic stable angina. A significant number of patients with T2DM do not survive their first myocardial

event (Cho *et al.*, 2002). Mortality rates for DM patients with acute MI are 1.5 - 2.0 times those of non-DM patients. In hospital and 6 month mortality rates after an acute MI are highest among DM patients receiving insulin therapy. The negative impact of DM on the outcomes is maintained across the ACS spectrum, including unstable angina and non-ST elevation myocardial infarction (NSTEMI) (Yusuf *et al.*, 2001b), ST elevation myocardial infarction (STEMI) treated medically (Mak *et al.*, 1997) and ACS undergoing percutaneous coronary intervention (PCI) (Roffi and Topol, 2004). DM patients have more progressive, diffuse and multi vessel coronary disease compared to non-diabetic patients and have poorer outcomes after both PCI (especially with bare metal stent [BMS]) and coronary artery bypass graft surgery (CABG), compared to non-diabetic patients (Flaherty and Davidson, 2005).

Following an acute cardiac event, patients with T2DM demonstrate higher incidence of recurrent cardiovascular events despite optimal secondary prevention therapy (Lee *et al.*, 2004). For example, the 7 year incidence of recurrent MI in a large population based study was 45% in diabetic patients versus 19% in non-diabetic patients. Cardiovascular mortality during that period was 42.0% and 15.4% in DM patients with and without history of acute MI, respectively (Haffner *et al.*, 1998). T2DM has been shown to be a strong independent predictor of mortality and recurrent cardiovascular events (Kotseva *et al.*, 2010). The advent of drug eluting stents (DES) has improved PCI outcomes but the problem of atherothrombotic complications, including stent thrombosis, persists in diabetic patients (Cola *et al.*, 2009). Patients with T2DM demonstrated higher rates of stent thrombosis following percutaneous coronary intervention (Machecourt *et al.*, 2007).

1.1.4 Risk factors for coronary artery disease in T2DM

Increased prevalence of conventional risk factors such as hypertension, dyslipidaemia, central obesity, smoking and endothelial dysfunction, all contribute to higher adverse cardiovascular events in patients with T2DM (Eberly *et al.*, 2003; Bhatt *et al.*, 2010; Griffin *et al.*, 2011) Hyperglycaemia may play an important role in increased atherothrombotic risk in DM patients. This has been supported by the Diabetes Mellitus, Insulin Glucose Infusion in Acute Myocardial Infarction (DIGAMI) trial. In this study, acute intensive glucose lowering therapy with insulin-glucose infusion led to a reduction in mortality after 3.4 years follow up in DM patients with acute myocardial infarction (Malmberg, 1997). In UKPDS follow up study, tighter control of

hyperglycaemia (HbA1c~7.0 %) reduced cardiovascular mortality over many years when begun in individuals with short-duration diabetes (Home, 2008; McGuire and Gore, 2009). However, in longstanding T2DM patients, long-term aggressive glucose lowering (glycated haemoglobin < 6.0 %) was associated with increased mortality in the Action of Control Cardiovascular Risk in Diabetes (ACCORD) study (Gerstein *et al.*, 2008). This was supported by ADVANCE trial and VADT trial data (Patel *et al.*, 2008; Duckworth *et al.*, 2009; Skyler *et al.*, 2009).

1.1.5 Ageing and Cardiovascular disease

Ageing is a continuous and extremely complex multifactorial process that involves the interaction of genetic and environmental factors, and in which the incidence of diseases and the possibility of dying increases (Tosato *et al.*, 2007). Ageing is a major cardiovascular risk factor and is a strong predictor of adverse outcomes after acute coronary syndrome. Coronary artery disease is the most common cause of death in the elderly. Due to increasing longevity and declining fertility, the geriatric population is rapidly expanding globally. At present, humans over age 65 years are more likely to be active and productive than at any other time in history, and life expectancy, disability rates, and health and wealth indicators have all shown significant improvement in the last 25 years.

The US census bureau reported that average life expectancy has gone up from about 47.3 years in 1900 to 77.9 years in 2004 and is estimated to increase to 79.2 years by 2015. This increase appears to be largely due to improvements in health care, nutrition, and overall standard of living for most people. Population ageing represents a shift in the distribution of a country's population towards greater ages. This shift is partly related to increased longevity, as a consequence of improvements in living conditions and health care. Considered as one of the most important human achievements, this shift is not only seen in the developed countries, but also in developing countries. The UN has projected that by 2025 there will be almost 1.2 billion elderly people living in the world, 71% of whom are likely to be in developing countries. Between 1950 and 2025, the "Old" Old (those who are 80 years and above) will grow twice as fast as the 60-plus-age group (Iliescu and Zanoschi, 2004). By 2030, 20% of the population will be aged 65 years or older (Muss, 2001). In 2020, the proportion of the population aged 80 years and above is expected to range between 3.7% and 7.5% (Anderson and Hussey, 2000).

Ageing is an inevitable part of life and is an independent risk factor for the development of atherosclerosis that plays a significant role in the development of many cardiovascular disorders including coronary artery disease (CAD). Given the rapid growth of an ageing population worldwide, an increasing proportion of morbidity and mortality related to cardiovascular disorders is likely to occur in elderly patients. Both the prevalence and severity of atherosclerotic CAD increase with age in men and women. Autopsy studies show that more than half of people older than 60 years of age have significant CAD with increasing prevalence of left main or triple-vessel disease with older age. Estimates from electronic medical records report almost half of men older than 80 years of age have CAD, as do one third of women (Steinman *et al.*, 2012).

Ageing unfortunately poses the largest risk factor for cardiovascular disease and CAD is the most common cause of death in the elderly. In fact, older people are not only more likely to develop acute myocardial infarction, but also more likely to die from it. Optimizing care for elderly patients with CAD constitutes both a central priority and a formidable challenge in contemporary medicine. The cost to treat cardiovascular diseases is estimated to get tripled in the next 15 years. Hence it remains vital that we understand why age is such a crucial component of CVD etiology.

1.1.6 Cardiovascular disease mortality and morbidity in elderly

The CRUSADE National Quality Improvement Initiative investigators 2005 report states that persons aged ≥ 75 years constitute <10% of the US population but they account for 35% of patients with non-ST segment elevation acute coronary syndromes (ACS). As expected, the prevalence of CAD is very high in the elderly population (Aronow, 1999). Elderly patients presenting with ACS including unstable angina and non-ST segment myocardial infarction (NSTEMI) have a very high prevalence of coronary risk factors (Woodworth *et al.*, 2002).

In the United Kingdom in 2008, more than twice as many individuals >75 years of age (n = 55,028) died from IHD compared to younger individuals <75 years (n = 25,540) (Nichols *et al.*, 2014). According to the UK Myocardial Ischemia National Audit Project (MINAP) database annual public report 2011-2012, there were 79,433 admissions with a final diagnosis of myocardial infarction (MI). Of these, 41% had ST-elevation myocardial infarction (STEMI). The mean age of patients presenting with MI in England

and Wales was 65 years in men and 73 years in women (68 years overall). Individuals' >70 years represented 49% of patients with an MI in the MINAP data. The average age for a first STEMI was 65 years, while that of NSTEMI was 70 years (21% of STEMI patients and 26% of NSTEMI patients were between 70 and 79 years of age, in comparison to 15% of patients with STEMI and 27% of patients with NSTEMI who were 80–89 years of age). Although cases of STEMI appear to be equally distributed around the age-range 60–69 years, for NSTEMI the majority are older than this at presentation (Gavalova and Weston, 2012). In the Global Registry of Acute Coronary Events (GRACE) (Avezum *et al.*, 2005), increasing age was associated with increased incidence of NSTEMI. NSTEMI was diagnosed in just over 30% of patients aged <65 years compared with 41% in those aged ≥85 years. STEMI was more frequent in younger patients (36.5% in 45-74 years vs 30.7% in >75 years). All in-hospital events after ACS were more frequent among elderly patients. Cardiogenic shock was nearly 6 times more common in the oldest compared with the youngest group (9.8% vs 1.6%, respectively). Rates of major bleeding were twice that in patients aged ≥85 years compared to <65 years ($p<0.0001$). Each 10-year increase in age resulted in a 75% increase in hospital mortality.

Age ≥75 years constitutes one of the seven prognostic variables in the Thrombolysis In Myocardial Infarction (TIMI) risk score (Antman *et al.*, 2000). In general, the management of ACS in the elderly patients does not differ a great deal from that the younger patients in terms of management according to the current guidelines (Braunwald *et al.*, 2002; Smith *et al.*, 2006).

As a result of higher prevalence of cardiac risk factors and impaired healing processes, elderly patients with ACS are at higher risk of recurrent ischaemic events and death, as well as treatment-related complications compared with younger patients. It has been reported that patients >75 years of age comprise one third of overall ACS episodes and that this age group accounts for around 60% of the overall mortality from ACS. Further, after allowance for confounding factors, the odds for in-hospital death from acute events increases by 70% for each 10-year increase in age (OR: 1.70; 95% CI: 1.52 to1.82). Atypical symptoms are more common in elderly patients presenting with ACS, dyspnoea, and confusion are relatively common, whereas ischaemic pain is less likely to be present or is present in an atypical location.

Despite these demographic realities, elderly persons are generally underrepresented in randomized controlled trials and recent data have suggested limited success in attempts at making cardiovascular trials more inclusive by including the elderly (Lee *et al.*, 2001). Current ACS treatment guidelines are based primarily upon studies largely comprising younger patients (Roe *et al.*, 2013). Although age may alter the balance of risk and benefit of therapeutic strategies, trials data regarding older patients with ACS have usually been limited to subgroup analyses rather than derived from dedicated age-specific studies. Registries have found that elderly patients presenting with an ACS less frequently receive evidence-based therapies highlighting the need for studies targeted specifically to the elderly.

1.1.7 Factors contributing to increased risk of CAD in elderly

Age dependent alterations of haemostasis suggest that certain coagulation proteins such as fibrinogen, factor V, VII, VIII, IX, XIII, high-molecular weight kininogen and prekallikrein levels increase with age (Franchini, 2006). Overall, the elderly experience a shift of the haemostatic balance towards increased clotting and decreased fibrinolysis. Ageing may also lead to intrinsic changes to platelets, thereby increasing platelet reactivity. This increased platelet reactivity has been correlated with a higher content of platelet phospholipids, suggesting an age related increase in platelet trans-membrane signalling (Bastyr *et al.*, 1990b). Blood stasis and endothelial dysfunction also play a key role contributing to increased platelet activation and arterial thrombosis in the elderly (Zahavi *et al.*, 1980; Loscalzo, 2001; Brandes *et al.*, 2005).

Ageing of the vasculature results in increased arterial thickening and stiffness as well as endothelial dysfunction. Clinically, these changes result in increased systolic pressure and present major risk factors for development of atherosclerosis, hypertension and stroke, and atrial fibrillation. The vasculature undergoes structure and function alterations with age that are well documented, such as luminal enlargement with wall thickening and a decline in endothelial cell function negatively affecting endothelium-dependent dilation and promoting vascular stiffness. In addition, endothelial cells lose their ability to proliferate and migrate after tissue injury. Furthermore, endothelial barriers become porous and vascular smooth muscle cells migrate into sub-endothelial spaces and deposit extracellular matrix proteins that result in intimal thickening. At the molecular level, as endothelial cells age, they exhibit a reduction in endothelial nitric oxide synthase (eNOS) activity, reducing the abundance

of nitric oxide (NO). NO is a critical vasodilator produced by endothelial cells, regulating vascular tone, in addition to inhibiting vascular inflammation, thrombotic events, and aberrant cellular proliferation. Loss of NO also promotes endothelial cell senescence. Numerous mechanisms can modulate eNOS activity. However, hemodynamic shear stress, the frictional force acting on endothelial cell surface as a result of blood flow, is one of the most potent inducers of eNOS activity. As vessels age, they are exposed to less hemodynamic stress due to reduced blood flow caused by decline in heart function; in addition, endothelial cells become less responsive to shear stress, resulting in a decline in the protective NO.

1.1.8 Ageing and bleeding

Age features as a risk factor in many bleeding risk stratifications. Indeed, bleeding risk is greater in elderly versus younger patients. In a recent meta-analysis of stroke prevention in elderly patients with atrial fibrillation, the risk of serious bleeding increased for each decade increase of age (HR: 1.61; 95% CI, 1.47 to 1.77). Furthermore, the use of several antiplatelet and anticoagulant therapies is associated with increased bleeding risk in the elderly, with a resulting reduction in net clinical benefit. For example, age is a predictor of intracranial haemorrhage in patients receiving antiplatelet or anticoagulant therapy. In the Assessment of the Safety and Efficacy of a New Thrombolytic Regimen (ASSENT)-III PLUS trial, enoxaparin as a conjunctive therapy to tenecteplase in patients with ST-segment elevation myocardial infarction was associated with an unacceptable risk of intracranial haemorrhage in the elderly population, warranting a dose reduction in patients aged >75 years.

1.1.9 Ageing and Diabetes Mellitus

The prevalence and incidence of T2DM are greater among elderly individuals compared to the younger population. From a study in France, the overall prevalence of DM was estimated at 4.4% in 2009. The prevalence increased with age to 14.2% in those aged 65-74 years, peaking at 19.7% in men and 14.2% in women aged 75-79 years (Fagot-Campagna *et al.*, 2005). From 2001 to 2005, the prevalence of DM increased by 3.1 % in men aged 70-79 years, 3.8 % in men aged 80-89 years and by 0.7 % and 3.0 % respectively in women in those age group (Pornet *et al.*, 2011). Elderly patients with T2DM are at increased risk of vascular complications, loss of cognitive function and mobility and, consequently, of dependency (Bourdel-Marchasson and

Berrut, 2005). Hence, the increasing number of older individuals with T2DM will lead to a greater public health burden (Bethel *et al.*, 2007; Sloan *et al.*, 2008). Ageing is accompanied by increased arterial stiffness and reduced myocardial compliance, contributing to adverse cardiovascular events. Patients with T2DM manifests these changes at a younger age, suggesting that mechanisms underlying these changes in vasculature during ageing is accelerated in diabetes. Advanced glycation end-products (AGEs) that form during the Maillard reaction (non-enzymatic reaction between glucose and proteins) are implicated in the complications of ageing and diabetes. The formation of AGEs on vascular wall collagen causes cross-linking of collagen molecules to each other. This leads to the loss of collagen elasticity, and subsequently a reduction in arterial compliance. This causes hypertension and might be contributing to increased cardiovascular events (Aronson, 2003; Aronson, 2004). Booth et al showed that diabetes confers an equivalent risk to ageing by 15 years. Elderly people with diabetes, on average seemed to be at high risk of CVD compared to young diabetics or elderly non-diabetics (Booth *et al.,* 2006).

1.2 Pathophysiology of coronary atherosclerosis

Pathophysiological mechanisms contributing to atherosclerosis are complex and inter linked. The 20th century has witnessed a remarkable evolution in the concepts concerning the pathogenesis of atherosclerosis. In the mid-19th century Rudolf Virchow viewed atherosclerosis as a proliferative disease whilst Karl Rokitansky believed that atheroma was derived from healing and resorption of thrombi (Mayerl *et al.*, 2006). Experiments in the early 20th century identified cholesterol as the main culprit (Steinberg). In the mid-20th century characterisation of human lipoprotein particles promoted the insudation of lipids as a cause of atherosclerosis. In late 20th century, studies have revealed significant involvement of inflammation and genetic propensity in various stages of atherosclerosis. We now recognise that elements of all these mechanisms contribute to atherogenesis with inflammation occupying a significant role in the initiation and progression of atherosclerotic lesions.

Atherosclerosis is a systemic dysfunctional endothelial, focal occurring or diffuse, chronic inflammatory, fibro-proliferative, prothrombotic, angiogenic, multifactorial disease of the arterial wall caused by the retention of modified low-density lipoproteins and hemodynamic and reductive-oxidative stress (Hayden and Tyagi, 2004).

Atherosclerosis was viewed as a localised narrowing of arteries secondary to cholesterol and smooth muscle cell deposits but this view has change in the last decade and atherosclerosis is now considered as a widespread vascular inflammatory process with varying clinical manifestations (Libby *et al.*, 2002). Atheroma or an atheromatous plaque is a focal atherosclerotic lesion in a coronary artery which is in a state of constant flux. It responds to changes mainly mediated by inflammation, immune, vasomotor and lipid environment in the intra and extra plaque milieu.

1.2.1 Coronary artery anatomy

An appreciation of the ultrastructure of coronary arteries is crucial in understanding the complex pathophysiology of atherothrombosis. Knowledge of coronary artery anatomy and pathogenesis of atherosclerosis has significantly evolved since the days of Rudolf Virchow.

The normal coronary artery has a well-developed trilaminar structure:

1. Tunica intima – innermost monolayer of endothelial cells abutting directly on a basal lamina containing non-fibrillar collagen types such as type IV collagen, laminin, fibronectin and other extra cellular matrix molecules. The internal elastic lamina bounds intima abluminally and serves as the border between the intimal layer and the underlying tunica media.

2. Tunica media – lies under the intimal layer and internal elastic lamina. This layer is made up of layers of SMCs interleaved with layers of elastin-rich extracellular matrix. In a normal artery the extracellular matrix neither accumulates nor atrophies due to a fine balance between the rate of matrix synthesis and dissolution. The external elastic lamina bounds the media abluminally, forming the border with the adventitial layer.

3. Tunica adventitia – outer layer consisting of collagen fibrils and other cells such as fibroblasts and mast cells. This layer also contains the neurovascular bundle with the vasa vasorum. Emerging evidence suggests a role of mast cells in atheroma in animal models, but their importance in humans remains speculative (Libby and Shi, 2007).

In general the endothelial monolayer forms a continuous sheet as the innermost layer of the arterial lumen, remains non thrombogenic and prevents the exposure of highly

thrombogenic collagen in tunica media from being exposed to flowing blood (Fuster *et al.*, 1992).

With ageing, human arteries develop a more complex intima, containing arterial smooth muscle cells (SMCs) and fibrillary forms of interstitial collagen (type I and III). This is termed as diffuse intimal thickening and characterizes most of the adult arteries. This does not necessarily go hand in hand with lipid accumulation and may occur in individuals without substantial atheroma burden.

1.2.2 Laminar flow and protection against atherosclerosis

Atheromatous changes in the coronary arteries are either focal or diffuse. Laminar blood flow, has been found to elicit various antiatherogenic homeostatic mechanisms (atheroprotective functions) and anti-inflammatory functions such that under usual conditions of laminar shear stress in normal coronary arteries, the endothelium is atheroprotective. Early atheromatous plaques in proximal segment of the coronary arteries at bifurcations or branch points, progresses to significant atherosclerotic plaque (Nigro *et al.*, 2011). This results in rapid changes to shear stress on the vessel wall and the plaque resulting in non-laminar flow. This triggers endothelial damage and dysfunction, mediated via the nuclear factor kappa B (NFKB) pathway (Tegos *et al.*, 2001; Cunningham and Gotlieb, 2005; Chiu and Chien, 2011).

1.2.3 Initiation of plaque formation – endothelial dysfunction

The initial steps in atherogenesis remains inconclusive. However, observations from tissues obtained from young humans and from experimental studies in animals points towards structural and functional changes in the endothelial layer as a marker of initiation of atherosclerosis.

All infants have focal thickening of the coronary artery intima due to vascular smooth muscle cell (VSMC) proliferation (Milei *et al.*, 2008). Although focal thickening is an important hallmark of the developing atherosclerotic plaque, this is considered to be an adaptive response to turbulent blood flow rather than to be pathological.

1.2.4 Fatty streak

Endothelial dysfunction initiated by risk factors permits the entry of lipids (low density lipoprotein - LDL) and inflammatory cells into the artery wall. LDL particles accumulate

over a period of time and undergo oxidative modification in the presence of free radicals. This oxidised and glycated LDL is more atherogenic and immunogenic (Tegos *et al.*, 2001; Kruth, 2002). Leucocyte recruitment and accumulation also occurs early in lesion generation (Muller, 2009). Normal endothelial lining generally resists adhesive interactions with leukocytes. However, at sites of endothelial dysfunction, the damaged endothelial cell responds by expressing adhesion molecules for leucocytes such as vascular cell adhesion molecule-1 (VCAM-1) and P-selectin as a repair mechanism.

Upon continuous insult by endogenous (high cholesterol) and exogenous (smoking) factors, these adhesion molecules are over expressed in coronary endothelium allowing the circulating leucocytes to adhere to the endothelium. They then move between the endothelial cell junctions or penetrate through endothelium (transcytosis) to enter the intima (Linton and Fazio, 2003). Once in the artery, monocytes differentiate into macrophages which take up the lipid and become foam cell macrophages. This results in the formation of lesions termed “fatty streaks”. These are small, slightly raised lesions caused by focal collections of foam cell macrophages in the intima. They may progress to larger atherosclerotic plaques or may regress. Chemoattractant cytokines or chemokines especially monocyte chemoattractant protein 1 (MCP-1) and interleukin-8 (IL-8) are involved in the process of leucocyte migration (Libby *et al.*, 2002; Gleissner *et al.*, 2008).

Fatty streak progresses to a more complex lesion due to the formation of a necrotic core and a fibrous cap. Foam cell macrophages, engorged with LDL, begin to die and their contents contributes to the formation of a necrotic core. The release of the cytoplasmic contents of the foam cells leads to the accumulation of extracellular lipids, cytokines, leukotrienes, prostanoids, histamine like substances and growth factors, all of which induce inflammation. These also impair nitric oxide synthesis which in turn reduces the functional and regenerating capacity of the endothelium (Stary *et al.*, 1995; Virmani *et al.*, 2000).

The occurrence of VSMC migration and proliferation results in the formation of a fibrous cap. VSMCs migrate into the intima where they proliferate and deposit extracellular matrix. The increase in cell number and presence of matrix causes augmentation of the bulk of the plaque, which now protrudes into the lumen. This is termed a stable advanced plaque (George and Dwivedi, 2004; Newby, 2006). The size and composition of the plaque determine its outcome.

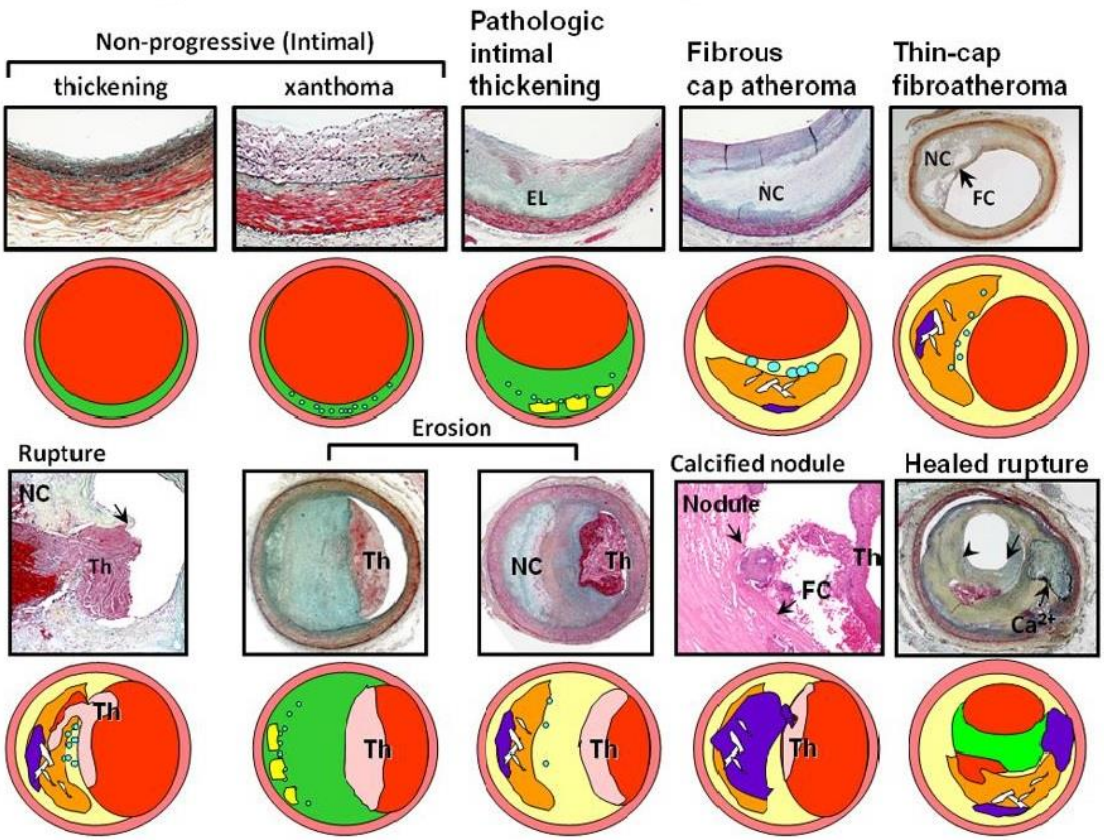

Figure 1.1 Progression of coronary atherosclerosis

Spectrum of representative coronary lesion morphologies seen in a sudden death population, forming the basis for the modified American Heart Association (AHA) descriptive classification. The 2 non-progressive lesions are intimal thickening and intimal xanthomas (foam cell collections known as fatty streaks, AHA type II). Pathological intimal thickening (PIT) (AHA type III transitional lesions) marks the first of the progressive plaques, as they are the assumed precursors to more advanced fibroatheroma (FA). Thin-cap fibroatheromas are considered precursors to plaque rupture. Essentially missing from the AHA consensus classification are alternative entities that give rise to coronary thrombosis, namely erosion and the calcified nodule. Erosions can occur on a substrate of PIT or FA, whereas calcified nodules depict eruptive fragments of calcium that protrude into the lumen, causing a thrombotic event. Luminal thrombi overlie areas lacking surface endothelium and so are in communication with the lipid-rich necrotic core. Lastly, healed plaque ruptures are lesions with generally smaller necrotic cores and focal areas of calcification where the surface generally shows areas of healing rich in proteoglycans. Multiple healed plaque ruptures are thought responsible for progressive luminal narrowing. Ca2+ = calcium; EL = extracellular lipid; FC = fibrous cap; NC = necrotic core; Th = luminal thrombus. From (Virmani *et al.*, 2000) Arterioscler Thromb Vasc Biol. 2000; 20(5):1262-75.

1.2.5 Progression and evolution of atheromatous lesion

Innate and adaptive immunity: Mechanisms of inflammation in atherogenesis

During the last decade, the role of inflammation in atherogenesis has been well established (Libby *et al.*, 2002; Hartvigsen *et al.*, 2009). The macrophage foam cells serve as the rich source of proinflammatory mediators including proteins such as cytokines, chemokines, various eicosanoids and lipids such as platelet activating factor. These can also elaborate large quantities of oxidant species such as superoxide anion, in the milieu of the atherosclerotic plaque. This ensemble of inflammatory mediators can promote inflammation in the plaque and contribute to the progression of lesions. The term innate immunity describes this type of amplification of the inflammatory response that does not depend on antigenic stimulation.

In addition to innate immunity, antigen-specific or adaptive immunity in plaque progression has also been identified (Andersson *et al.*, 2010). Macrophages and dendritic cells in the atherosclerotic lesions can present antigens to the T cells. These antigens include modified lipoproteins, heat shock proteins, beta2-glycoprotein 1b and infectious agents (Tsimikas *et al.*, 2005; Chou *et al.*, 2008). The antigen presenting cells allow the antigen to interact with T cells in a manner that triggers their activation. These activated T cells then can secrete copious quantities of cytokines that can modulate atherogenesis.

The helper T cells (bearing CD4) fall in to two general categories:

i. T helper 1 (Th1) subtype secretes proinflammatory cytokines such as interferon-y (IFN-y), lymphotoxin, CD40 ligand and tumour necrosis factor-α (TNF-α) which in turn activates vascular wall cells leading to plaque destabilization and heightened thrombogenicity.

ii. T helper 2 (Th2) subtype secretes cytokines such as interleukin-10 which can inhibit inflammation in the context of atherogenesis (Ait-Oufella *et al.*, 2009).

The cytotoxic T cells (bearing CD8) can express Fas ligand and other cytotoxic factors that can promote cytolysis and apoptosis of target cells, including smooth muscle cells (SMCs), endothelial cells and macrophages. The death of these cell types can occur in the atherosclerotic lesion and may contribute to plaque progression and complication.

The regulatory T cells (Treg – bearing CD4 and CD25) can elaborate transforming growth factor-β (TGF-β) and interlukin-10. These cytokines can exert anti-inflammatory effects. Several experimental studies have suggested an antiatherosclerotic function of Treg cells in vivo (Taleb *et al.*, 2008; Andersson *et al.*, 2010).

Smooth muscle cell migration and proliferation

Evolution of atheroma into more complex plaques involves SMCs in addition to endothelial dysfunction and leucocyte recruitment (Manabe and Nagai, 2003; Mulvihill *et al.*, 2004). Intimal SMCs in atheroma contain rougher endoplasmic reticulum and fewer contractile fibres than do the normal medial SMCs. SMCs are recruited from the underlying tunica media into the intimal layer in advanced atheroma. This recruitment is facilitated by chemoattractants such as platelet-derived growth factor (PDGF) secreted by activated macrophages. Although replication of SMCs in the steady state appears infrequent in mature atheroma, bursts of SMC replication may occur during the life history of a given atheromatous lesion. These can occur during episodes of plaque disruption with thrombosis, when the intimal SMCs are exposed to mitogens including the coagulation factor thrombin. Thus accumulation of SMCs and their growth in the intima does not occur in a continuous linear fashion but bursts of SMC replication or migration may occur.

Smooth muscle cell death during atherogenesis

In addition to replication, death of these cells also participate in complication of the atherosclerotic plaque (Clarke *et al.*, 2006). Some SMCs in advanced atheroma exhibit fragmentation of their nuclear DNA that is characteristic of programmed cell death or apoptosis. Soluble proinflammatory cytokines act in conjunction with Fas ligand expressing T cells in the atheromatous lesion and this leads to SMC apoptosis (Kavurma *et al.*, 2008). Thus, SMC accumulation in a progressing atheromatous plaque probably results from a tug-of-war between cell replication and cell death (Geng and Libby, 2002). Contemporary cell and molecular biologic research has identified candidates that mediate replication and attrition of SMCs.

Arterial extracellular matrix

Extracellular matrix makes up much of the volume of an advanced atherosclerotic plaque. These molecules include interstitial collagens (types I and III) and proteoglycans. SMCs produce these matrix molecules and the main stimuli include

PDGF and TGF-β. Elastic fibers may also accumulate in atherosclerotic plaques. Similar to accumulation of SMCs, extracellular matrix is also in a state of constant flux. The biosynthesis of matrix molecule is counterpoised by catabolic enzymes known as matrix metalloproteinases (MMPs). Dissolution of extracellular matrix molecules undoubtedly plays a crucial role in the migration of SMCs into the intima from media traversing the elastin-rich internal elastic lamina (Dollery and Libby, 2006).

Extracellular matrix breakdown also is likely to play a role in arterial remodelling that accompanies atheroma growth. During the early life of an atheromatous lesion, plaques grow outwardly, in an abluminal direction, rather than inwardly. Luminal stenosis starts to occur only after the plaque burden exceeds around 40% of the cross-sectional area of the artery.

Angiogenesis in plaques

Atherosclerotic plaques develop their own microvasculature as they grow because of endothelial migration and replication. These microvessels probably form in response to angiogenic peptides overexpressed in the atheroma. These factors include vascular endothelial growth factor (VEGF), placental growth factor (PGF) and oncostatin M. As a consequence of the presence of microvessels, there is increased trafficking of leucocytes, prominence of adhesion molecules like VCAM-1 and increased growth of plaque overcoming diffusion limitations on oxygen and nutritional supply (Dollery and Libby, 2006). The plaque microvessels may be friable and prone to rupture. Haemorrhage and thrombosis in situ could promote local SMC proliferation and matrix accumulation in the area adjacent to the microvascular disruption. This in turn helps progression of atheromatous plaque.

Plaque mineralisation

Plaques develop areas of calcification as they evolve. Some subpopulation of SMCs may foster calcification by enhanced secretion of cytokines such as bone morphogenic proteins (BMP), homologues of TGF-β. Receptor activation of NF-kB ligand (RANKL), a member of TNF-α family, appears to promote SMC mineral formation through the BMP4 dependent pathway. Osteoprotegerin can inhibit plaque mineralisation by inhibiting RANKL signalling. Genetic absence of osteoprotegerin augments calcification of mouse atheromas and administration of exogenous osteoprotegerin limits it (Bennett *et al.*, 2006; Morony *et al.*, 2008). Transcription factor Runx-2,

activated by inflammatory mediators and oxidative stress also promote SMC mineral formation (Aikawa *et al.*, 2007; Byon *et al.*, 2008).

1.2.6 Plaque vulnerability and rupture

Atherosclerotic plaque morphology has been classified into 7 types based on lipid core, ratio of fibrous tissue to lipid core, fibrous tissue lining of the plaque and the presence of thrombus. This pathological classification is based on the vulnerability and rupture of the plaque (Farb *et al.*, 1996; Virmani *et al.*, 2000). This final common end point of an atheromatous plaque is plaque rupture and thrombosis (Arai *et al.*, 2010).

Fracture of the fibrous cap, accounts for two thirds of acute myocardial infarctions. Another mechanism involves superficial erosion of intima. The rupture of the fibrous cap reflects an imbalance between the forces that impinge on the cap and the mechanical strength of the fibrous cap. Interstitial collagen provide most of the biomechanical resistance to disruption of the fibrous cap and hence collagen metabolism is thought to participate in regulating the propensity of plaque rupture (Libby, 2008). T cell derived cytokine INF-y potently inhibits SMC collagen synthesis, thereby impairing the ability of the plaque to repair and maintain the fibrous cap. On the other hand, certain mediators released from platelet granules during activation, including TGF-β and PDGF, increase collagen synthesis and reinforce the plaque's fibrous structure.

Increased catabolism of extracellular matrix macromolecules that compose the fibrous cap can also contribute to weakening of this structure, rendering it susceptible to rupture and hence thrombosis. Macrophages in advanced atheroma overexpress MMPs and elastolytic cathepsins that can breakdown the collagen and elastin of the arterial extracellular matrix (Dollery and Libby, 2006; Libby, 2008). The strength of the fibrous cap undergoes dynamic regulation, linking the inflammatory response in the intima with the molecular determinants of plaque stability and hence thrombotic complications of atheroma.

Another feature of vulnerable plaque is a relative lack of SMCs. As describe earlier, inflammatory mediators, can produce apoptosis of SMCs. Loss of SMCs from regions of local inflammation within plaques probably explains the relative lack of SMCs at places where plaque ruptures. Due to lack of SMCs there is reduced collagen production that is needed to repair and maintain the matrix of the fibrous cap. This

contributes to the weakening of the fibrous cap and increases the propensity for plaque rupture (Clarke *et al.*, 2008).

A prominent accumulation of macrophages and a large lipid milieu is a third micro-anatomical feature of the so-called vulnerable atherosclerotic plaque. The activated macrophages produce cytokines and matrix degrading enzymes thought to regulate matrix catabolism and SMC apoptosis. These macrophages and SMCs can generate particulate tissue factor, a potential instigator for microvascular thrombosis after plaque disruption.

The success of lipid lowering therapy in reducing the incidences of acute myocardial infarction or unstable angina in patients at risk may result from a reduced accumulation of lipid and decrease in inflammation and plaque thrombogenicity. Plaque rupture exposes thrombogenic lipids and collagen to flowing blood and can result in either occlusive or non-occlusive thrombus leading to myocardial necrosis (Lafont, 2003).

The pathobiology of superficial erosion is much less well understood. Most of the superficial erosions do not result in clinically apparent myocardial infarction but are responsible for bursts in growth of atheromatous plaques (Fuster *et al.*, 2005; Virmani *et al.*, 2006). In humans, superficial erosion appears more likely to cause fatal acute myocardial infarction in women and in individuals with hypertriglyceridemia and diabetes mellitus but the underlying molecular mechanisms remain obscure. Apoptosis of endothelial cells contributing to desquamation of endothelial cells and the role of MMPs in degrading the nonfibrillar collagen in the basement membrane have been postulated for superficial erosion (Kramer *et al.*, 2010).

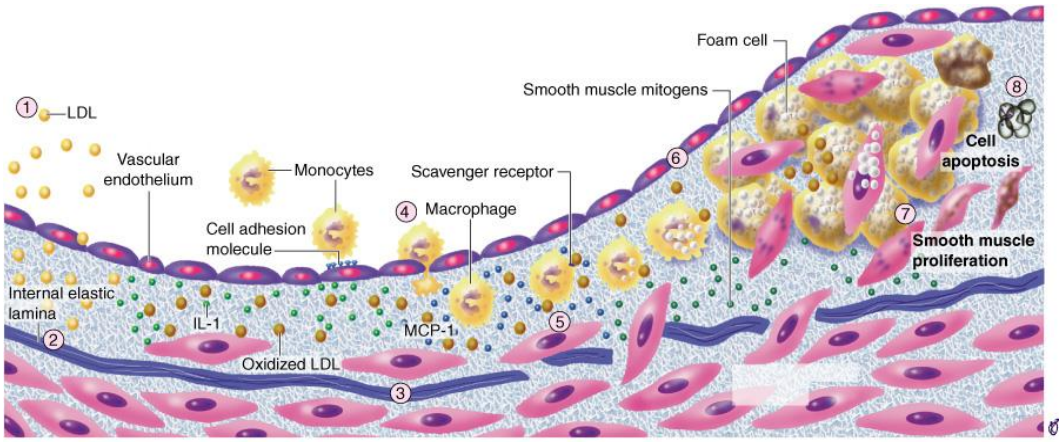

Figure 1.2 Schematic of the evolution of the atherosclerotic plaque.

1: Accumulation of lipoprotein particles in the intima. The modification of these lipoproteins is depicted by the darker colour. Modifications include oxidation and glycation.

2: Oxidative stress, including products found in modified lipoproteins, can induce local cytokine elaboration.

3: The cytokines thus induced increase expression of adhesion molecules for leukocytes that cause their attachment and of chemoattractant molecules that direct their migration into the intima.

4: Blood monocytes, upon entering the artery wall in response to chemoattractant cytokines such as MCP-1, encounter stimuli such as macrophage colony stimulating factor (M-CSF) that can augment their expression of scavenger receptors.

5: Scavenger receptors mediate the uptake of modified lipoprotein particles and promote the development of foam cells. Macrophage foam cells are a source of mediators such as further cytokines and effector molecules such as hypochlorous acid, superoxide anion (O2-), and matrix metalloproteinases.

6: Smooth muscle cells in the intima divide other smooth muscle cells that migrate into the intima from the media.

7: Smooth muscle cells can then divide and elaborate extracellular matrix, promoting extracellular matrix accumulation in the growing atherosclerotic plaque. In this manner, the fatty streak can evolve into a fibrofatty lesion.

8: In later stages, calcification can occur (not depicted) and fibrosis continues, sometimes accompanied by smooth muscle cell death (including programmed cell death, or apoptosis) yielding a relatively acellular fibrous capsule surrounding a lipid-rich core that may also contain dying or dead cells and their detritus.

Abbreviation: IL-1 = interleukin-1; LDL = low-density lipoprotein. Adapted from (Libby *et al.*, 2010).

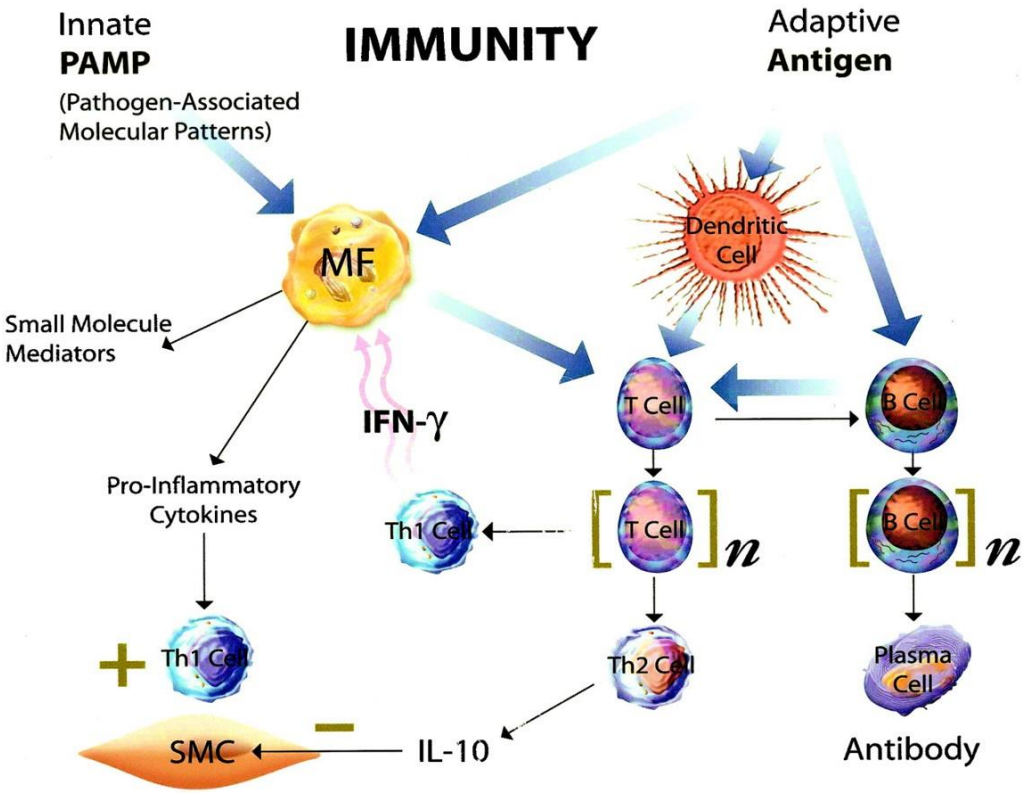

Figure 1.3 Interplay between adaptive and innate immunity during atherogenesis.

The principal effector cell of innate immunity, the macrophage (MF), secretes cytokines that critically regulate many functions of atheroma-associated cells involved with disease initiation, progression, and complication, as well as thrombosis. IFN-γ, a product of the activated T cell, activates a number of these functions of the macrophage. In turn, the activated macrophage expresses high levels of MHC class II antigens, needed for antigen-dependent activation of T cells.

Abbreviation: IFN-γ = interferon- γ; MF = macrophages; Th = T helper cell

From (Hansson *et al.*, 2002)

1.3 Blood Haemostasis and thrombus formation

Thrombus formation is the most effective protective mechanism by which haemostasis is maintained in a closed, high pressure circulatory system after vessel wall damage. Haemostasis is nature's first and most efficient response to control bleeding and preserve the integrity of the circulatory system. This process remains inactive but readily gets activated following vascular injury to minimise extravasation of blood from the vasculature. Following this activation, it is critical to contain this thrombus formation so that it is localised to the site of injury and to modulate thrombus size to be appropriate to the injury. Thus, there exists a very fine balance between pathways that initiate thrombus formation and that regulate / modulate thrombus formation (Furie, 2009).

1.3.1 Mechanisms of thrombosis

At sites of vascular injury, platelets rapidly adhere to the exposed sub-endothelial extracellular matrix, gets activated and together with the coagulation system form a thrombotic plug that seals the lesion (Furie and Furie, 2008). A highly specified and efficient haemostatic system consisting of cellular (platelets) and non-cellular (coagulation factors) components is responsible for three main steps in thrombus formation as listed below:

i. Initiation of thrombosis

ii. Propagation of thrombus

iii. Autolysis of thrombus

Initiation of thrombosis

The vessel wall with intact endothelium is crucial to the maintenance of a patent vasculature. The endothelium contains three thromboregulators — nitric oxide, prostacyclin and the ectonucleotidase CD39 — which together provide a defence against thrombus formation. Collagen and tissue factor facilitate the maintenance of a closed circulatory system. When the vessel wall is injured or the endothelium is disrupted, collagen and tissue factor gets exposed to the flowing blood, thereby initiating thrombus formation.

Two distinct pathways act in parallel to activate platelets. In one, exposed collagen triggers the accumulation and activation of platelets, whereas in the other exposed tissue factor initiates thrombin generation, which not only converts fibrinogen to fibrin but also activates platelets.

The interactions of platelet glycoprotein VIb with the collagen of the exposed vessel wall and of platelet glycoprotein Ib-V-IX with collagen-bound von Willebrand factor (vWF) result in adhesion of platelets to the site of injury (Ruggeri, 2000). In addition to its role in the adherence of platelets to collagen, glycoprotein VIb is the major agonist for initial platelet activation and granule release. The platelet integrin $\alpha_2\beta_1$ plays a supportive but not essential role in the interaction between platelets and collagen (Nieswandt *et al.*, 2001).

Tissue factor (TF) triggers a second independent pathway that initiates platelet activation. Tissue factor forms a complex with coagulation factor VIIa, the enzymatically active form of factor VII, and this tissue factor–factor VIIa complex activates coagulation factor IX, thereby initiating a proteolytic cascade that generates thrombin (Steffel *et al.*, 2006). Thrombin cleaves protease-activated receptor 1 (PAR 1) on the platelet surface, thereby activating platelets and causing them to release adenosine diphosphate (ADP), serotonin, and thromboxane A_2 (Vu *et al.*, 1991). In turn, these agonists activate other platelets, and in doing so, amplify the signals for thrombus formation.

Von Willebrand factor synthesised by endothelial cells and megakaryocytes, promotes platelet adhesion to subendothelium. Elevated levels of vWF indicates endothelial damage and correlates with cardiovascular risk factors including T2DM and dyslipidaemia (Kessler *et al.*, 1998). vWF plays a crucial role in thrombosis by binding to fibrillar and non-fibrillar collagen, heparin and sulfatide in the sub endothelium. vWF interacts with platelet GPIb receptor and initiates platelet adhesion. It also triggers the expression of GPIIb/IIIa and binds to it, thereby promoting irreversible adhesion and aggregation of platelets (Kessler *et al.*, 1998; Reininger *et al.*, 2006)

Propagation of thrombus formation

A developing thrombus recruits unstimulated platelets and within the thrombus, activation occurs only in a subgroup of the recruited platelets (Dubois *et al.*, 2007). Other platelets remain loosely associated with the thrombus but do not undergo

activation and may ultimately disengage from the thrombus. In short, thrombus formation is a dynamic process in which some platelets adhere to and others separate from the developing thrombus, and in which shear, flow, turbulence, and the number of platelets in the circulation greatly influence the architecture of the clot.

Following adhesion and platelet activation, cyclooxygenase-1 (COX-1)-dependent synthesis and release of thromboxane A_2 (TXA_2) and release of ADP from storage granules occur. TXA_2 is a potent vasoconstrictor and it locally activates platelets and recruits them to the site of injury. This results in expansion of the platelet plug. To activate platelets, TXA_2 and ADP binds to their respective receptors on the platelet membrane. The role of ADP receptors in platelet function and the pharmacology of drugs directed against these receptors are described in detail later. Activated platelets promote assembly of coagulation factor complexes. These complexes trigger a burst of thrombin generation and subsequent fibrin formation. In addition to converting fibrinogen to fibrin, thrombin amplifies platelet activation further and helps anchor the platelet aggregates at the site of injury.

This is followed by platelet aggregation, the final step in the formation of the platelet plug. Platelet aggregation links platelets to each other to form clumps. Glycoprotein IIb/IIIa (GP IIb/IIIa) mediates these platelet to platelet linkages. On nonactivated platelets, GP IIb/IIIa exhibits minimal affinity for its ligands (Lefkovits and Topol, 1995). On platelet activation it undergoes a conformational transformation, which reflects transmission of signals from its cytoplasmic domain to its extracellular domain. This transformation enhances the affinity of GP IIb/IIIa for its ligands, fibrinogen under low shear and vWF under high shear conditions (Italiano *et al.*, 2008). Fibrinogen and vWF serve as bridges and bind adjacent platelets together. Fibrin, the final end product of the coagulation cascade, tethers the platelet aggregates together and anchors them to the site of injury. The viscoelastic property of thrombus is derived from fibrin to fibrin cross linkage and overlapping of longitudinal fibrin fibres. By forming a mesh-like structure due to crosslinking of individual fibres, cellular elements in the blood are trapped in thrombus resulting in cellular thrombus (Collet *et al.*, 2003; Weisel, 2005). Circulating monocytes are attracted towards this organising thrombus. Leukocyte-platelet adhesion is mediated by inflammatory cytokines, microparticles and P-selectin from activated platelets (Zarbock *et al.*, 2007).

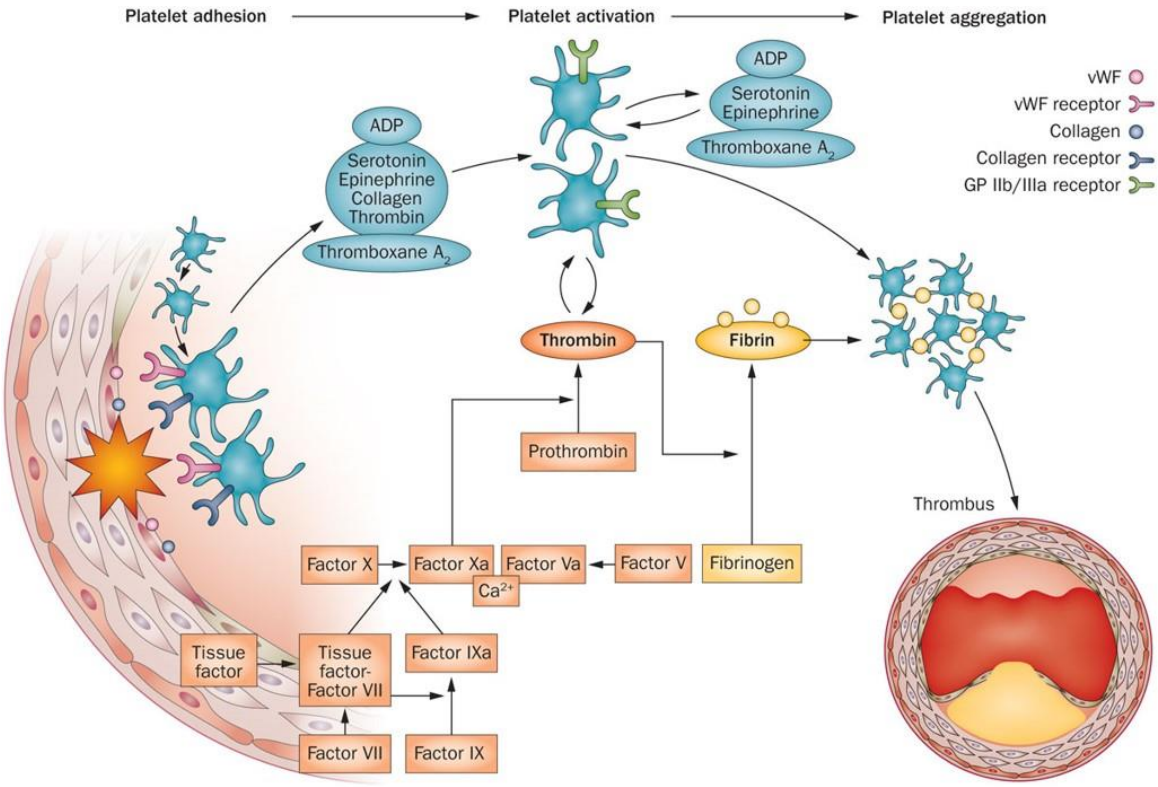

Figure 1.4 Mechanism of thrombus formation.

Plaque rupture exposes subendothelial components. Platelet adhesion is mediated by interactions between vWF and GP Ib/V/IX receptor complexes located on the platelet surface, and between platelet collagen receptors (GP VI and GP Ia) and collagen exposed at the site of vascular injury. Binding of collagen to GP VI induces the release of activating factors (ADP, thromboxane A2, serotonin, epinephrine, and thrombin), which promote interactions between adherent platelets, as well as further recruitment and activation of circulating platelets. Platelet activation leads to changes in platelet shape, expression of proinflammatory molecules, platelet procoagulant activity, and activation of platelet integrin GP IIb/IIIa. Activated GP IIb/IIIa binds to the extracellular ligands fibrinogen and vWF, leading to platelet aggregation and thrombus formation. Vascular injury also exposes subendothelial tissue factor, which forms a complex with factor VIIa and sets off a chain of events that results in formation of the prothrombinase complex. Prothrombin is converted to thrombin, which subsequently converts fibrinogen to fibrin, generating a fibrin-rich clot. Abbreviations: GP, glycoprotein; vWF, von Willebrand factor. From (Franchi and Angiolillo, 2015)

Autolysis of formed thrombus

Autolysis is a natural mechanism by which haemostasis is regulated and maintained by removal of excess thrombus formation. An early but weaker thrombus retraction mediated by platelets is followed by a stronger fibrinolysis mediated by fibrinolytic enzymes. Thrombus retraction involves rearrangement of internal actin cytoskeletons in the platelet cell wall resulting in platelet contraction (Carroll *et al.*, 1981).

Thrombus retraction is followed by fibrinolysis where plasminogen activators convert plasminogen to plasmin, which then degrades insoluble fibrin into soluble fibrin degradation products. Two immunologically and functionally distinct plasminogen activators, namely endothelial tissue plasminogen activator (t-PA) and hepatic urokinase-type plasminogen activator (u-PA) (Cesarman-Maus and Hajjar, 2005) are present in the circulation. Tissue plasminogen activator mediates intravascular fibrin degradation and u-PA mediates both proteolysis and tissue remodelling and repair (Medcalf, 2007).

Regulation of fibrinolysis occurs at two levels. Plasminogen activator inhibitor-1 (PAI-1) and to a lesser extent PAI-2, inhibits plasminogen activator whereas α_2-antiplasmin inhibits plasmin. Endothelial cells synthesise PAI-1 which inhibits both t-PA and u-PA. Monocytes and placental cells synthesise PAI-2 which specifically inhibits u-PA. A dynamic equilibrium is maintained between t-PA and PAI-1 on the luminal surfaces of the vessels to determine net local fibrinolytic activity. Either increased expression of PAI-1 or decreased expression of t-PA or both can lead to decreased fibrinolytic activity predisposing to thrombosis (Vaughan, 2005). The other fibrinolysis inhibitors include α_2-antiplasmin, α_2-macroglobulin and thrombin-activated fibrinolysis inhibitor (TAFI).

Macrophages are also involved in thrombus resolution. Following completion of fibrinolytic process, platelets and other cellular elements of thrombus are resorbed by macrophages. Some of these cellular components undergo calcification resulting in giant cell formation. Macrophage induced calcification is the hallmark of old and recurrent thrombus (Fuster *et al.*, 2005; Medcalf, 2007).

1.3.2 Platelets in thrombogenesis

Platelets are anucleate particles released into the circulation after fragmentation of bone marrow megakaryocytes. Once they enter the circulation, they have a life span of 7 to 10 days (Kaushansky, 2005). Platelets are involved in haemostasis, wound

healing and inflammation. They contribute to haemostasis by activation, adhesion and aggregation. Under physiological conditions, platelets circulate in a quiescent state, protected from untimely activation by inhibitory mediators released from intact endothelial cells, mainly NO and prostaglandin I_2 (PGI_2, prostacyclin) which increase the platelet content of cyclic AMP (cAMP) and cyclic GMP (cGMP) respectively. Endothelial dysfunction and changes in release of antiplatelet factors may lead to increased platelet activation followed by their interaction with neutrophils and monocytes, and increased platelet adhesion and aggregation (Frenette *et al.*, 1995; Frenette *et al.*, 1998).

Upon activation by thrombin, ADP or TXA2, platelets undergo shape change, and secrete the contents of α- and dense granules (Holmsen, 1994). Rearrangement of cytoskeletal proteins, including the disassembly of a microtubule ring, occurs as one of the very first steps and results in a shape change from a disc shaped cell into an intermediate spherical shape cell. This is followed by actin polymerization and extension of pseudopodia (Bearer, 1995).

Platelet secretion

Activated platelets secrete a number of potent inflammatory and mitogenic substances into the local microenvironment. These mediators modulate functions of other platelets, leukocytes, and endothelial cells. Platelets secrete:

i. chemokines (CCL3, 5, 7, 17, CXCL1, 4, 5, 7, and 8)

ii. cytokines (e.g., IL-1b, CD40 ligand, b-thromboglobulin)

iii. growth factors (e.g., PDGF, TGF-b, EGF, VEGF, bFGF),

iv. coagulation factors (e.g., factor V, factor XI, PAI-1, plasminogen, protein S).

These factors participate in cell survival, proliferation, coagulation and fibrinolysis, chemotaxis, and cell adhesion.

Platelet chemokines play a key role in the activation of different cell types and can induce adhesion by activating integrins (Carveth *et al.*, 1989; Detmers *et al.*, 1990). Most of the platelet chemokines are stored in α-granules and can be released upon platelet activation. CXCL4 (platelet factor 4) is a chemokine that is constitutively and abundantly expressed in platelets.

The platelet cytokine CD40 ligand (CD40L), a transmembrane protein, was originally described on stimulated CD4+ T cells and also found on stimulated mast cells as well as basophils (Vishnevetsky *et al.*, 2004). Preformed CD40L is stored in platelets and rapidly translocated to the cell surface upon activation. CD40L, surface-expressed or secreted by platelets, can bind to endothelial CD40 and induces chemokine secretion and up-regulation of adhesion molecules. This process leads to recruitment to and extravasation of leukocytes at the site of injury and thereby immediately links haemostasis to the inflammatory system.

Activated platelets secrete IL-1β, a major activator of endothelial cells (Hawrylowicz *et al.*, 1991). Interaction of activated platelets with endothelial cells induces an IL-1β dependent secretion of IL-6, CXCL8, CCL2 (monocyte chemoattractant protein-1) from endothelial cells. In addition to the induction and release of these inflammatory mediators, IL-1β induces increased expression of adhesion molecules, such as E-selectin, VCAM-1, ICAM-1, alpha2b/beta3 integrin and others (Kaplanski *et al.*, 1994; Gawaz *et al.*, 2000). Two other important platelet agonists released upon activation are ADP and serotonin. ADP acts through P2Y1 receptor and produces calcium mobilisation, shape changes and initial transient activation. It also interacts with the P2Y12 receptor, which mediates potentiation of platelet secretion and irreversible aggregation. Serotonin is an agonist of the $G\alpha_q$-coupled 5HT2A-receptor and amplifies the platelet response (Gachet, 2001a).

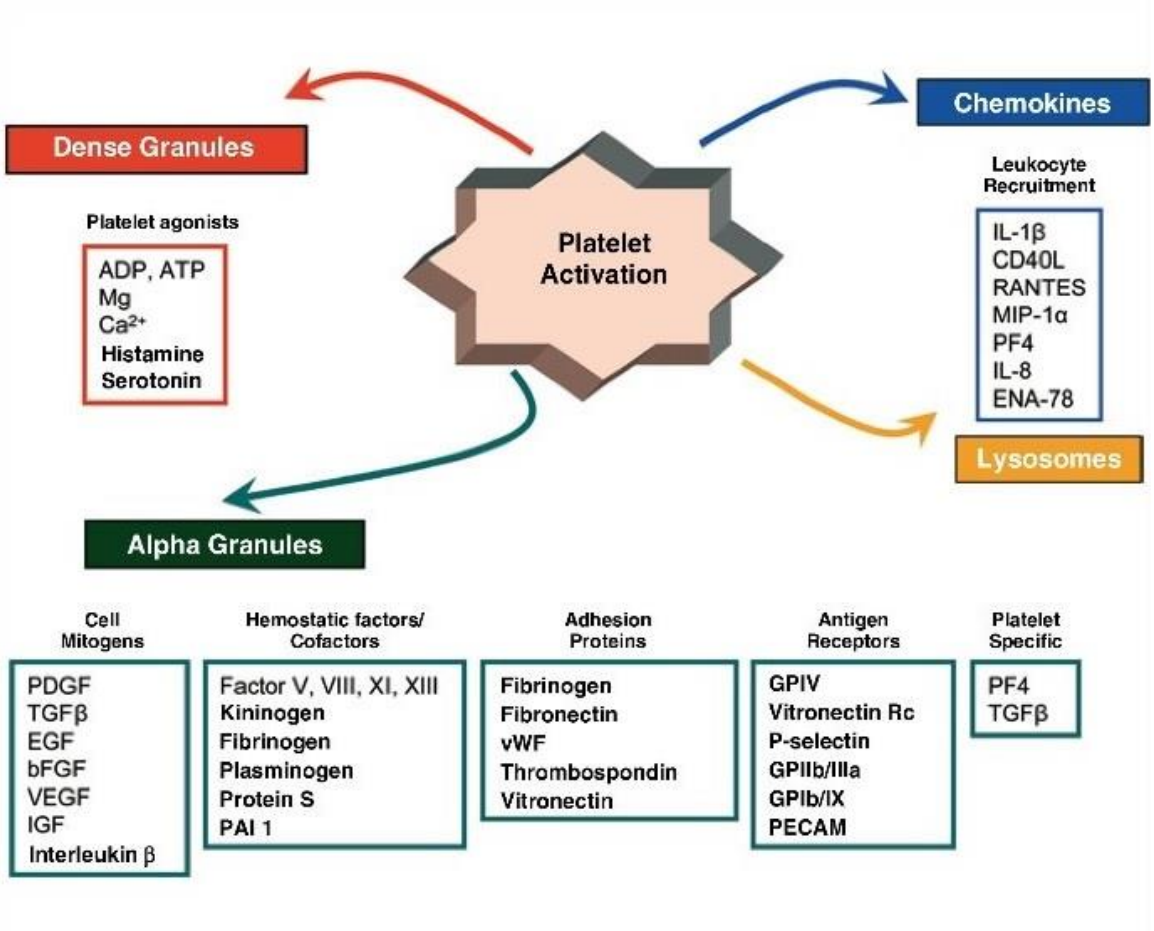

Figure 1.5 Platelet components implicated in coagulation and atherosclerotic process.

ADP, adenosine diphosphate; bFGF, fibroblast growth factor; EGF, endothelial growth factor; ENA, neutrophil-derived peptide activator of epithelial cells; GP, glycoproteins; IGF, insulin-like growth factor; IL, interleukin; MIP-1, macrophage inflammation protein 1; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; PECAM, platelet and endothelial cell adhesion molecule; PF4, platelet factor 4; TGFb, transforming growth factor b; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor. From (Badimon *et al.*, 2009)

1.3.3 Platelet-neutrophil interaction

Polymorphonuclear leukocytes (PMN) play an important role in host defence and in the pathogenesis of various diseases. The recruitment of PMN into inflammatory tissue follows a distinct recruitment pattern. During these different recruitment steps, PMN become activated and subsequently release mediators into the surrounding tissue (Zarbock *et al.*, 2007). In many experimental animal models, blockade of PMN recruitment or PMN depletion leads to attenuation of organ damage. In addition to observations in animal models, clinical studies show a positive correlation between the number of PMNs and the risk of acute myocardial infarction as well as recurrence (Ernst and Matrai, 1987; Danesh *et al.*, 1998).

In addition to classical neutrophil recruitment, platelets bound to activated endothelial cells can interact with leukocytes, and induce 'secondary capture' which induces interactions of neutrophils with platelets first, followed by neutrophil-endothelial interaction (Mine *et al.*, 2001). Under high shear stress as may be encountered in arterioles or stenotic arteries, platelets can adhere to subendothelial vWF. The interaction of platelets with vWF is mediated by the GPIb/IX/V complex. These interactions induce the activation of GPIIb/IIIa with a subsequent binding of GPIIb/IIIa to immobilized vWF, fibrinogen, and other ligands (Yuan *et al.*, 1999; Zaffran *et al.*, 2000). The binding of integrin $\alpha_5\beta_1$ to fibronectin can also mediate stable adhesion.

Neutrophil rolling on platelets is mostly mediated by platelet P-selectin binding to P-selectin glycoprotein ligand (PSGL) on leukocytes. Firm adhesion of leukocytes to platelets is achieved by CD11b/CD18 and CD11a/CD18. Further mechanisms involved in firm adhesion include the simultaneous binding of fibrinogen to GPIIb/IIIa on platelets and CD11b/CD18 on leukocytes and the binding of GPIbα to CD11b/CD18. Hence, GPIIb/IIIa antagonists do not prevent the formation of platelet-neutrophil-aggregates in patients (Zhao *et al.*, 2003). Upon adhesion of PMN to platelets, activation of PMN is induced through PSGL-1 and chemokine and lipid mediators presented by platelets.

In addition to interacting with neutrophils, platelets interact with other leukocyte subpopulations. Platelets present chemokines to and thereby activate monocytes (Huo *et al.*, 2001). Activated platelets increase monocyte binding to inflamed endothelium, which is important in atherosclerosis. The interaction between endothelial cells,

platelets, and monocytes leads to increased monocyte recruitment and accelerates the development of atherosclerotic lesions (Huo *et al.*, 2003).

The end result of platelet activation is conversion of soluble fibrinogen into insoluble fibrin and also activation and recruitment of other platelets. Platelets along with other cells such as erythrocytes and monocytes are trapped in the fibrin mesh formed by fibrin polymerisation resulting in growth and stabilisation of thrombus.

1.3.4 Platelet procoagulant activity

Activated platelets further activate other resting platelets and interact with other coagulant factors, eventually promoting thrombogenesis (Ilveskero *et al.*, 2001). This inherent property of platelets is known as platelet procoagulant activity (PPA). Activated platelet adhesion is mediated by vWF and GPIb interaction as described earlier. This interaction also initiates PPA which comprises of changes in platelet membrane architecture, intra cytoplasmic enzyme activation and release of powerful procoagulants from the platelets.

The plasma membrane of activated platelets provides this surface for the assembly of the "tenase" and "prothrombinase" complexes that activate factor X and prothrombin, respectively. Activation of factor X and conversion of prothrombin to thrombin are the most powerful steps in the coagulation cascade. Platelet plasma membrane asymmetry is maintained by two enzymatic activities: a "flippase" directing the inward transport of lipids and a "floppase" that directs the transport of choline- and amino-phospholipids to the outer leaflet. Following an agonist-stimulated rise in intraplatelet calcium (Ca^{2+}), membrane asymmetry is lost with exposure of anionic phospholipids on the platelet surface. Platelets then round off to balloon-like structures as well as developing membrane blebs termed membrane vesicles or microparticles. The significance of microparticles is described elsewhere.

High shear forces can increase PPA. In high shear conditions, despite blocking vWF and GPIb receptors platelet activation was present. Increased release of ADP and intraplatelet accumulation of Ca^{2+} in activated platelets under high shear conditions help sustain further procoagulant activity thereby enhancing thrombogenesis (Goto *et al.*, 2003; Chen *et al.*, 2010). Activated platelets contribute to coagulation activity by releasing several compounds including thrombin, factor V, factor XIII, fibrinogen, vWF, calcium ions and ADP from their α-granules and dense granules. These in turn activate

the resting platelets and this vicious cycle helps to maintain and increase platelet procoagulant activity (Jennings, 2009).

1.3.5 Coagulation physiology and factors

The coagulation system, together with endothelial cells and platelets, is responsible for maintaining blood in a fluid state, but when activated rapidly results in the development of a fibrin clot by conversion of the fibrinogen to the insoluble polymer fibrin by the key enzyme, thrombin (Mann, 1999; Dahlback, 2000). The coagulation system comprises of proenzymes that typically reside in the intravascular space in an inactivated state together with cofactors, cations and cell-associated phospholipids. Coagulation can be activated by two principal mechanisms, the intrinsic and the extrinsic pathways that converge to produce thrombin by the common pathway through a series of inter-related enzymatic reactions (Davie and Ratnoff, 1964; Macfarlane, 1964).

The intrinsic pathway

One mechanism of coagulation activation is the intrinsic pathway, so called because its components, factors XII, XI, IX, VIII, prekallikrein (PK), and high molecular weight kininogen (HMWK), are all plasma proteins and are “intrinsic” to the lumen of the blood vessel. The intrinsic pathway can be activated when factor XII undergoes auto-activation to factor XIIa on a negatively charged surface through a process called “contact activation” (Gailani and Renne, 2007) (Figure 1.6). Negatively charged surfaces include the artificial reagents in the APTT assay, such as kaolin, celite, and silica, which explains the dependence of the APTT on the contact activation factors. However, the intrinsic pathway can be activated in vivo by substances such as articular cartilage, endotoxin, L-homocysteine, and the developing thrombus (Schmaier and McCrae, 2007). Activation of factor XII leads to conversion of PK to kallikrein, facilitated by the cofactor HMWK, with kallikrein further stimulating the activation of factor XII. Activated factor XII subsequently activates factor XI, with factor XIa activating factor IX. Factor IXa, together with factor VIIIa, phospholipids, and calcium from the tenase complex that activates factor X. The intrinsic pathway can also be activated in a cell-based process, as the components can assemble on endothelial cells, platelets, and granulocytes. HMWK-PK binds to a cell-based receptor complex, which includes a binding protein for the globular head domains of complement component C1q, designated gC1qR, cytokeratin 1 (CK1) and urokinase plasminogen activator receptor

(u-PAR) (Schmaier and McCrae, 2007). Prolylcarboxypeptidase (PRCP) bound to the complex activates PK to form plasma kallikrein, resulting in factor XII activation. Apart from activation of factor XI, this complex is involved in other physiological activities through formation of bradykinin, which participates in fibrinolysis activation and the production of antiplatelet molecules, nitric oxide and prostacyclin, from endothelial cells.

The Extrinsic Pathway

The so-called Extrinsic Pathway of coagulation, comprising tissue factor (TF) and factor VII, is activated by tissue injury or cellular activation and is likely the primary mechanism for in vivo haemostasis (Dahlback, 2000; Mackman *et al.*, 2007). TF is an intrinsic membrane glycoprotein expressed on many vascular wall cells, such as endothelial cells, vascular smooth muscle cells, pericytes, fibroblasts (Day *et al.*, 2005) and blood borne cells including platelets and monocytes (Giesen *et al.*, 1999; Steffel *et al.*, 2006). TF is a 236-residue membrane-bound glycoprotein which is not normally present in or exposed to the circulation. Constitutively expressed cell-based tissue factor can be exposed to the blood following vascular injury, but tissue factor expression can also be induced on vascular endothelial cells and leukocytes by thrombin and inflammatory stimuli. This induction is well described for monocytes, but also may occur in neutrophils and eosinophils. Activation of many cells leads to production of minute membrane-bound microparticles, which may be a source of circulating tissue factor activity (Aras *et al.*, 2004). Recent studies have shown that platelets can be stimulated to produce TF mRNA and synthesize TF protein (Panes *et al.*, 2007).

Fibrin Formation and Common Pathway Activation

The purpose of coagulation is the formation of an insoluble fibrin polymer as a hemostatic plug. To this end, the transformation of plasma-based fibrinogen to cross-linked insoluble fibrin is accomplished by several mechanisms and is tightly regulated.

Fibrinogen is a plasma protein synthesised by liver which helps blood thrombogenesis and platelet aggregation. This is also an acute phase reactant and a marker of inflammation. It is present in blood at a concentration of 2.0 to 5.0 g/dl. Fibrinogen binds with high affinity to the α_{2b}/β_{3a} integrin receptor on activated platelets, and acts as a bridge to link platelets. Fibrinogen dimer is composed of two mirror-image groups

of three polypeptide chains, the Aα, Bβ, and γ chains which are held together by a group of central disulfide bonds (McKee *et al.*, 1966). Fibrinogen has a trinodular structure, with the central disulfide bonded area forming the central E domain, and the flanking polypeptides forming two lateral D domains (Yang *et al.*, 2000) (Figure 1.7). Serum fibrinogen level is a well-known independent risk factor for coronary artery disease. In a longitudinal observational study over 13 years, elevated plasma fibrinogen was associated with higher rates of subclinical cardiovascular disease (Danesh *et al.*, 1998).

Fibrin is formed by polymerisation of fibrinopeptides. Thrombin cleaves off small fibrinopeptides from the Aα and Bβ chains, converting fibrinogen to fibrin monomer. Binding sites in the central region of the fibrin monomer, termed A-knobs and B-knobs, are exposed. This allows the “knobs” on one monomer to bind to γC and βC “holes” in the D domains in another monomer in a half-staggered overlap pattern, facilitating non-covalent assembly into a fibrin protofibril polymer (Weisel, 1986; Yang *et al.*, 2000). The protofibrils then associate laterally into bundles that form thicker fibers. Concurrent with the conversion of fibrinogen to fibrin, thrombin catalyses the activation of factor XIII, a transglutaminase enzyme that stabilizes the fibrin polymer by forming covalent crosslinks between γ chains in the D domains of adjacent fibrin molecules (Chen and Doolittle, 1969). After reaching a sufficient length (600-800 nm) protofibrils elongate laterally by binding to their adjacent fibrils to form mesh like fibrin network (Cohen *et al.*, 1983; Weisel, 1986; Ryan *et al.*, 1999). Twisting of individual fibrin fibres limits its length and lateral growth thereby reducing the available binding sites for fibrinolytic agonists. Increased thickness of fibrin strands in this way limits the growth of fibre (Wolberg, 2007). Fibrin is characterised by stiffness (representing its elastic properties) and compliance (representing its inelastic properties).

Thrombin, which plays a key role in the formation of fibrin and its crosslinking, is a serine protease that is formed from prothrombin by the action of factor Xa and the prothrombinase complex, a phospholipid membrane-based complex consisting of factors Xa and Va together with calcium (Lane *et al.*, 2005). Apart from fibrin formation, thrombin is associated with propagation of coagulation through activation of factors V and VIII, thus accelerating the activity of the prothrombinase and tenase complexes, respectively (Brummel *et al.*, 2002) (Figure 1.8). Thrombin also exhibits direct activation of factor XI. Thrombin also activates platelets and other cells, such as endothelial cells and monocytes via G-coupled protease-activated receptors (PARs)

(Martorell *et al.*, 2008). Upregulation of PARs in smooth muscle cells play a key role in the pathogenesis of atherosclerosis (Barnes *et al.*, 2004). Due to its wide-ranging effects, thrombin activity is closely regulated. The activation of thrombin requires the action of factor Xa, in the prothrombinase complex resulting in cleavage of a peptide fragment, prothrombin fragment F1 + 2, from prothrombin (Tracy *et al.*, 1985).

The formation of active factor Xa from the proenzyme factor X is considered the start of the common pathway and can be triggered by both TF/factor VIIa from the extrinsic pathway and by the tenase complex (factors IXa, VIIIa, calcium, and phospholipids) from the intrinsic pathway (Dahlback, 2000). Having two separate mechanisms for factor Xa activation is thought to be associated with two temporal stages of blood coagulation (Butenas *et al.*, 1997; Butenas *et al.*, 2007), the initiation and propagation stages. In the initiation stage, factor X is initially activated by TF/factor VIIa. This produces small amounts of thrombin, which activates factors V and VIII, leading to further thrombin production (Brummel *et al.*, 2002).

1.3.6 Regulation of coagulation

The major regulation of TF/factor VIIa activity is through an inhibitor, tissue factor pathway inhibitor (TFPI). TFPI, a serine protease then downregulates the ability of tissue factor/VIIa to activate factor X (Broze *et al.*, 1993; Lu *et al.*, 2004). TFPI may also further downregulate coagulation by causing TF-expressing cells to internalise surface TF/factor VIIa complexes (Iakhiaev *et al.*, 1999). Coagulation then enters the propagation phase, directing the activity of tissue factor/VIIa toward activation of factor IXa, resulting in a switch of the primary activation of factor X to be via the intrinsic pathway tenase complex. Further thrombin production leads to the activation of factor XIa, which amplifies the propagation phase further.

Levels of TFPI correlate positively to levels of TF in patients with dyslipidaemia (Hansen *et al.*, 2001) and acute coronary syndrome (Falciani *et al.*, 1998; Gori *et al.*, 2002). Hyperactivation of the coagulation system in ischaemic heart disease patients may, in part be compensated for by TFPI but may not be sufficient to attenuate the elevation of TF (Viles-Gonzalez and Badimon, 2004). A rise in TFPI is a marker of hyperactivation of the coagulation system and offers some but not full protection against future thrombotic events (Leurs *et al.*, 1997; Badimon *et al.*, 1999a).

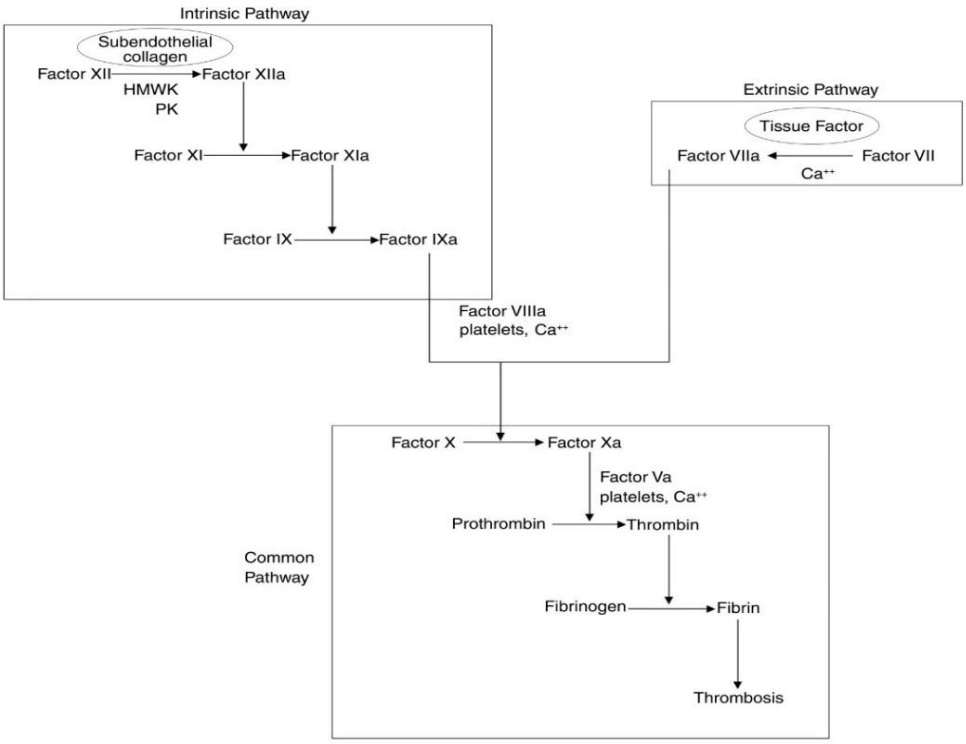

Figure 1.6 The coagulation cascade.

Subendothelial collagen and tissue factor, both released during vessel intimal injury, initiate the intrinsic and extrinsic pathways, respectively, of the cascade. Ca++ = calcium, HMWK = high-molecular-weight kininogen, PK = prekallikrein

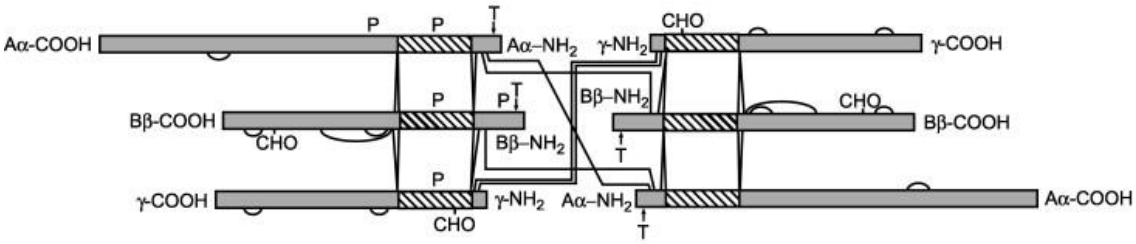

Figure 1.7 Schematic diagram of the three pairs of polypeptide chains

of fibrinogen.

The Aα, Bβ, and γ chains are represented by bars with lengths proportional to the number of amino acid residues in each chain and the N- and C-terminal ends of the chains are labelled. The coiled-coil regions are indicated by the diagonally striped boxes, while the intra- and inter- chain disulfide bonds are indicated by solid lines. Carbohydrate attachment sites are labelled with CHO, while thrombin and major plasmin cleavage sites are indicated by T and P, respectively. From (Weisel, 2005)

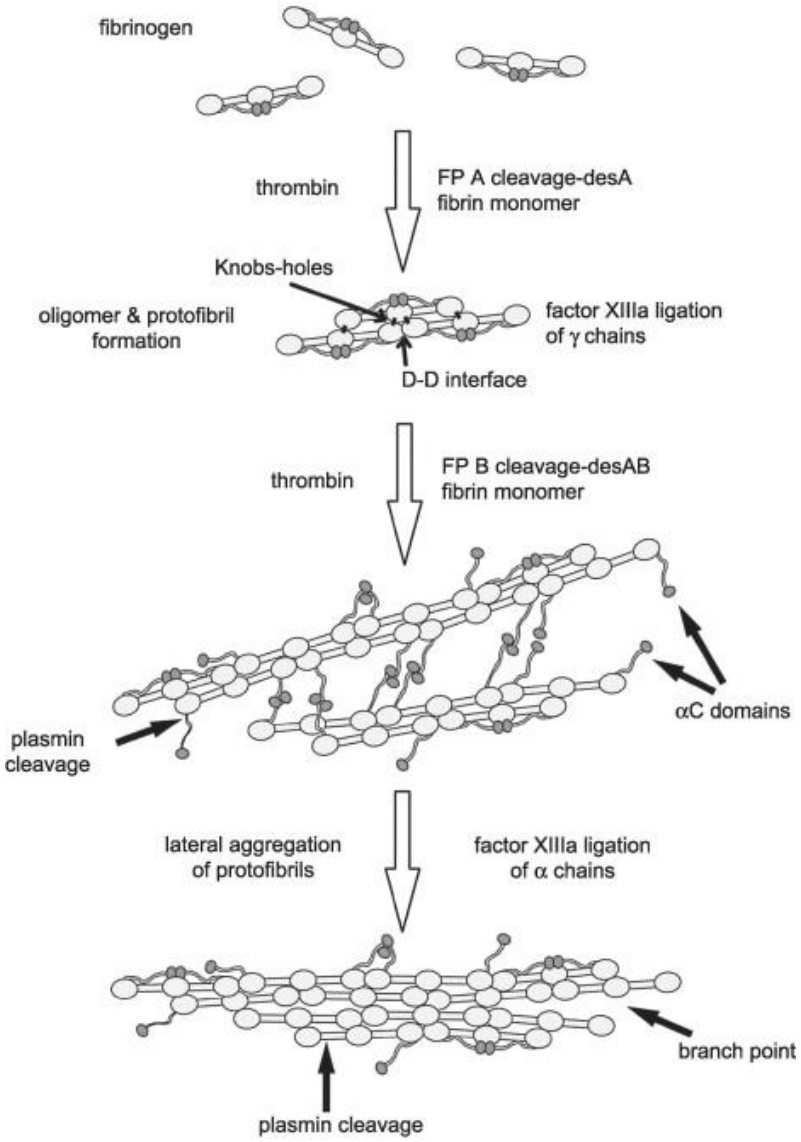

Figure 1.8 Schematic diagram of fibrin polymerization.

Fibrinopeptide A is cleaved from fibrinogen, producing desA fibrin monomers, which aggregate via knob-hole interactions to make oligomers. Fibrinopeptide B is cleaved primarily from polymeric structures. The oligomers elongate to yield protofibrils, which aggregate laterally to make fibers, a process enhanced by interactions of the αC domains. Factor XIIIa crosslinks or ligates γ chains more rapidly than α chains. Plasmin cleaves the αC domains and Bβ1-42 and then cuts across the fibrin in the middle of the coiled coil. At the bottom of the diagram, a branch point has been initiated by the divergence of two protofibrils. From (Weisel, 2005).

1.4 Blood thrombogenicity in Diabetes and Ageing

Higher cardiovascular mortality has been attributed to higher thrombogenicity in patients with T2DM (Osende *et al.*, 2001; Berry *et al.*, 2007). In vitro studies have demonstrated an inherent prothrombotic state in patients with T2DM. Endothelial dysfunction, coagulation factors, hyperactive platelets, inflammatory milieu and impaired fibrinolysis results in higher blood thrombogenicity in patients with T2DM (Balasubramaniam *et al.*, 2012). Antiplatelet therapy reduces this thrombogenicity but less so in those with T2DM compared to those without (Natarajan *et al.*, 2008a). Hypo-responsiveness to current antiplatelet therapy may also be contributing towards excess mortality seen in patients with T2DM (Angiolillo, 2009b).

The incidence of thrombotic cardiovascular disease dramatically increases with age (Tracy and Bovill, 1992), and recent studies have begun to address the important clinical problem of “ageing and thrombosis” (Wilkerson and Sane, 2002). Age-related changes may occur in the vascular and hemostatic systems, which includes platelets, coagulation, and fibrinolytic factors as well as endothelial changes. Ageing-associated sclerotic changes in the vascular wall may also contribute to the increased incidence of thrombosis in the elderly (Kiechl *et al.*, 1999). The hypercoagulability of the blood in the elderly may be yet another cause of the increased thrombotic tendency. For example, platelet activity is enhanced with advancing age, and ageing is associated with increased plasma levels of several blood coagulation factors (e.g., factor VII, factor VIII, and fibrinogen) (Balleisen *et al.*, 1985), all of which have been shown to be risk factors for thrombotic diseases (Koster *et al.*, 1994). Advanced age is associated with elevated interleukin-6 (IL-6) and C-reactive protein (CRP) levels, indicating an inflammatory state that may also be an important stimulus for thrombus formation in the elderly. On the other hand, a proportional increase in natural anticoagulant factors (e.g., protein C, protein S, antithrombin, tissue factor pathway inhibitor, etc.) has not been observed in the elderly (Sagripanti and Carpi, 1998). The fibrinolytic system is impaired by ageing since a progressive prolongation of the lysis time (Abbate *et al.*, 1993) and an increase in plasminogen activator inhibitor-1 (PAI-1), a principal regulator of fibrinolysis (Loskutoff, 1988), have been observed with ageing (Hashimoto *et al.*, 1987). Thus, increases in coagulation factors without a proportional increase in anticoagulant factors is the likely contributing factor for increased thrombotic events in the elderly. As the population ages, further studies are warranted to define the mechanisms for thrombosis in the elderly.

1.4.1 Platelets in Diabetes - "Angry Diabetic" platelets and Ageing platelets

ACS is precipitated by the ischaemic effect of an occlusive intra-coronary thrombus that develops over a ruptured atheromatous plaque as a result of platelet adhesion and aggregation (Badimon *et al.*, 1999b). Platelet function is significantly altered favouring a thrombotic tendency in patients with T2DM (Vinik *et al.*, 2001; Natarajan *et al.*, 2008b; Ferroni *et al.*, 2009). Platelets in patients with T2DM constantly remain in a state of hyperactivation and hence have been referred to as 'angry platelets' (Bhatt, 2008). Platelet synthesis is also enhanced in patients with T2DM. As diabetic platelets respond more frequently to sub-threshold stimuli, they soon become exhausted, consumed and finally incorporated into thrombus. This relative excess consumption of platelets results in accelerated thrombopoiesis in the bone marrow mediated by thrombopoietin and the release of 'fresh' hyper-reactive platelets (Watala *et al.*, 1999; Watala *et al.*, 2005).

Among diabetic individuals increased platelet aggregation and adhesion are due to:

- Reduced platelet membrane fluidity due to changes in the lipid composition or glycation of membrane proteins (Papanas *et al.*, 2004)
- Increased production of thromboxane A_2 (TXA_2) which increases platelet sensitivity (Halushka *et al.*, 1981)
- Increased expression of platelet adhesion molecules such as CD31, CD36, CD49b, CD62P and CD63 (Eibl *et al.*, 2004)
- Upregulation of platelet ADP $P2Y_{12}$ receptor signalling leading to increased adhesion, aggregation and pro-coagulant activity
- Increased expression of platelet surface receptors such as P-selectin, Glycoprotein (GP) Ib and GP IIb/IIIa (Lefkovits *et al.*, 1995)
- Increased generation of platelet dependent thrombin
- Decreased sensitivity to the effects of PGI_2 and NO (Ferroni *et al.*, 2004)
- Disorder in calcium and magnesium haemostasis. Increased intracellular calcium and decreased intracellular magnesium have been linked to platelet hyperaggregability and adhesiveness (Gawaz *et al.*, 1994; Lee *et al.*, 2001)

- Increased platelet content of cytokines and chemokines such as platelet factor-4, interleukin-1β and CD40L contribute to inflammation and atherogenesis alongside a pro-coagulant milieu (Ross, 1999; Lindemann *et al.*, 2001; Cipollone *et al.*, 2002; Weyrich *et al.*, 2003)

- Accelerated platelet turnover results in the presence of 'young' activated platelets in the circulation which demonstrate reduced responsiveness to antiplatelet agents and augment their response to naturally circulating agonists (Ferreiro and Angiolillo, 2011)

These platelet characteristics may contribute to the poorer outcomes observed in DM patients despite compliance with the recommended secondary prevention therapy.

A significant age related elevation in the markers of platelet activation such as β thromboglobulin and platelet factor 4 has been observed (Zahavi *et al.*, 1980). Platelets of individuals aged 60 years showed greater aggregation in response to ADP and collagen than did the platelets from younger individuals (Kasjanovova and Balaz, 1986). A significant reduction in the bleeding time and an increase of eicosanoid biosynthesis has been reported in older individuals, suggesting that platelet activation increases with age (Reilly and Fitzgerald, 1986). The increase of platelet activity with age is also correlated with higher content of platelet phospholipids, suggesting an age related increase in platelet transmembrane signalling (Bastyr *et al.*, 1990a).

1.4.2 Microparticles in Diabetes and Ageing

In addition to platelets, microparticles (MPs) are also involved in diabetic atherothrombosis. MPs are small membrane coated vesicles (size: 0.02 to 0.10 μm) that emerge by exocytosolic budding from their parental cells upon activation or apoptosis and which carry procoagulant activity (Burnier *et al.*, 2009). They retain at least some functions of their cells of origin, which can include platelets, endothelial cells and various leukocytes. MPs have the ability to activate the coagulation cascade with consequent thrombosis formation (Morel *et al.*, 2010). Platelet-derived MPs (PMPs) expose negatively charged phospholipids which act as binding sites for activated coagulation factors (George, 2008). Platelet MPs also bind to the sub endothelial matrix and act as a substrate for further platelet adhesion via GP IIb/IIIa fibrinogen bridging (Morel *et al.*, 2008). Monocyte-derived MPs (MMPs) exposing TF have P-selectin glycoprotein ligand-1 which interacts with P-selectin on the surface of

activated platelets and helps in further stabilization of thrombus. Other possible pathways regulated by MPs include production of lysophosphatidic acid (a strong platelet agonist), endothelial and leukocyte activation, recruitment of monocytes within the plaque, stimulation of neoangiogenesis, induction of apoptosis in endothelial or smooth muscle cells and increase in T XA_2 release which in turn causes vasoconstriction (Ardoin *et al.*, 2007; Leroyer *et al.*, 2008).

Increased levels of platelet-derived MPs and their role in macrovascular complications have been reported in patients with T2DM patients (Nomura *et al.*, 1995). Elevated levels of endothelial cell-derived MPs (EMPs) are predictive for the presence of coronary artery lesions, and are a more significant independent risk factor than the duration of DM, lipid levels and history of hypertension (Nomura *et al.*, 2004). In patients with T2DM and ACS, increased EMPs have been linked to non-calcified atheromatous lesions as detected by multi-detector computed tomography (Bernard *et al.*, 2009). Increased levels of procoagulant TF positive MPs have been demonstrated within the occluded coronary artery of patients with STEMI (Morel *et al.*, 2009). Beneficial effects of statins in T2DM and atherothrombosis are possibly due to their effects on MPs (Sommeijer *et al.*, 2005; Koh *et al.*, 2007; Diamant *et al.*, 2008; Nomura *et al.*, 2009). All this evidence indicates that MPs are not only a reliable marker for vascular injury but they also actively participate in promoting atherothrombotic complications in T2DM (Morel *et al.*, 2010).

Age was shown to be associated with reduced basal levels of EMP but with preservation of MP procoagulant potential (Forest *et al.*, 2010). In stable conditions, despite a reduction in EMP, procoagulant activity was maintained using PMP, RBC-MP, TF-bearing MP or annexin V MP in the elderly (Forest *et al.*, 2010). These results suggest that MP still carry their procoagulant activity with age, possibly due to reduced ability to maintain energy consuming plasma membrane phospholipid asymmetry, resulting in increased exposure of the anionic procoagulant phospholipid phosphatidylserine on the external layer.

In this context, drugs that may reduce the release of MPs and/or their thrombogenicity may have the potential to improve the effects of current antiplatelet therapy, resulting in lower adverse event rates in elderly patients and those with T2DM.

1.4.3 Platelet leukocyte aggregates (PLA) in Diabetes and Ageing

Platelets and leukocytes from patients with DM are hyperreactive and express more adhesion molecules. P-selectin is one of the markers of platelet activation and is the main link for platelet adhesion to circulating leukocytes (Vandendries *et al.*, 2004). Platelet P-selectin interacts with leukocyte P-selectin glycoprotein ligand (PSGL-1) leading to the formation of PLA (Rinder *et al.*, 1991; Hidari *et al.*, 1997). Consequently, activated leukocytes secrete several pro-inflammatory cytokines and express a pro-thrombotic membrane phenotype. Elevated levels of circulating PLA have been reported in DM population, thereby suggesting their involvement in the pathogenesis of atherothrombosis. Elevated circulating PLA levels are linked with vascular injury in DM patients (Vandendries *et al.*, 2004). A significant increase in circulating platelet-polymophonuclear aggregates (PPA) and platelet-monocyte aggregates (PMA) percentages was demonstrated in DM patients (Elalamy *et al.*, 2008). Interestingly, levels of circulating PPA and PMA were significantly higher in DM with vascular injury compared to DM without vascular injury, suggesting the involvement of inflammatory leukocytes in vascular damage (Hu *et al.*, 2004; Elalamy *et al.*, 2008).

Platelet aggregation in platelet-rich plasma (PRP) is increased in the elderly, relative to young individuals, in response to the agonists ADP, epinephrine, collagen and arachidonic acid. Platelet reactivity, aggregation, adherence to thrombogenic surfaces and plasma levels of the secreted platelet α-granule protein thromboglobulin are increased in elderly. However, there are no published data comparing circulating PLA levels among older and younger individuals.

1.4.4 Coagulation in Diabetes and Ageing

TF and Factor VII (F VII) initiate the thrombotic process, resulting in the generation of thrombin. This subsequently helps in the conversion of fibrinogen into a three-dimensional network of fibrin fibres which forms the skeleton of the blood clot (Alzahrani and Ajjan, 2010). TF is an integral prothrombotic transmembrane protein expressed by both vascular and non-vascular cells, including monocytes, macrophages and platelets (Breitenstein *et al.*, 2010). In T2DM, TF expression is upregulated due to the presence of low grade inflammation. Levels of TF in atherosclerotic plaques in patients with unstable angina are higher compared with those who have stable angina (Annex *et al.*, 1995). Plasma TF levels are raised in

subjects with CAD, particularly ACS, further emphasising the role of TF in atherothrombosis (Suefuji *et al.*, 1997; Soejima *et al.*, 1999). Patients with T2DM have higher circulating TF levels which are directly modulated by glucose and insulin, and the two appear to have an additive effect (Boden *et al.*, 2007). In T2DM, increased levels of advanced glycation end products and reactive oxygen species activate NFκB which in turn leads to increased TF production (Breitenstein *et al.*, 2010).

Factor VII is a vitamin-K dependent coagulation factor synthesised in the liver. F VII coagulant activity (F VII: c) has been associated with fatal cardiovascular events (Folsom *et al.*, 1991; Heinrich *et al.*, 1994; Scarabin *et al.*, 1998). T2DM subjects have elevated F VII: c levels (Heywood *et al.*, 1996). An association between triglyceride levels and F VII: c levels has been demonstrated; this appears to be independent of obesity and insulin resistance (Karatela and Sainani, 2009).

Thrombin concentration influences fibrin clot formation and also determines the clot structure and stability (Wolberg and Campbell, 2008). High thrombin concentration results in denser and less permeable clots which are more resistant to lysis. Thrombin generation is enhanced in T2DM secondary to low grade coagulation system activation (Boden *et al.*, 2007; Ceriello *et al.*, 2009).

Plasma fibrinogen is a well-known independent CVD risk factor and is used as a surrogate marker for CVD risk (Corrado *et al.*, 2010). High fibrinogen levels predict silent myocardial ischaemia in patients with T2DM (Guardado-Mendoza *et al.*, 2009). High levels of IL-6 in T2DM stimulate fibrinogen synthesis by hepatocytes, representing a link between inflammation and the prothrombotic state (Ajjan and Grant, 2006). Insulin resistance is also associated with increased hepatocyte fibrinogen synthesis (Barazzoni *et al.*, 2003; Tessari *et al.*, 2006). Overall, in patients with T2DM there is increased TF production, increased F VII: c levels, increased thrombin generation, high levels of IL-6 and fibrinogen.

Fibrinogen levels increase with advancing age from approximately 18 to 85 years old (Tracy *et al.*, 1992). Plasma concentrations of IL-6, F VII, factor VIII, factor IX activation peptide, factor X activation peptide, prothrombin fragments 1+2, thrombin-antithrombin (TAT) complex and fibrinopeptides A (FPA) were found to positively correlate with advancing age (Bauer *et al.*, 1987; Mari *et al.*, 1995).

1.4.5 Fibrinolysis in Diabetes and Ageing

Fibrinolysis is initiated by the conversion of plasminogen to plasmin and this is largely mediated by tissue plasminogen activator (tPA). Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of fibrinolysis by binding to tPA and forming PAI-1/tPA complex. In a long term 18 year study, glycated haemoglobin (HbA_{1C}) correlated positively with PAI-1 and negatively with tPA. This demonstrates an association between hyperglycaemia and elevated PAI-1 levels (Seljeflot *et al.*, 2006). Hyperinsulinaemia also has been shown to increase PAI-1 levels, which may account for elevated PAI-1 levels in insulin resistant states (Alessi and Juhan-Vague, 2008; Stegenga *et al.*, 2008).

A study indicated that clots derived from fibrinogen purified from plasma from 150 subjects with T2DM had a more compact structure characterised by smaller pore size, increased fibrin thickness and number of branch points than that from 50 healthy controls (Dunn *et al.*, 2005). Clot lysis from diabetic patients was slower when compared to controls due to elevated PAI-1 levels.

There is strong evidence that PAI-1 increases with ageing (Wilkerson and Sane, 2002). A significant age related decrease in fibrinolytic activity, documented by an increase in PAI-1 levels has been reported (Mehta *et al.*, 1987). Age related increase of plasmin-antiplasmin complex, D-dimer and thrombin activatable fibrinolysis inhibitor (TAFI) have also been reported (Mari *et al.*, 1995; Sakkinen *et al.*, 1999; Schatteman *et al.*, 1999).

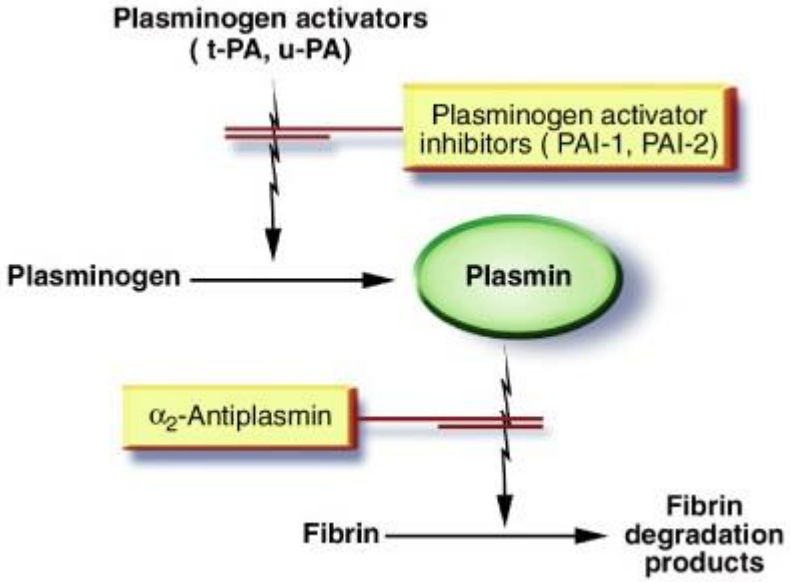

Figure 1.9 Fibrinolytic system and its regulation.

Plasminogen activators convert plasminogen to plasmin. Plasmin then degrades fibrin into soluble fibrin degradation products. The system is regulated at two levels. PAI-1 regulates the plasminogen activators, whereas α_2-antiplasmin serves as the major inhibitor of plasmin. From Jeffrey I, Haemostasis, thrombosis, fibrinolysis and cardiovascular disease, Braunwald's Heart Disease. 9th edition.

1.4.6 Inflammation and thrombosis in Diabetes and Ageing

The presence and extent of inflammation, procoagulant state and composition of the atherosclerotic plaque have been strongly associated with an increased risk of future cardiovascular events. Thus, the perpetuation of the inflammatory response likely plays a pivotal role in the pathobiology and vulnerability of the atherosclerotic plaque. Inflammatory markers once thought to be passive observers are now being investigated as active participants in the progression of atherosclerosis and therefore targets for future pharmacological intervention. Inflammation up-regulates procoagulants and down-regulates anticoagulants and fibrinolysis.

Subclinical chronic low-grade inflammation is linked to insulin resistance and is involved in the pathogenesis of type 2 diabetes (Stern, 1995; Herder *et al.*, 2005). Inflammatory and insulin signalling pathways are tightly linked, both of which lead to insulin resistance and endothelial dysfunction, contributing to cardiovascular complications including coronary events (Hotamisligil, 2006). As mentioned earlier, it is possible that inflammation is the hidden but potent 'common soil' for the prothrombotic state in T2DM (Omoto *et al.*, 2002; Koga *et al.*, 2005).

Adipose tissue is an active endocrine and paracrine organ that releases a large number of cytokines and bioactive mediators, such as leptin, adiponectin, IL-6 and TNF-α that influence insulin resistance, inflammation and atherosclerosis (Hauner, 2005; Halberg *et al.*, 2008). Obesity is also associated with more generalized, systemic inflammation involving circulating inflammatory proteins such as CRP, IL-6, PAI-1, P-selectin, VCAM-1, and fibrinogen. Adhesion molecule expression is induced by proinflammatory cytokines such as IL-1β, TNF-α, and CRP produced by the liver in response to IL-6 (Szmitko *et al.*, 2003). A study was performed in offspring of patients with T2DM who are at high risk of developing diabetes and CVD. This demonstrated the presence of insulin resistance, an excess of intra-abdominal fat mass, hypoadiponectinemia, and multiple defects in glucose and energy metabolism in these individuals (Salmenniemi *et al.*, 2004). This study also demonstrated high levels of high-sensitivity CRP (hsCRP), IL-6, IL-1β, IL-1 receptor antagonist, and adhesion molecules (P-selectin, ICAM-1) among these pre-diabetic subjects, indicating that low-grade inflammation and markers of endothelial dysfunction are characteristic findings in subjects at high risk of T2DM and CVD (Ruotsalainen *et al.*, 2008).

Tumour necrosis factor α (TNFα) is a pleiotropic proinflammatory cytokine present in atherosclerotic lesions. This appears to be one of the most important influences on the progression of atherosclerosis. Its upregulation is known to mediate and amplify a multitude of interactions resulting in progressive inflammation, plaque destabilisation and prothrombotic tendencies (Rus *et al.*, 1991). Plasma TNFα levels are higher in T2DM and have been linked to insulin resistance and complications of T2DM such as retinopathy and nephropathy (Mavridis *et al.*, 2008). Suppression of TNFα either by biological antagonists or by physical measures such as weight reduction and exercise improves glycaemic control in T2DM. Treatment with biological antagonists to TNFα has been shown to suppress inflammation in patients with rheumatoid arthritis and has also been proven to be associated with the rapid down-regulation of a spectrum of cytokines (IL-6), cytokine receptors and acute phase proteins (amyloid A, haptoglobin and fibrinogen) (Charles *et al.*, 1999). This potent suppression of markers and mediators of inflammation may have tremendous potential in preventing progression of atherosclerosis.

Interleukins, especially IL-6 and IL-8 circulating levels are significantly higher in patients with T2DM compared to those without even after adjustment for confounding factors including visceral obesity. IL-6 increases platelet production and indirectly increases thrombogenicity since young platelets are more thrombogenic compared to the old platelets (Lim *et al.*, 2004). IL-6 increases fibrinogen and PAI-1, promotes adhesion of neutrophils and myocytes during myocardial reperfusion (Miyao *et al.*, 1993). Elevated levels of IL-6 in subjects with unstable angina were associated with higher 6- and 12- month mortality which was independent of troponin levels (Lindmark *et al.*, 2001). IL-8 is a powerful trigger for firm adhesion of monocytes to vascular endothelium. In addition, it may play a potential atherogenic role by inhibiting local inhibitors of metalloproteinases in atherosclerotic plaques and by stimulating smooth muscle cell migration (Yue *et al.*, 1993; Gerszten *et al.*, 1999). Inflammatory mediators induce expression of protease activated receptors (PARs) on the endothelium of patients with T2DM which in turn increases leukocyte adhesion molecules on the cell surface (Dangwal *et al.*, 2011).

Adipokines, such as leptin, adiponectin, TNF-α, IL-6, resistin, visfatin, and retinol-binding protein 4, have been suggested to be associated with insulin resistance (Van Gaal *et al.*, 2006). Adiponectin has important anti-atherogenic, antidiabetic, and anti-inflammatory properties and is expressed abundantly in adipocytes. In subjects with

an excess of intra-abdominal fast mass, adiponectin levels are low, which might be explained by an increase in TNF-α secretion from visceral fat. High adiponectin level correlates with high insulin sensitivity (Kadowaki *et al.*, 2008). Adiponectin inhibits the expression of ICAM-1, VCAM-1, and E-selectin through the inhibition of nuclear factor-κB (NF-κB) activation and has several antiatherogenic and anti-inflammatory properties (Kadowaki *et al.*, 2008).

Ageing is associated with increased inflammatory activity in the blood, including increased circulating levels of TNF-*α* (Bruunsgaard *et al.*, 1999), IL-6 (Ershler *et al.*, 1993; Cohen *et al.*, 1997), cytokine antagonists, acute-phase proteins and neopterin (Ballou *et al.*, 1996). Increased inflammatory activity in the elderly may reflect age-related pathological processes. Thus, atherosclerosis is an age-related inflammatory disease reflected by secretion of cytokines such as TNF-*α*, IL-1, IL-6, and IFN-γ and the presence of large numbers of macrophages and activated $CD4^+$ T cells within inflammatory atherosclerotic plaques.

High TNF-*α* levels in centenarians are associated with a low ankle-brachial arterial pressure index, indicating peripheral atherosclerosis. Furthermore, atherosclerosis and increased risk of thromboembolic complications have been associated with several parameters which are related to TNF α, e.g. increased circulating levels of IL-6, acute-phase proteins such as C-reactive protein (CRP) and fibrinogen, intercellular adhesion molecule-1 (ICAM-1), leucocytes, and a lipid profile including increased levels of triglycerides, total cholesterol (TC), and low-density lipoproteins (LDL), decreased concentrations of high-density lipoproteins (HDL), and a low HDL/TC ratio. Thus, TNF-*α* is an early mediator of the acute-phase response and involved in the production of chemokines, IL-6, and CRP as well as the recruitment of leucocytes during inflammatory reactions. TNF-*α* is also known to induce smooth muscle proliferation and to increase adherence of leucocytes to endothelial cells by inducing the expression of cell adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Furthermore, TNF-*α* induces the expression of a wide range of cytokines, including chemokines and IL-6 by endothelial cells. TNF-*α* also has an important role in lipid metabolism by decreasing the activity of 7 *α*-hydroxylase and lipoprotein lipase and by stimulating the liver production of triglycerides.

1.4.7 Markers of inflammation

Previous studies have shown that high levels of CRP, IL-6, and TNF-α predict subsequent development of T2DM (Schmidt *et al.*, 1999). In one study, hsCRP levels were measured in 1,045 subjects with T2DM. Subjects with hsCRP >3 mg/l had a higher risk for CHD death than patients with hsCRP ≤3 mg/l, even after the adjustment for confounding factors (Soino *et al.*, 2006). This study confirmed hsCRP as an independent risk factor for CHD deaths in patients with T2DM. In another recent study, hsCRP was independently associated with short-term mortality risk in individuals with T2DM and in those without a previous diagnosis of CVD (Bruno *et al.*, 2009). The American Heart Association suggested in their guidelines on cardiovascular risk factors that higher levels of CRP are a hallmark of T2DM and may warrant preventive therapy for cardiovascular events even in patients with pre-diabetes (Sabatine *et al.*, 2007).

CRP has been suggested to play a more direct role in atherothrombosis involving the endothelium, platelets, and leukocytes as well as vascular smooth muscle cells (Mazer and Rabbani, 2004; Paffen and Demaat, 2006). Evidence now suggests that CRP may possess procoagulant activity, as it has been shown to decrease tPA activity while increasing PAI-1 levels in human aortic endothelial cells (Devaraj *et al.*, 2003; Singh *et al.*, 2005). CRP also inhibits endothelial nitric oxide synthase and prostacyclin activity, thus promoting a prothrombotic state. CRP increases TF synthesis on monocytes, promotes platelet adhesion via up-regulation of P-selectin, promotes monocyte-platelet aggregation, increases vascular smooth muscle cell migration and proliferation, as well as neointima formation (Danenberg *et al.*, 2007).

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1.5 Antiplatelet therapy in patients with CAD

Platelets play a central role in thrombosis and hence anti-platelet agents are vital in prevention of acute coronary events in high risk patients. A clear benefit of antiplatelet agents in the prevention of atherothrombotic events in those at high risk is well established. Multiple genetic, iatrogenic and environmental factors influence platelet responsiveness to these agents. Three different classes of antiplatelet agents are approved for treatment and/or prevention of ACS:

i. Cyclooxygenase-1 (COX-1) inhibitors (Aspirin)

ii. ADP P2Y12 receptor antagonists (thienopyridines – Clopidogrel, Prasugrel and Ticagrelor)

iii. Platelet GP IIb/IIIa inhibitors (Figure 1.10).

Commonly used antiplatelet agents in patients with CAD and T2DM have been aspirin and clopidogrel. Prasugrel and Ticagrelor is being used more often than clopidogrel in ACS setting. Clopidogrel is still being used in patients who have contraindications to prasugrel or ticagrelor, elderly population or patients at high risk of bleeding.

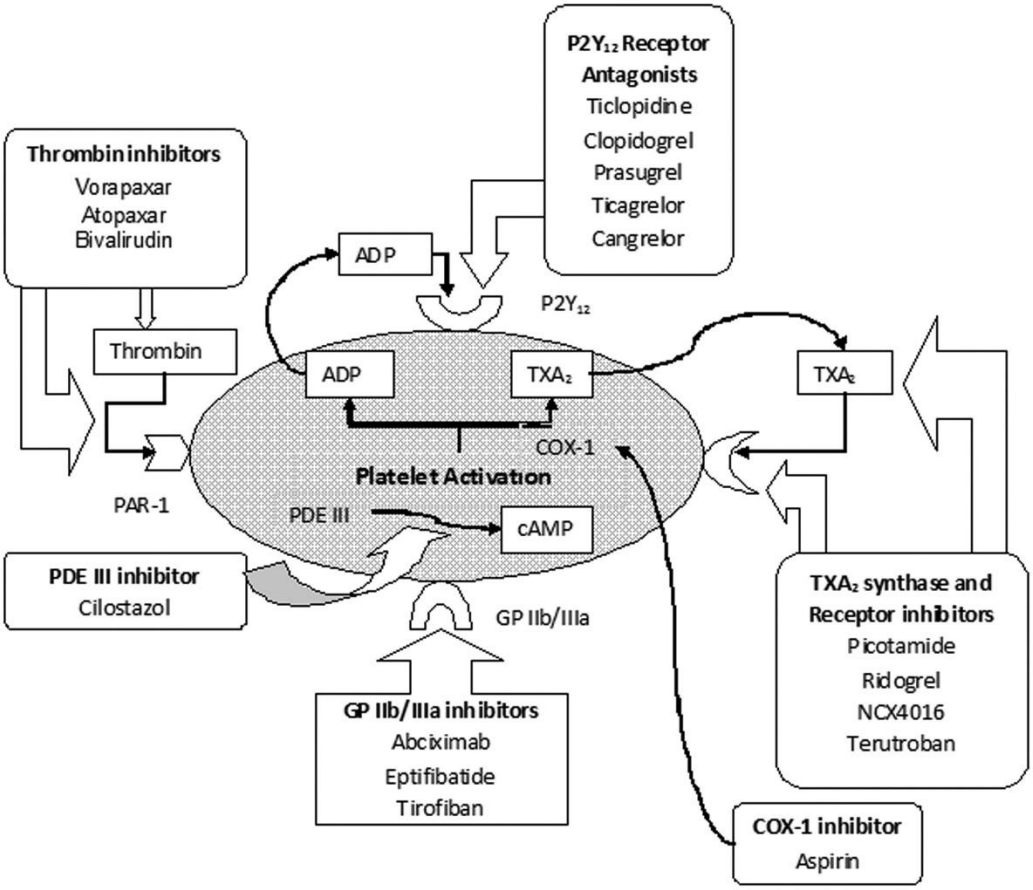


Figure 1.10 Schematic representation of mechanisms of action of antiplatelet agents.

PAR-1: Protease activated thrombin receptor – 1. Adapted from (Schafer, 1996)

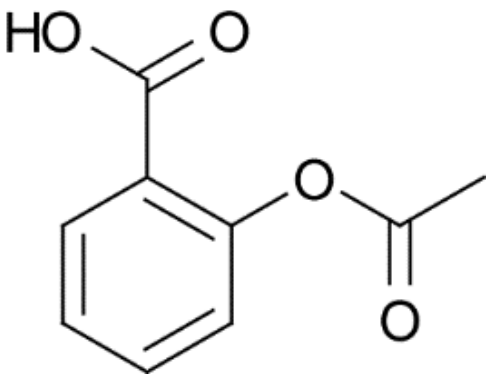


Figure 1.11 Structure of aspirin

1.5.1 Structure of aspirin

Aspirin is the most widely used antiplatelet for prevention of atherothrombotic events. Low dose aspirin (e.g. 75mg daily) has been used as an antiplatelet therapy since 1960 (Spectre and Varon, 2009). Aspirin (acetyl salicylic acid) is prepared from salicylic acid, by acetylation with acetic anhydride (Figure 1.11) (Vane and Botting, 2003).

1.5.2 Pharmacokinetics of aspirin

After oral intake, non-dissociated acetylsalicylic acid passively diffuses across the gastric and intestinal mucosa. Enteric coated aspirin has delayed absorption time (peak plasma levels in 3-4 hours) as compared to uncoated aspirin (peak plasma levels in 30-40 minutes). In the liver, it is hydrolysed by esterases. Aspirin has a half-life of about 15-20 minutes in human circulation but its antiplatelet effects last for the entire lifespan of platelets due to irreversible inhibition of COX-1, allowing the use of a once a day regimen despite the very short half-life of the drug (Reilly and Fitzgerald, 1987). The mean life span of human platelets is approximately 10 days. About 10% of circulating platelets are replaced each day and 5-6 days following aspirin ingestion approximately 50% of the platelets function normally. Aspirin thus exhibits a dissociation of pharmacokinetics and pharmacodynamics. Platelet function recovers after stopping aspirin faster than the predicted 10 days due to a non-linear relationship between thromboxane A_2 (TXA_2) biosynthesis and COX-1 inhibition (Perneby *et al.*, 2006).

1.5.3 Mechanism of action of aspirin

After rapid absorption from the stomach and upper small intestine, aspirin first comes in contact with platelets in the portal circulation, where it leads to irreversible inactivation of the cyclooxygenase activity of prostaglandin H synthase 1 and synthase 2, referred to as COX-1 and COX-2 respectively. These enzymes catalyse the conversion of arachidonic acid to prostaglandin H_2, which is then converted to prostaglandins, prostacyclin and TXA_2 by tissue-specific isomerases. TXA_2 induces potent platelet aggregation and vasoconstriction, whereas prostaglandins inhibit platelet aggregation and induces vasodilatation. In addition, TXA_2 augments the actions of platelet agonists, is pro-atherogenic and a potent stimulus for vascular smooth muscle cells to express adhesion molecules (Vane and Botting, 2003).

After diffusing through the cell membrane, aspirin enters the COX channel, first binds to an arginine 120 residue, and then acetylates the serine 529 residue in COX-1 and serine 516 residue in COX-2. This prevents access of arachidonic acid to the COX site. As the anuclear platelets cannot synthesise new enzymes, this aspirin effect remains for the rest of their life span. In contrast, the inhibition of COX-2 dependent pathophysiological processes such as hyperalgesia and inflammation, mediated by nucleated cells, require higher doses of aspirin and frequent dosing because higher levels of aspirin are required to inhibit COX-2 than to COX-1 (Patrono *et al.*, 2005a). In addition, unlike platelets, nucleated cells can repeatedly synthesise fresh COX-2, explaining the approximate 100-fold variation in daily doses of aspirin when it is used for anti-inflammatory rather than antithrombotic purposes.

1.5.4 Dosage of aspirin for coronary artery disease

Aspirin is commonly used at doses 75mg to 100mg for primary and secondary prevention of cardiovascular diseases. At this dose, it has maximal antiplatelet effect and reduces major cardiovascular events without excessively increasing the bleeding risk. Complete suppression of COX-1 mediated platelet activity has been shown to be achieved at doses 50 to 81mg (Patrono *et al.*, 2005b; Lordkipanidze *et al.*, 2007; Qayyum *et al.*, 2008). Doses higher than 100mg have not been shown to provide additional benefit but increase bleeding risk.

Low dose aspirin is effective as antiplatelet therapy in patients with CAD due to the following reasons:

i. Complete suppression of COX-1 mediated platelet activity at low doses
ii. Lack of significant clinical benefits at higher doses
iii. Dose dependent side effects as explained below

1.5.5 Side effects of aspirin

In a sex-specific meta-analysis of the six primary prevention trials, aspirin use was associated with 70% increased risk of major bleeding events in men and women; this translated into causing one major bleeding event per 400 women and per 303 men over 6.4 years of aspirin use (Berger *et al.*, 2006). Gastrointestinal tract ulceration, bleeding and perforation are expected adverse effects, in that, they are at least partly explained by inhibition of prostaglandin synthesis produced by aspirin. Gastrointestinal bleeding risk is dose-dependent; higher doses are associated with higher bleeding

rates. Concomitant use of NSAIDs, steroids, Helicobacter pylori infection, history of previous gastric ulcer and age more than 70 years increase the risk of gastrointestinal side effects of aspirin (Dutch, 1991; Farrell *et al.*, 1991). A case-control study has suggested that proton pump inhibitors are more effective than H_2 receptor antagonists in preventing upper gastrointestinal bleeding associated with aspirin use (Lanas *et al.*, 2007). Enteric-coated aspirin has been shown to be associated with endoscopically documented decreased risk of gastric mucosal injury in at least five randomised controlled trials (Walker *et al.*, 2007). Meta-analysis by Antithrombotic Trialists' Collaboration (ATC) found an odds ratio of 1.6 for major extracranial bleeding, e.g. gastric bleeding, in patients taking aspirin (Antithrombotic *et al.*, 2009).

Aspirin hypersensitivity is an uncommon adverse effect and desensitisation therapy may be successful in permitting the continued use of aspirin.

1.5.6 Aspirin resistance

The term "aspirin resistance" suffers from lack of consensus definition and validity. It has been varyingly defined as recurrence of major thrombotic events such as acute coronary events, stroke or peripheral vascular acute ischaemic events despite prescription of a regular therapeutic dose of aspirin ("clinical aspirin resistance") or persistent ex vivo platelet activation and aggregation, measured by platelet function tests, despite aspirin use ("biochemical" or "laboratory aspirin resistance"). The reported prevalence of biochemical aspirin resistance ranges widely from 1% to 61% (Hankey and Eikelboom, 2006). A recent meta-analysis showed that patients with laboratory aspirin resistance have higher risk of clinical aspirin resistance (Snoep *et al.*, 2007). Three studies followed individuals with failure to suppress thromboxane production by aspirin and found they were 2.2 to 4.3 times at risk of developing cardiovascular events (Sciulli *et al.*, 2006; Santilli *et al.*, 2009; Dragani *et al.*, 2010). No study has shown clear benefit from routine screening for aspirin resistance. There is some indication that genetic factors such as polymorphism PLA1/A2 of the gene encoding for glycoprotein IIIa might be associated with altered platelet function and increased risk of cardiovascular events.

In addition, non-compliance to aspirin intake is more common (up to 40%) than true aspirin resistance and hence any laboratory or clinical assessment of aspirin resistance should be adjusted for compliance to aspirin therapy (Cotter *et al.*, 2004;

Schwartz *et al.*, 2005). Other factors like cigarette smoking, concomitant use of NSAIDs including ibuprofen and indomethacin have been shown to antagonise the antiplatelet effect of aspirin.

1.5.7 Aspirin and T2DM

Patients with T2DM and CAD, have an increased risk of atherothrombotic events despite being on aspirin. Genetic variations, increased platelet activation, enhanced thrombopoiesis, increased thromboxane production and persistent thromboxane-dependent activation of platelets have all contributed to aspirin unresponsiveness. This warrants the need for intensive platelet-inhibiting strategies in these high risk groups to reduce further ischaemic risk. Low dose aspirin at doses ranging from 75mg to 325mg is currently the preferred antiplatelet agent for secondary prevention in individuals with T2DM and CAD. Low dose aspirin as a primary prevention measure in T2DM is controversial (Antithrombotic *et al.*, 2009; De Berardis *et al.*, 2009). Although in vitro studies demonstrate that doses of aspirin less than 75mg daily are sufficient to achieve full COX-1 inhibition, ex vivo pharmacodynamic studies have suggested that patients at high risk, such as those with T2DM, may have improved pharmacodynamic responses with higher doses of aspirin. Hence, it has been argued that patients with T2DM may need higher doses of aspirin for secondary prevention therapy.

Mortensen et al demonstrated that patients with T2DM and CAD on aspirin 75mg OD had higher platelet reactivity compared to non-diabetic patients with CAD, as measured by two different point of care tests (Mortensen *et al.*, 2010). T2DM is a significant risk factor for residual platelet reactivity in patients treated with aspirin after adjusting for confounding factors, and residual platelet activity positively correlates with clinical cardiovascular events (Geisler *et al.*, 2010).

1.5.8 Aspirin and acute coronary syndrome

Benefits of aspirin in the early management of ACS patients including unstable angina/NSTEMI (Lewis *et al.*, 1983; Theroux *et al.*, 1988; Group, 1990) and STEMI (Investigators, 1988; Roux *et al.*, 1992) have been demonstrated consistently in various studies. Aspirin should be given as early as possible at an initial dose of 162 to 325 mg followed by a daily maintenance dose of 75 to 162 mg (Antman *et al.*, 2004; Anderson *et al.*, 2007). Withdrawal of aspirin can lead to recurrence of ACS (Senior, 2003). Two large meta-analyses performed by the Antiplatelet Trialists' Collaboration included 287 studies and involved 212000 high risk patients (with acute or previous vascular disease or other predisposing condition increasing the risk of occlusive vascular disease) ('Collaborative overview of randomised trials of antiplatelet therapy: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration,' 1994; 'Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients,' 2002). Aspirin was the most frequently used antiplatelet agent at doses ranging from 75 to 325 mg daily in these trials. The incidence of vascular events on aspirin was reduced from 22.3% to 18.5% in the DM cohort (P<0.002) and from 16.4% to 12.8% (P<0.00001) in non-DM cohort. The incidence of vascular events was much higher in DM patients but the benefit of antiplatelet therapy was consistent regardless of the DM status ('Collaborative overview of randomised trials of antiplatelet therapy: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration,' 1994). Low dose aspirin (75 to 150 mg) was as effective as higher daily doses but with significantly lower bleeding complications ('Collaborative overview of randomised trials of antiplatelet therapy--I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration,' 1994; 'Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients,' 2002). The Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events – Organisation to Assess Strategies in Ischaemic Syndromes (CURRENT/OASIS-7) trial is the first large scale randomised study comparing high and low dose aspirin. This study randomised ACS patients who were scheduled to undergo angiography within 72 hours of hospital arrival (Mehta *et al.*, 2010a; Mehta *et al.*, 2010b). With a 2x2 factorial design, patients were

randomised (double blinded) to high or standard dose clopidogrel for a month and in open label fashion to high dose (300 to 325 mg daily) or low dose (75 to 100 mg daily) aspirin. There was no significant difference in the rates of the primary outcome (cardiovascular death, MI or stroke after 30 days) between high and low dose aspirin (4.1% versus 4.2%; hazard ratio [HR] =0.98; p=0.76) in DM and non-DM patients. A trend towards higher gastrointestinal bleeding rates (0.38% versus 0.24%; p=0.051) in the high dose group was observed (Mehta *et al.*, 2010a).

1.5.9 Aspirin and elderly population

Although a clear excess of adverse events has been shown with aspirin even at lower dosage in elderly patients when compared to the young (Naschitz *et al.*, 1990; Segal *et al.*, 2003), only very few primary and secondary prevention trials have specifically addressed the benefit-risk ratio of aspirin in the elderly population and these are frequently derived from large clinical trials in which data are stratified by age. The magnitude of absolute benefits and risks with aspirin for primary prevention is not fully known. Although patients with high baseline atherothrombotic risk are more likely to benefit from aspirin, bleeding complications including intracranial and gastrointestinal bleeding are common in the elderly and this might counteract the small benefit from aspirin in those at lower risk (Capodanno and Angiolillo, 2010).

1.5.10 Structure and metabolism of clopidogrel

Clopidogrel is a thienopyridine and acts by inhibition of P2Y12 receptors on platelet membranes. It is one of the most widely used and carefully studies antithrombotic agents. It is estimated that more than 100,000 patients have been enrolled in various randomised trials involving clopidogrel. Platelet ADP signalling pathways mediated by P2Y1 and P2Y12 play an important role in platelet activation and aggregation. P2Y12 activation leads to sustained platelet aggregation and stabilisation of the aggregated platelets (Gachet, 2001b; Storey *et al.*, 2001). Thienopyridines are non-direct, irreversible P2Y12 antagonists.

Clopidogrel is well absorbed from the upper small intestine and enters the portal circulation. It is inactive in vitro, but is converted to its active metabolite by the Cytochrome P450 pathway in the liver. When platelets come in contact with this active metabolite, their P2Y12 receptors are blocked irreversibly and they become permanently inactivated. Only about 15% of the absorbed drug is metabolised by

cytochrome P450 (CYP450) into its active metabolite. About 85% of the drug is hydrolysed by an esterase to create an inactive carboxylic acid derivative.

Clopidogrel is converted to its active metabolite by cytochrome enzymes in the liver by a two-step process:

i. CYP450 oxidises the thiophene ring of clopidogrel to 2-oxoclopidogrel (Lau *et al.*, 2004)

ii. Thiophene ring opens to form a thiol and carboxyl group. The thiol derivative of clopidogrel binds with cysteine residues (cys17 and cys20) of P2Y12 receptors resulting in irreversible platelet inhibition (Savi *et al.*, 2000; Ding *et al.*, 2003)

Platelet inhibition prevents expression of GPIIb/IIIa receptors on platelet membranes and thereby inhibits fibrin mediated platelet aggregation. Collagen- and thrombin-induced platelet aggregation are also inhibited by clopidogrel. Clopidogrel reduces platelet-leukocyte aggregate formation, levels of CRP, p-selectin and CD40L, and also the rate of thrombin formation (Gurbel *et al.*, 2007).

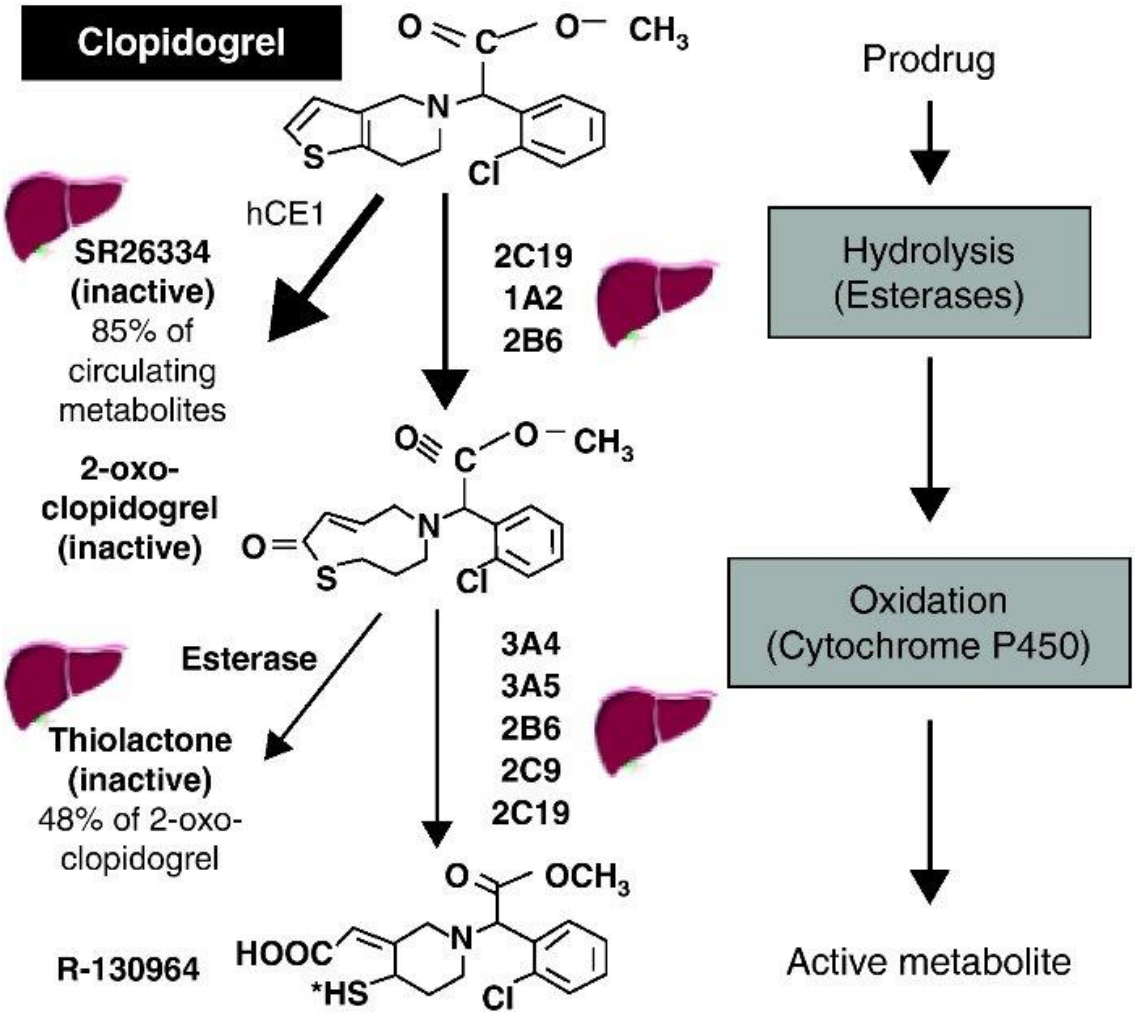


Figure 1.12 Structure and metabolism of clopidogrel

Clopidogrel is a prodrug, and its activation is complex. Some 85% of the dose administered is hydrolyzed in the liver, where it is converted into an inactive metabolite (SR 26334); the remainder is metabolized by CYP2C19 and to a lesser degree by CYP1A2 and CYP2B6 to 2-oxo-clopidogrel. Around half of this metabolite, which is also inactive, is hydrolyzed and converted into an inactive thiolactone, while the other half is metabolized by the 3A4, 3A5, 2B6, 2C9 and 2C19 isoenzymes, finally producing the active metabolite (R-130964). The fact that a significant proportion of the absorbed drug is wasted (converted to inactive metabolites), the complex activation of the prodrug requiring two oxidation steps, and its heavy dependence on the function of the CYP2C19 isoenzyme (whose reduced-function genetic variants have a prevalence of around 25%), are the main pharmacokinetic factors affecting the variability of response to clopidogrel. From (Aguiar, 2012)

1.5.11 Clinical studies of clopidogrel in coronary artery disease

Clopidogrel has been studied in several disease areas including CAD, cerebrovascular disease, PVD either separately or in combination, ACS, PCI and atrial fibrillation.

The Clopidogrel versus aspirin in patients' at risk of ischaemic events (CAPRIE) trial was the first study to show safety and efficacy of clopidogrel. This study randomised 19,185 patients with a history of prior stroke, MI or PVD to either clopidogrel 75mg or aspirin 325mg. The annual rates of death, stroke, or MI over the average 2 years of follow-up were 5.3% and 5.8% respectively (HR: 0.91; 95% CI: 0.83 – 0.97; p=0.04). The overall incidence of haemorrhagic events did not differ between treatment groups (Gent *et al.*, 1996) (CAPRIE steering committee, 1996). Although there was a slight benefit of clopidogrel over aspirin, there was no change in practice. Importantly CAPRIE established the efficacy and safety of clopidogrel as a useful antiplatelet agent and an alternative if patients cannot tolerate aspirin.

The next major trial was Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) study which enrolled 12,562 patients with non ST elevation acute coronary syndrome (NSTE-ACS) and randomised them to clopidogrel plus aspirin (loading dose clopidogrel 300mg followed by 75mg daily or aspirin alone (75 – 300mg). After a mean duration of treatment of 9 months, the risk of cardiovascular death, stroke or MI was 9.3% and 11.4% respectively (HR: 0.80; 95% CI: 0.72 – 0.90; p<0.001). There was an increase in major bleeding events in the clopidogrel group (OR: 1.39; 95% CI: 1.14 to 1.70). This was a landmark study and established clopidogrel along with aspirin as standard of care antiplatelet therapy in patients after NSTE-ACS (Yusuf *et al.*, 2000).

The Clopidogrel as Adjunctive Reperfusion Therapy (CLARITY) – Thrombolysis in Myocardial Infarction (TIMI) 28 trial and ClOpidogrel and Metoprolol in Myocardial Infarction Trial (COMMIT) also known as Second Chinese Cardiac Study (CCS-2) were complimentary trials evaluating clopidogrel plus aspirin for management of patients with ST elevation acute coronary syndrome (STE-ACS). CLARITY was an angiographic study in patients treated with thrombolysis as the method of reperfusion and enrolled 3491 patients randomised to clopidogrel plus aspirin and aspirin alone. Rates of occluded arteries (TIMI flow grade 0 or 1) were 11.7% and 18.4% respectively (HR: 0.59; 95% CI: 0.48 to 0.72; p<0.001). This was the main driver of the primary outcome composite assessed at angiography occluded infarct related artery, death or MI. There was no difference in bleeding between the two groups. Clopidogrel plus

aspirin improved patency of the infarct related artery compared to aspirin alone (OR: 1.36; 95% CI: 1.18 to 1.57) (Sabatine *et al.*, 2005a).

COMMIT was a large pragmatic study of 45,852 patients with mainly STE-ACS enrolled in China, randomised to clopidogrel 75mg plus aspirin 162mg or aspirin alone for 4 weeks. The primary outcome of death, stroke or MI occurred in 9.2% in clopidogrel plus aspirin group and 10.1% in aspirin alone group (HR: 91; 95% CI: 0.86 to 0.97; p=0.002), and there was a significant reduction in all-cause mortality (HR: 0.93; 95% CI: 0.83 to 0.99; p=0.03). There were no excess major bleeding events or cerebral haemorrhage in the clopidogrel group (COMMIT collaborative group) (Chen *et al.*, 2005a). These two trials established clopidogrel as a standard approach for patients with STE-ACS treated with thrombolysis. Previous smaller trials had shown benefits of clopidogrel in patients undergoing PCI and therefore clopidogrel usage became standard practice for patient with STE-ACS undergoing primary PCI.

The Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management, and Avoidance trial (CHARISMA) compared combination of clopidogrel and aspirin to aspirin alone in providing greater vascular protection in a wide range of stable patients in at risk of vascular events. Eligibility criteria included patients who had a clear diagnosis of coronary, cerebrovascular or peripheral arterial disease (without a definite prior stroke or MI), or if they had a combination of risk factors such as diabetes, hypertension, hypercholesterolemia or smoking (without a clear prior history of vascular disease). The trial enrolled 15,603 patients randomised either to clopidogrel (75mg daily) plus aspirin (75 to 162mg daily) or to placebo plus aspirin. There was a non-significant reduction in the primary end point of MI and death from cardiovascular cause (OR: 0.92; 95% CI: 0.81 to 1.04). There was a non-significant increase in major bleeding (OR: 0.92; 95% CI: 0.81 to 1.04) (Chen *et al.*, 2005a).

The Clopidogrel and Aspirin Optimal Dose Usage to Reduce Recurrent Events-Seventh Organization to Assess Strategies in Ischaemic Syndromes (CURRENT-OASIS 7) trial was a complex design study and showed that there was no difference in the primary outcome (cardiovascular death, stroke and MI) in patients assigned to double dose clopidogrel (150mg) as compared to standard dose (75mg) (HR: 0.94; 95% CI: 0.83 to 1.06). Major bleeding occurred at similar rates in both the groups and 150mg of clopidogrel was associated with a significant reduction in stent thrombosis (CURRENT-OASIS7 Investigators, 2010) (Mehta *et al.*, 2010b).

1.5.12 Dosage of clopidogrel

Without a loading dose, clopidogrel 75 mg daily induces inhibition of ADP-induced platelet aggregation as early as 2 hours after the first dose but requires 3 to 7 days to achieve maximal inhibition of platelet aggregation (Thebault *et al.*, 1999). The 3- to 7-day delay can be shortened to 5 to 6 hours with a loading dose of 300mg and can be maintained for 24 hours. Doubling the loading dose from 300 to 600mg achieves platelet inhibition at 2 to 3 hours and a further increase in platelet inhibition is reported at this dose (L'Allier *et al.*, 2008). Doses higher than 600mg are not recommended for loading since only a limited increase in platelet inhibition occurs at higher doses.

Dosage regimes differ based on the timing and clinical indication of PCI

Current guidelines for clopidogrel loading dose are as follows:

i. Patients undergoing elective PCI – 300mg is recommended, to be administered at least 24 hours before PCI

ii. Patients who have acute coronary syndrome – 300mg loading dose is recommended

iii. Patients undergoing non elective PCI and have never had clopidogrel (clopidogrel naïve), 600mg loading dose is recommended. If they have already been preloaded with 300mg, a further 300mg dose is recommended

iv. Doses higher than 600mg are not currently recommended

Following this, a daily dose of 75mg is recommended for maintenance therapy. The antiplatelet response to clopidogrel is highly heterogeneous. Variability in clopidogrel response is widely recognised and well documented, and is associated with higher adverse clinical events as described later.

1.5.13 Side effects of clopidogrel

Clopidogrel in general was well tolerated in clinical trials and fatal complications have not been reported. Serious side effects at loading dose of 300mg or 600mg or maintenance dose of 75mg are rare in clinical practice.

Several clinical trials (CAPRIE, CURE, CREDO, CLARITY TIMI-28, COMMIT, ISAR-CHOICE, MATCH and others) focused mainly on bleeding as a major side effect of

clopidogrel. Some studies reported an increase in the incidence of bleeding following dual clopidogrel and aspirin therapy compared to aspirin alone (CURE study, 1.6% vs. 1.0%) (Yusuf *et al.*, 2001b), while others did not. Gastrointestinal haemorrhage requiring hospitalisation was less frequent with clopidogrel compared to aspirin in CHARISMA trial (2.0% vs 2.7%) (Berger et al 2010). In the large CAPRIE trial (1996), major haemorrhage occurred in 1.4% of clopidogrel treated patients; this percentage was not significantly different from that seen in aspirin-treated patients (1.6%).

Other important but less frequent side effects include severe neutropenia as observed in CAPRIE trial, thrombocytopenia in CURE trial (Yusuf *et al.*, 2000), severe multisystem thrombotic microangiopathy, thrombotic thrombocytopenic purpura (TTP) (Bennett *et al.*, 2000; Hankey, 2000), drug-associated thrombotic thrombocytopenic purpura-haemolytic uremic syndrome (Medina *et al.*, 2001). Clopidogrel in rare instances has been associated with many other life threatening side effects such as severe bone-marrow suppression manifested as toxic bone marrow failure, aplastic anaemia (Meyer *et al.*, 2001), thrombocytopenia, neutropenia or even pancytopenia (Trivier *et al.*, 2001). Furthermore, Meyer et al. reported another case of fatal aplastic anaemia, which led to the patient's death from septicaemia (Meyer *et al.*, 2001). Non-haematological side effects of clopidogrel treatment have also been reported. These include, membranous nephropathy, a major cause of nephrotic syndrome in adults (Tholl *et al.*, 1999), gastroenteritis (e.g., abdominal pain, dyspepsia, gastritis, diarrhoea and constipation), acute arthritis (Garg *et al.*, 2000), angioedema (Fischer *et al.*, 2003), urticarial rash (Khambekar *et al.*, 2004) and hepatocellular and cholestatic liver injury (Goyal, 2009). Dyspeptic symptoms were more frequent in clopidogrel therapy in CAPRIE study (27.1%) but resulted in drug withdrawal only in very few cases (3.2%) (1996).

1.5.14 Variability of clopidogrel response

The antiplatelet effect of clopidogrel varies significantly from one person to another. The mechanisms leading to variability in clopidogrel responsiveness are not fully elucidated and similar to aspirin, are multifactorial. The variability is attributable to patient related clinical factors, genetic factors and cellular factors (Figure 1.13).

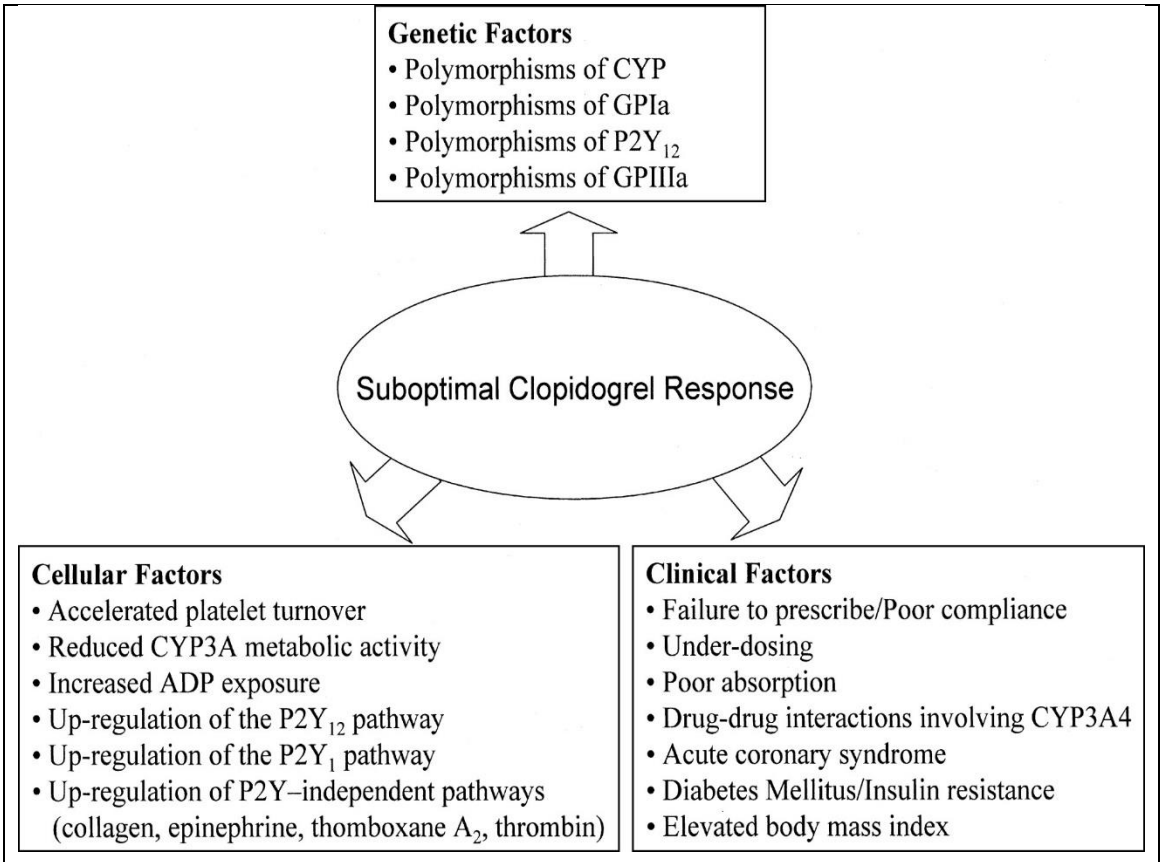


Figure 1.13 Proposed Mechanisms Leading to Variability in Individual Responsiveness to Clopidogrel

ADP = adenosine diphosphate; CYP = cytochrome P450; GP = glycoprotein

1.5.15 Pharmacogenomics of clopidogrel

The response to clopidogrel is influenced by pharmacokinetic variables such as intestinal absorption and metabolic activation in the liver, both of which are affected by genetic polymorphisms (Giusti and Abbate, 2010). Three different types of genetic polymorphisms of clinical interest in patients taking clopidogrel include gene series involved in modulating intestinal absorption of clopidogrel (ABCB1 gene), metabolic activation of clopidogrel (CYP3A5 and CYP2C19), and biologic activity (P2RY12 and ITGB3). Although the active metabolite of clopidogrel arises from complex biochemical reactions that involves a number of different hepatic CYP enzymes, there is accumulating evidence that CYP2C19 plays a dominant role in clopidogrel activation (Mega *et al.*, 2010). The CYP2C19 gene is located on chromosome 10 (10q24.1-q24.3) and consists of 490 amino acid residues. At least 25 genetic variants in CYP2C19 have been identified. Hulot et al. conducted a pharmacogenomics study using young healthy volunteers who were treated with clopidogrel and provided novel evidence that loss-of-function CYP2C19*2 allele was associated with marked decrease in platelet responsiveness and hence proposed that this gene was an important contributor to clopidogrel resistance in clinical setting (Hulot *et al.*, 2006). In a systematic meta-analysis of 10 studies involving 11,959 patients, Hulot et al. demonstrated that carriers of the loss-of-function CYP2C19*2 allele were at 30% higher risk of major adverse clinical events compared to non-carriers (9.7% vs. &.8%; OR: 1.29; 95% CI: 1.12 to 1.49; P<0.001). CYP2C19*2 alone was also associated with increased mortality (1.8% vs. 1.0%; OR: 1.79; 95% CI: 1.10 to 2.91; P=0.019; n=6225) and stent thrombosis (2.9% vs. 0.9%; OR: 3.45; 95% CI: 2.14 to 5.57; P<0.001; n=4905). This increased risk was apparent in both heterozygotes and homozygotes and was independent of the baseline cardiovascular risk (Hulot *et al.*, 2010).

The US Food and Drug Administration (FDA) has added a boxed warning to the clopidogrel label which suggests that individuals with poor metabolizer genotypes (i.e., CYP2C19*2 and *3 carriers) may be at increased risk for adverse cardiovascular outcomes and should consider other antiplatelet medications or alternative dosing strategies (Holmes *et al.*, 2010).

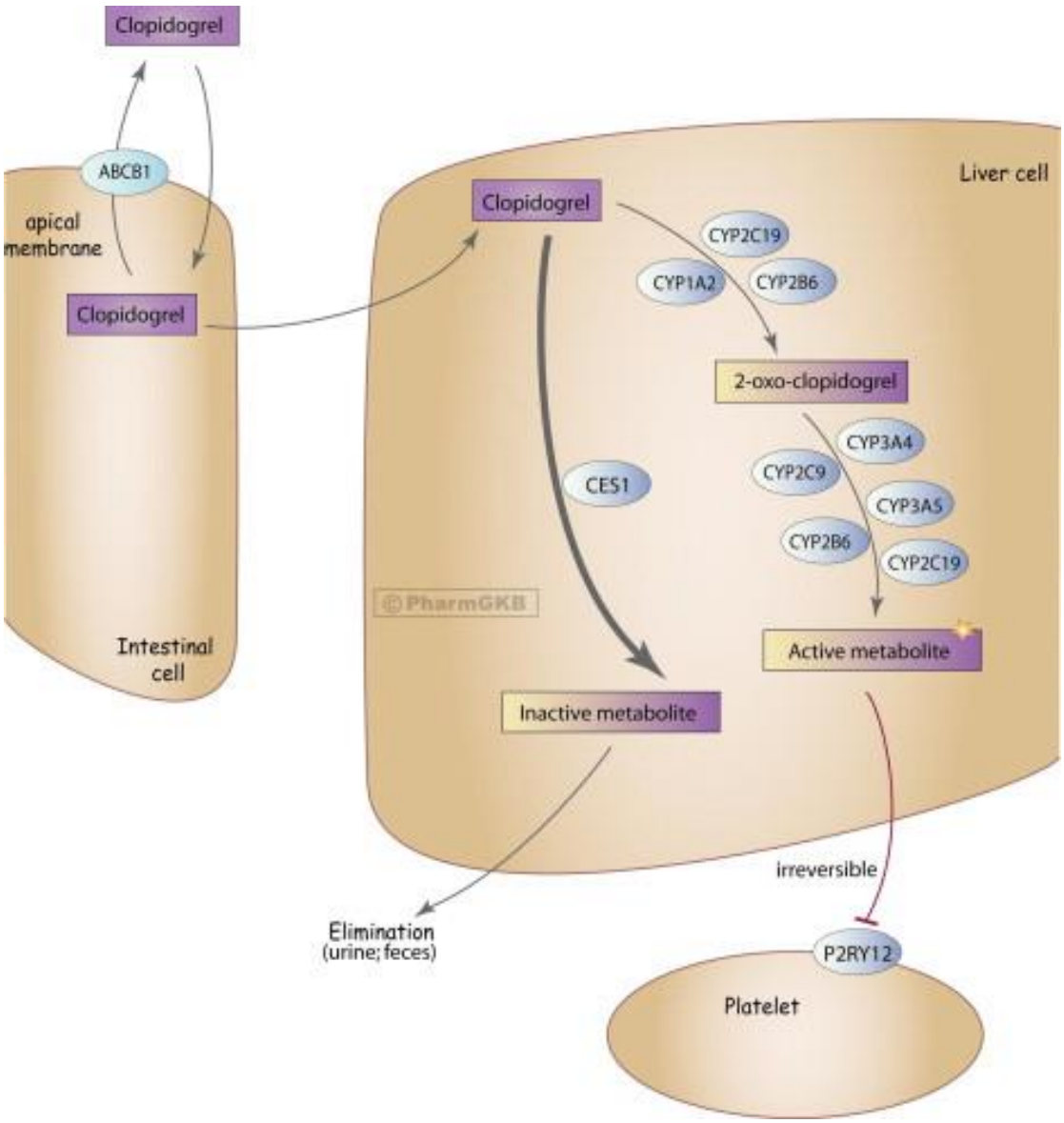


Figure 1.14 Pharmacogenomics of clopidogrel.

Representation of the candidate genes involved in the metabolism of clopidogrel and its primary mechanism of action. Intestinal absorption of the prodrug clopidogrel is limited by an intestinal efflux pump P-glycoprotein coded by the ABCB1 gene. The majority of the prodrug is metabolized into inactive metabolites by ubiquitous esterases. The minority is bio-activated by various cytochrome P450 (CYP) isoforms into active metabolites. These metabolites irreversibly antagonize the adenosine diphosphate (ADP) receptor (coded by the P2RY12 gene), which in turn inactivates the fibrinogen receptor (the glycoprotein [GP] IIb/IIIa receptor coded by the ITGB3 gene) involved in platelet aggregation.

From (Simon *et al.*, 2009; Beitelshees *et al.*, 2011)

1.5.16 Clopidogrel and T2DM

There is plenty of evidence to support the superiority of clopidogrel compared to aspirin in patients with diabetes mellitus (DM). CAPRIE trial included 3837 patients with DM. The primary end point (combined incidence of CV death, stroke or MI) occurred less frequently in the clopidogrel group compared to the aspirin group (15.6% vs 17.7%; RR: 0.87; 95% CI: 0.76 to 0.99; p=0.042). For every 1000 DM patients treated with clopidogrel, 21 vascular events were prevented (Bhatt *et al.*, 2002). CURE study sub group analysis of patients with T2DM showed that addition of clopidogrel to aspirin resulted in fewer adverse clinical cardiovascular events (14.2% vs. 16.7%; RR: 0.84; 95% CI: 0.70 to 1.02) (Yusuf *et al.*, 2000). CHARISMA trial found no benefit of dual aspirin and clopidogrel therapy in the long term in a wide range of non-ACS patients (n=15 603) with risk factors for, and established atherothrombotic disease including DM patients (n=6555; 42% of the study population) (Bhatt *et al.*, 2007).

Nevertheless, clinical benefits of clopidogrel are lower in T2DM compared to those without diabetes (Angiolillo, 2009a). Elevated plasma fibrinogen levels, presence of DM and BMI ≥ 25 kg/m^2 are associated with lower platelet inhibition with clopidogrel therapy in subjects with CVD. A significant interaction between elevated plasma fibrinogen and DM exists, identifying elevated plasma fibrinogen as a unique factor associated with lower platelet inhibition in diabetic patients. Increased BMI also remains independently associated with lower platelet inhibition after clopidogrel therapy.

The aetiology of clopidogrel resistance in T2DM is multifactorial, and insulin resistance and systemic inflammation play a significant role. Patients with T2DM show lower response to clopidogrel both in the loading phase and in the maintenance phase. Patients who are on insulin have the highest degree of platelet reactivity whilst on dual antiplatelet therapy. Impaired response to clopidogrel in T2DM has been shown to be an independent marker of cardiovascular mortality after adjusting for confounding factors (El Ghannudi *et al.*, 2011). Persistent high platelet activity in DM patients despite current recommended antiplatelet therapy has raised interest in identifying strategies to optimise platelet inhibition in this high risk population.

1.5.17 Antiplatelet therapy in the elderly

The physiological changes accompanying ageing have an important impact on the effects of therapeutic agents including antithrombotic medications. Given that atherothrombotic disease processes increase with age and that prevalence of the elderly population is continuously growing, understanding the effects of antithrombotic therapy in this high risk cohort is of key importance (Capodanno and Angiolillo, 2010). This is further emphasized by the fact that antithrombotic therapy used to reduce ischaemic events in the elderly is counterbalanced by their increased risk of bleeding. Numerous factors influence the effect of antiplatelet therapy in the elderly. These include renal function, hepatic metabolism, body mass distribution, platelet dysfunction, coagulation disorders and drug-drug interaction due to polypharmacy. Lack of dedicated research studies in the elderly, who are excluded from many large scale clinical trials, often leads to either no recommendations or arbitrary assumptions regarding the most appropriate antithrombotic therapy. Hence more data from large scale clinical trials and dedicated studies in the elderly assessing the safety and efficacy of antithrombotic agents are strongly warranted.

1.5.18 Dose modification of antiplatelet agents

Suboptimal responsiveness to clopidogrel is a well-established clinical entity and this phenomenon has been associated with recurrence of ischaemic events including stent thrombosis. Stent thrombosis may lead to severe consequences and has become a major concern in the era of drug-eluting stents. Diabetes mellitus has been identified as an independent predictor of stent thrombosis.

The Optimizing Antiplatelet Therapy in Diabetes Mellitus **(**OPTIMUS) study was the first study which showed marked improvement in platelet inhibition when 150 mg maintenance dose of clopidogrel was used in DM patients with CAD (Angiolillo *et al.*, 2007). This study was not powered to evaluate the risk of bleeding with the use of high-dose clopidogrel in association with aspirin and the study was not designed to measure clinical outcomes.

In the Gauging Responsiveness With Verify Now Assay: Impact on Thrombosis and Safety (GRAVITAS) trial, high clopidogrel dose (600mg loading dose followed by 150mg daily maintenance dose for 6 months) was given to patients with inadequate response to standard clopidogrel dose. At six months of follow-up, the composite end

point of cardiovascular death, MI or stent thrombosis was identical in both groups, at 2.3% (HR=1.01; 95% CI, 0.58-1.76; p=0.97). Stent thrombosis occurred in 0.5% of the high-dose group and 0.7% of the standard-dose group, a non-significant difference. There was also no difference in bleeding (Price *et al.*, 2011b). The results of GRAVITAS did not support a uniform treatment strategy of high-dose clopidogrel in patients with high on-treatment platelet reactivity. Thus, at present there is no evidence that increasing the dose of clopidogrel will improve the outcomes in patients with T2DM. Alternative treatment strategies incorporating platelet function testing merit further investigation.

1.5.19 Dual antiplatelet therapy in stable coronary artery disease

There is little clear evidence to routinely add a thienopyridine to aspirin in patients with stable coronary, cerebrovascular or symptomatic PVD and no evidence for this regimen for primary prevention in patients without evidence of vascular disease. CAPRIE data clearly supports the use of clopidogrel in patients who need vascular protection but cannot tolerate aspirin.

The CHARISMA trial was designed to examine the effect of DAPT on vascular protection compared to aspirin monotherapy in patients with stable CAD (n=15 603). Patients with diabetes comprised 42% of the study population (n=6555). Data on the effects of clopidogrel plus aspirin in the subgroup of patients with CAD have not been published from the CHARISMA trial, but presentations of these data and interpretation from the available publications suggest that an expected proportional reduction of about 15% for CAD patients is consistent with the CAPRIE like cohort subgroup. In the main publication of CHARISMA, a separate subgroup analysis was performed on patients with previous myocardial infarction, ischaemic stroke or symptomatic peripheral arterial disease (PAD). In this cohort, DAPT decreased the rate of cardiovascular death, myocardial infarction or stroke (7.3% vs. 8.8%; HR, 0.83; 95%CI, 0.72 to 0.96; p=0.01), and the risk of hospitalisations for ischaemia (11.4% vs. 13.2%; HR, 0.86; 95%CI, 0.76 to 0.96; p=0.008) when compared with aspirin and placebo. This benefit was associated with increased risk of GUSTO defined moderate (2.0% vs. 1.3%; HR, 1.60; 95%CI, 1.16 to 2.20; p=0.004), but not severe bleeding (1.7% vs. 1.5%; HR, 1.12; 95%CI 0.81 to 1.53; p=0.50). This analysis suggested that high risk patients with history of previous MI, ischaemic stroke or symptomatic PAD may benefit from intensified antithrombotic therapy (dual antiplatelet therapy with aspirin and

clopidogrel). Those without prior MI did not demonstrate any significant benefit (Bhatt *et al.*, 2006).

A CHARISMA subgroup analysis comparing patients with age<75 years and age≥75 years did not demonstrate any significant difference in clopidogrel response both in terms of primary outcomes and bleeding complications. Another subgroup analysis of the CHARISMA trial also showed that the benefits of clopidogrel are greater in the population with T2DM and a previous cardiovascular event (relative risk reduction: 17.1%; 95% CI: 4.4 – 28.1). Overall, the CHARISMA trial demonstrated a small but significant benefit in the subgroup of patients with known atherothrombosis and a potential role for intensification of antithrombotic therapy beyond aspirin alone (long-term dual antiplatelet therapy) in certain high-risk groups.

The REACH registry (n=68,000), which ran concurrently with the CHARISMA trial with similar eligibility criteria but an observational study design, demonstrated that patients with vascular disease in more than one territory (coronary, cerebrovascular or peripheral) have a greater risk of adverse outcomes than patients with either no documented vascular disease or disease in a single territory (Steg *et al.*, 2007). Annual average risk of death, stroke or MI in the REACH registry was between 4% and 5% compared to 3% overall risk in CHARISMA, suggesting that future studies on dual antiplatelet therapy for secondary prevention should focus on patients with a prior vascular event, symptomatic PVD or patients with evidence of disease in more than one territory. MATCH trial, one of the largest secondary prevention stroke trials, did not confirm the benefits of dual antiplatelet therapy compared to clopidogrel alone (Diener *et al.*, 2004).

From all the above discussion, it is clear that in elderly patients and in patients with T2DM, rates of recurrent cardiovascular events remain higher compared to younger individuals and in those without T2DM. Despite being aware of these increased risks in high risk population group, the current guidelines from international societies recommend similar antiplatelet and antithrombotic therapy for all patients in ACS setting and after coronary stent insertion. These increased risks persist in elderly and T2DM patients even in stable CAD setting but the current recommended antiplatelet therapy is aspirin monotherapy for stable CAD and clopidogrel monotherapy if the patients are intolerant or allergic to aspirin. Thus further work to reduce the risk of further adverse cardiovascular events in these high risk groups is essential. As

discussed in detail above, CHARISMA trial did demonstrate modest benefit of DAPT in selected population (previous MI, ischaemic stroke or symptomatic PAD) but cost-effectiveness might have been one of the reasons for not adapting DAPT in those high risk patients. Because of increased platelet reactivity in elderly and T2DM, It is possible that these groups of patients may benefit from DAPT when continued long-term. Proportion of hyporesponders to aspirin and clopidogrel are much higher in elderly and in T2DM. Hence other ways of inhibiting platelet functions also need to be developed to improve outcomes in these high risk groups.

1.5.20 Introduction to Rafigrelide – a novel platelet lowering agent

Successful antithrombotic therapy is a balancing act between maximising efficacy that yields reduction in adverse outcomes, and minimising bleeding risk that would lead to adverse events. Persistent high platelet activity in elderly and T2DM patients despite current recommended antiplatelet therapy has raised interest in identifying strategies to optimise platelet inhibition in these high-risk population groups.

Hence in my second study, in healthy volunteers I assessed the effects of Rafigrelide, a novel, highly selective platelet lowering agent, on platelet count, platelet dependent thrombus formation and viscoelastic strength of the clot. Rafigrelide is a chemical analogue of anagrelide, which is currently used to reduce the platelet count in patients with myeloproliferative disorders. Rafigrelide has two distinct pharmacological activities similar to anagrelide: inhibition of megakaryocyte maturation and differentiation which gives rise to the platelet lowering effect (4 to 6 fold less effect than anagrelide) and inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase III (PDE III) characterised by positive inotropic and chronotropic effects resulting in vasodilation. The clinical symptoms of PDE III inhibition manifest mainly as headaches and cardiovascular side-effects including tachycardia and palpitations. Rafigrelide is stated to be better tolerated than anagrelide since the PDE III inhibition is 200 fold less effect than anagrelide.

Chapter 2 Hypothesis and Objectives

2.1 Study 1: Elderly stable CAD study

2.1.1 Hypothesis

Dual antiplatelet therapy with aspirin (75mg) and clopidogrel (75mg) inhibit platelet-dependent thrombus formation more powerfully in subjects aged ≥75 years than in younger individuals. In the presence of T2DM, the effect of age is reduced.

2.1.2 Primary Objectives

i. To determine the effect of age and age plus T2DM on the inhibition of platelet-dependent thrombus formation by dual oral antiplatelet therapy in patients with stable CAD.

2.1.3 Secondary Objectives

i. To identify platelet hyporesponsiveness to dual antiplatelet therapy (DAPT) and effect of age and T2DM on platelet hyporesponsiveness

ii. To evaluate the effect of age and T2DM on viscoelastic properties of thrombus role in patients after initiation of DAPT

iii. To study the role of inflammation in thrombogenicity and effect of age and T2DM on

    a. platelet reactivity upon stimulation with platelet agonists

    b. inflammation

    c. platelet activation markers

iv. To assess the ultrastructural changes to thrombus after initiation of DAPT

v. To evaluate the relationship between platelet reactivity measured by “point of care” platelet function assays and platelet dependant thrombus

2.2 Study 2: Rafigrelide (new platelet lowering agent) study – Phase 1 study

2.2.1 Hypothesis

1. Rafigrelide induced reduction in the platelet count will inhibit platelet dependent thrombus formation
2. Thrombus characteristics alter with the change in the platelet count

2.2.2 Primary Objectives

i. To assess the relationship between platelet count and platelet dependent thrombus formation with and without aspirin; using rafigrelide administration to lower platelet counts

ii. To evaluate the effect of platelet count on viscoelastic properties of thrombus

2.2.3 Secondary Objectives

i. To assess the safety and tolerability of rafigrelide administered with and without aspirin in healthy volunteers

Chapter 3 Methods

3.1 General methods

I conducted two hypothesis led clinical studies namely, Study 1: Elderly stable CAD study (a non-randomised, open labelled study) and Study 2: Rafigrelide study (an open-label, phase 1, single-sequence crossover study)

3.2 Study 1: Elderly stable CAD study design

This was a single-centre, non-randomised, open-labelled study assessing the efficacy of clopidogrel in patients with established CAD, with and without T2DM and over a wide age range. Patients who had stable, established CAD were recruited in three groups as below:

i. Age ≥ 75 years with type 2 diabetes mellitus (T2DM)

ii. Age ≥ 75 years without diabetes

iii. Age < 75 years without diabetes

Briefly, eligible patients with stable CAD were studied on day 1 to assess baseline blood thrombogenicity. They all were treated with clopidogrel 75mg once daily for 7 days and studied again on the eighth day. All patients took their routine prescribed medications as per standard practice.

The 4th group used for comparison included patients aged<75 years with T2DM and stable CAD. This group was recruited as a part of a previous research study by my senior research associate, Dr G Viswanathan. Similar inclusion / exclusion criteria were used to enrol patients in that study. They underwent Badimon chamber study, thromboelastography (TEG®) and platelet mapping™, VerifyNow® and Multiplate® assays at baseline and 1 week after clopidogrel therapy, using procedures identical to mine. For thrombus area from Badimon chamber study, I analysed all the images and recalculated the thrombus area for consistency and comparison with my data. I used the results from all three point of care platelet function assays for comparison (Viswanathan *et al.*, 2012b).

3.2.1 Patient selection

Eligibility criteria

Inclusion criteria

1. Patients with CAD and T2DM as defined below:
   CAD: Presence of any one of the following: history of angina and positive exercise tolerance test, history of (enzyme and/or Q wave) positive myocardial infarction, angiographic evidence (>50% stenosis of one vessel) or prior history of percutaneous or surgical coronary revascularisation
   T2DM: Diagnosed according to the WHO criteria, and on treatment
2. Patients who were taking aspirin 75mg once daily.
3. Patients who could provide written informed consent for participation in the trial prior to any study-specific procedures or requirements.

Exclusion criteria

1. Contraindications to clopidogrel, as per Summary of Product Characteristics (http://www.medicines.org.uk/emc/medicine/24207, accessed August 8th 2015) such as history of hypersensitivity, severe hepatic impairment and major / active pathological bleeding
2. Current smokers
3. Malignancy (currently diagnosed or under investigation)
4. Haematological disorders (Anaemia, malignancy, bleeding disorders)
5. Women of child-bearing potential as clopidogrel is contraindicated in pregnancy
6. Use of corticosteroids or non-steroidal anti-inflammatory drugs as it could increase bleeding risks and might affect the thrombotic milieu
7. Chronic liver disease (Cirrhosis, malignancy and patients with abnormal liver function tests, as clopidogrel bio-availability will be unpredictable)
8. End stage renal disease on dialysis as clopidogrel can be removed from the blood by haemodialysis
9. Patients who were already on clopidogrel for other clinical indications such as stroke and coronary intervention
10. Patients who were taking aspirin at doses other than 75mg once daily
11. Patients who were taking other antithrombotic agents such as dipyridamole, warfarin, low molecular weight heparin and newer oral anti-coagulants
12. Unable to consent

13. Use of other investigational study drugs within 1 year prior to entry into our study

3.2.2 Screening and recruitment

The study was approved by Regional Ethics Committee, Sunderland. All the appropriate approvals for recruitment from various centres list below where obtained. All participants were recruited from the Cardiology outpatient clinics in the Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle Diabetes Centre, Gateshead Diabetes centre, Diabetes clinics in general practice of Tyneside primary care trusts and cardiovascular community rehabilitation centres in Newcastle and Gateshead. Potential participants were identified by me and eligibility was verified from information documented in their clinical records. The patient information sheet, consent form and a letter of invitation were all posted at least 1 week prior to their planned outpatient appointments. Eligible patients were then approached in person at a suitable time point during their visit and I provided detailed verbal information regarding the study and answered any questions. Patients who provided written consent were given a further minimum of 24 hours to reflect on their agreement to participate in the study. Participants voluntarily agreed to participate in the study and no financial reimbursements were offered to them. Participants attended two appointments, visit 1 (Day 1) and visit 2 (Day 8) at Clinical Research Facility.

Visit 1 (Day 1)

Patients attended the research facility in the morning of Day 1. They fasted overnight and delayed taking their usual medications until study specific procedures were completed.

All subjects underwent the following assessments:

- Eligibility, understanding and consent to participate in the study were reconfirmed
- Subject number was assigned to each participant sequentially starting from 601
- Demographic data including height, weight, waist and hip circumference, resting heart rate and blood pressure were recorded
- Participant's medical history and routine prescription was checked
- Rose angina questionnaire was completed
- Badimon perfusion chamber experiment was then performed as described.

    - An 18G cannula was inserted in the antecubital fossa.
    - The first 2ml of blood was discarded to avoid activated platelets by venepuncture.
    - Ten ml blood was then collected for serum separation and Badimon perfusion chamber experiment was performed.
    - Effluent blood sample (5ml) from the chamber was collected for serum separation.
    - Another 5ml of venous blood was collected using the same cannula after disconnecting the chamber. This was used for platelet function assays and biomarker analysis.
    - The cannula was then removed.
- Participants were provided with breakfast
- Participants were observed to swallow the first dose of their IMP with at least 100ml of water under supervision
- Prior to discharge from the facility, subjects were given instructions for at-home self-administration of IMP
- A wallet sized card with information regarding the IMP, emergency contact details of myself and principal investigator, subject number and sponsors of the study was provided to the participants

Visit 2 (Day 8)

Participants returned to the research facility having fasted overnight and delayed taking their usual medications until study specific procedures were completed. All subjects underwent the following assessments:

- Returned investigational medicinal product collected to determine compliance
- Assessment of AEs and concomitant medications
- Badimon perfusion chamber experiment was then performed exactly as performed in Visit 1

3.2.3 Study specific procedures

Demographic data

Demographic data were collected during visit 1 to Clinical research facility. They were collected in a clinical research proforma and then stored electronically in a password protected computer. The following details were recorded:

- Participant's age was calculated as total number of completed years on the day of chamber study
- Height was measured in centimetres (cm)
- Weight in kilograms (kg)
- Body mass index (BMI) was calculated as body weight (kg) divided by height squared (m^2)
- Waist circumference (cm) was measured at midpoint between the lower rib margin and the iliac crest
- Hip circumference (cm) was measured at level of the widest circumference over the greater trochanters
- Waist hip ratio (WHR) was calculated as waist circumference divided by hip circumference

Vital signs

Blood pressure and pulse were measured in the sitting position after the participants rested for at least 5 minutes. The left arm was used for blood pressure measurements.

General Biochemistry

Patient's blood samples were analysed at the nationally accredited clinical biochemistry laboratory at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. Samples were identified by study number, participants name and date of birth. Quality control and quality assurance were in keeping with local laboratory protocols.

The following parameters were assessed:

Fasting plasma glucose, mmol/L HbA1c (DCCT aligned), %

Blood urea mmol/L Serum creatinine, µmol/L

Serum high sensitivity C reactive protein, mg/dl

Lipid profile (fasting) including total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides, mmol/L

General Haematology

Patient's blood samples were analysed at nationally accredited general haematology and coagulation laboratories at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. Quality control and quality assurance were in keeping with local laboratory protocol. Blood was drawn into a tube containing potassium ethylene diamine tetra acetic acid (EDTA) anticoagulant for full blood count analysis and the following parameters were assessed:

Haemoglobin, g/L	Haematocrit, %
Red blood cells (RBC), 10^{12}/L	Platelet count, 10^{9}/L
Prothrombin time, sec	Activated partial thromboplastin time, sec

Plasma fibrinogen was measured using the von Clauss quantitative method. Citrated tubes were preferred over heparinised tubes for coagulation analysis including fibrinogen as citrate preserves labile coagulation factors thus ensuring better reproducibility. The von fibrinogen assay is the most reliable method for use in laboratories according to the British Society of Haematology guidelines (Mackie *et al.*, 2003).

3.2.4 Investigational Medicinal Product

This was a non-randomised, open labelled study. Hence there was no randomisation or blinding involved.

Clopidogrel bisulphate 75mg (Plavix®, Sanofi-Aventis) was used as the investigational medicinal product (IMP). This was provided by the pharmacy production unit at Royal Victoria Infirmary, Newcastle upon Tyne (MHRA Unit IMP license number 17736), as individual subject dose packs. These supplies were open-label, packaged as a commercial pack containing 8 blistered tablets.

All supplies were labelled with the study name, subject number, medication identification number, dosage form (including product name and quantity in pack), directions for use, storage conditions, expiry date, batch number or packing reference number, the statements 'For clinical trial use only', "'Keep out of reach of children', and the Sponsor's name and address.

As per the protocol, participants took IMP from Day 1 to Day 7 (Seven tablets in total), but I chose to provide them with 8 tablets for two reasons: 1) pill counting to monitor compliance 2) additional spare IMP to be used by participants if they lose one tablet.

3.2.5 Pharmacovigilance and data monitoring

An adverse event (AE) was defined as any untoward medical occurrence which does not necessarily have a causal relationship with the treatment. “Treatment” includes all investigational products administered during the course of the study. Medical conditions/diseases present before starting study treatment are only considered adverse events if they worsened after starting study treatment.

Any serious adverse event was deemed to be recorded throughout the duration of the trial until 5 days after cessation of study drug. Non-serious adverse reactions were recorded and monitored throughout duration of trial until 5 days after cessation of study drug. Participants were provided with a wallet sized card with contact details of myself and principal investigator if they experienced any further adverse events after the final visit.

I was responsible for monitoring and reporting all adverse events. The intensity (mild, moderate, severe), relationship to investigational product, and outcome were recorded for all AEs. All AEs were followed to closure, and reviewed, confirmed, and classified by myself. I was also delegated the task of informing MHRA and REC of all serious adverse events / reaction [SAE/SAR including Suspected Unexpected Serious Adverse Reaction (SUSAR)] occurring during the study. A study data monitoring committee and adverse event monitoring committee oversaw the safety of the study.

3.2.6 Statistical methods

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc, NY, USA) and Graphpad prism version 6 (Graphpad software, CA, USA). Graphs were constructed after data analyses using Graphpad software.

Elderly stable CAD study was a non-randomised open labelled study comparing four study groups. For continuous variables, mean and standard deviations were calculated; for dichotomous variables number and percent are shown. For variables that were normally distributed with equal variances, one way analysis of variance (ANOVA) was performed to measure statistical significance across the four groups and

post hoc test was performed with Bonferroni correction to determine which pairs of groups were significantly different. For variable that were not normally distributed, Kruskal-Wallis H Test was performed to determine statistical differences across the four groups and Mann-Whitney U test was performed to determine which groups were significantly different.

To assess the effect of age at baseline and after the addition of clopidogrel, patients were divided into two groups (age<75 vs. age≥75). Age category was the grouping variable and all the other data from the study such as thrombus area, platelet function test indices, coagulation and inflammatory biomarker levels were the test variables. I performed analysis adjusting for sex, body mass index, waist-hip ratio and basic laboratory tests (creatinine, lipids). Independent sample t test (normally distributed variables) and Mann-Whitney U test (non-parametric) were used to look for statistical differences between the groups. Similar test were repeated for T2DM vs. Non-DM, using Diabetes category as the grouping variable.

The volunteers in each group were carefully chosen and were well matched except for the difference in age and diabetic status. Hence I analysed the effect of the intervention in each group without including covariates in the analysis. For continuous variables mean and standard deviations were calculated and paired sample t test was used to measure statistical significance. For dichotomous variables, the percentage in each group was measured and chi-square estimates were used to calculate statistical significance. For highly skewed variables, I used non-parametric paired t test and repeated the analysis with log-transformation.

For correlation analysis, Pearson's method and linear regression analysis were employed for normal data and Spearman's method and non-linear regression analysis method were employed for non-normally distributed data.

Data from our previous study were used to estimate the sample size needed for this study. In order to detect a 20% reduction in thrombus area from baseline 1 week after clopidogrel therapy with 80% power, we estimated that a sample size of 45 patients per group was required.

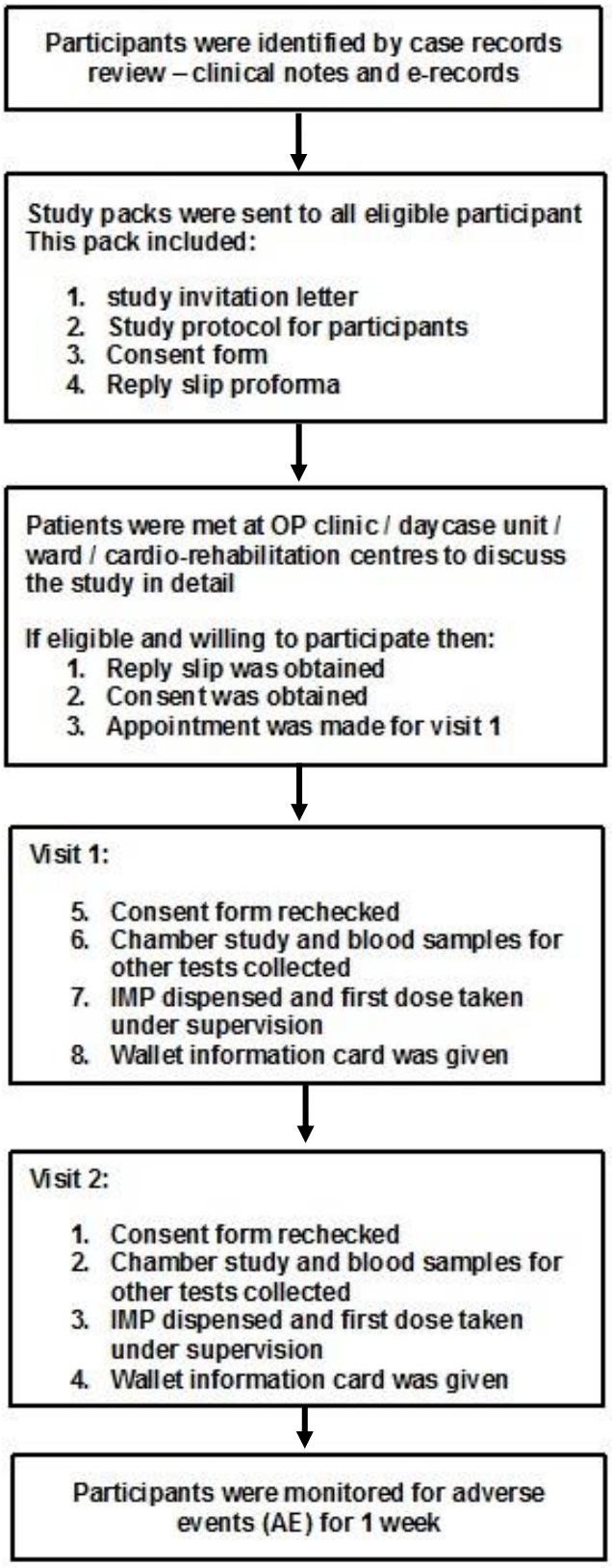


Figure 3.1 Participant Pathway for Elderly stable CAD study

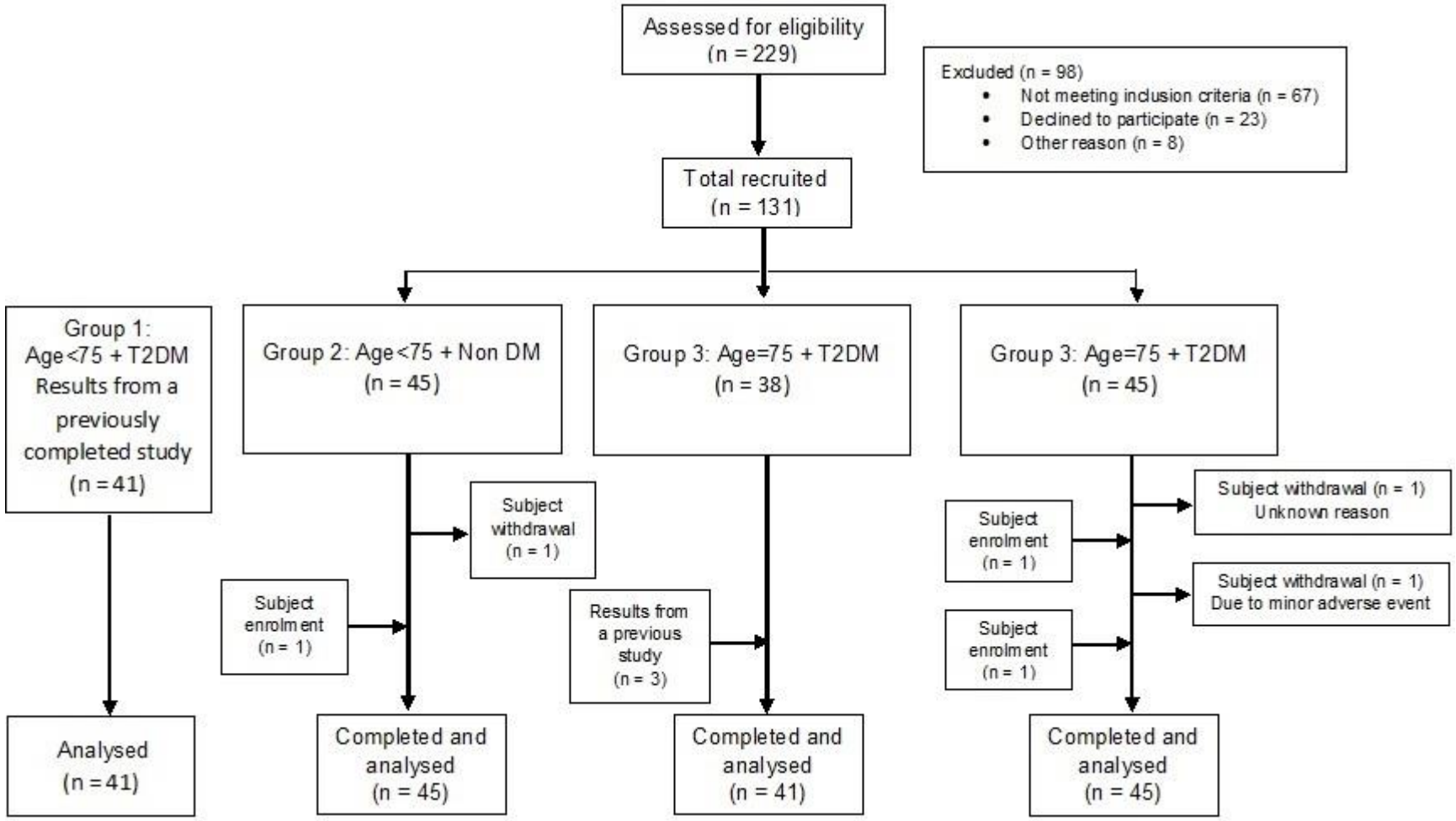


Figure 3.2 Elderly stable CAD study – Flow chart

3.3 Study 2: Rafigrelide study design

This study used a Phase 1, non-randomised, open-label, single-sequence, crossover design to investigate the effect of rafigrelide with or without aspirin on thrombus formation and clot kinetics. This study was conducted in healthy subjects and comprised of 2 treatment periods where subjects received rafigrelide for 14 days either alone (Treatment Period 1) or in combination with aspirin (Treatment Period 2). In each period, subjects underwent blood thrombogenicity studies which included the ex-vivo Badimon perfusion chamber and thromboelastography (TEG) along with tests for plasma drug concentrations, and platelet count assessments.

Upon qualifying for the study, subjects were alternately assigned to 1 of 2 schedules (Panel A or Panel B) (Figure 3.3), for the assessment of pharmacokinetic (PK) and pharmacodynamic (PD) measures. The subjects followed the same panel assignment for both treatment periods.

In both treatment periods, subjects received 4mg/day of rafigrelide for 14 consecutive days starting on the morning of Day 1. In treatment period 2, subjects also received 75mg/day of aspirin administered from Day 1 until Day 20 (Panel A) or Day 17 (Panel B).

In both treatment periods, subjects returned to the clinical research facility for 6 visits. For 4 of these visits subjects underwent pharmacokinetic and/or pharmacodynamic assessments. For these visits, subjects were admitted to the clinical research facility on the evening prior to the assessment day and were discharged the next day (the visit day), following the completion of all assessments. On the other 2 visit days, subjects undergo “haematology” assessments on an outpatient basis. The day of each visit varied according to the assigned test panel. An overview of visit days is outlined in Table 1.

During the remainder of the treatment period (when not confined in the Clinical Research Facility), subjects self-administered investigational product at home, and recorded daily dosing information (dates/times), adverse events (AEs), and any concomitant medications in subject diary cards.

3.3.1 Patient selection

Eligibility criteria

Inclusion Criteria

Subjects meeting all of the criteria listed below were included in the study:

1. Male volunteers aged between 18-50 years willing to fully comply with study procedures and restrictions
2. Satisfactory medical assessment as determined by medical history, physical examination, vital signs, 12-lead ECG, and clinical laboratory evaluation (haematology, biochemistry, urinalysis)
3. Ability to provide written informed consent
4. Body Mass Index (BMI) between 18.5-30.0 kg/m^2
5. Haemoglobin of 12.0g/dL or greater and platelet count between 200-400x109/L
6. Ability to swallow all investigational medicinal products

Exclusion Criteria

Subjects were excluded from the study if any of the following criteria were met at Screening or at baseline (Day –1 [or Day 1 when specified] of treatment period 1) (if reassessed):

1. Current or recurrent disease that could affect the action, absorption or disposition of rafigrelide, aspirin, or clinical laboratory assessments

2. Current or relevant history of physical or psychiatric illness, any medical disorder that may require treatment or make the subject unlikely to fully complete the study, or any condition that presents undue risk from the investigational medicinal product (IMP) or study procedures

3. Use of any prescription medication, non-prescription medication or over-the-counter medicine (including multivitamin, herbal or homeopathic preparations) within 14 days prior to the first dose of investigational medicinal product

4. Use of anti-aggregant, anti-platelet or anti-coagulant therapy, including aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), and paracetamol within 14 days prior to the first dose of investigational medicinal product

5. Abnormal clotting parameters as assessed by both TEG® and platelet mapping on initial Screening and Day 1 of Treatment Period 1
6. Known or suspected intolerance or hypersensitivity to the investigational medicinal product(s), including aspirin, closely related compounds, or any of the stated ingredients
7. History of alcohol or other substance abuse within the last year
8. A positive screen for alcohol or drugs of abuse
9. Consumption of more than 21 units of alcohol per week or 3 units per day
10. Use of an investigational medicinal product within 30 days prior to receiving the first dose of investigational medicinal product or active enrolment in another drug or vaccine clinical trial
11. A positive human immunodeficiency virus (HIV) antibody screen, Hepatitis B surface antigen (HBsAg), or Hepatitis C virus (HCV) antibody screen
12. Donation of blood or blood products (e.g. 450mL or more of plasma or platelets) within 60 days prior to receiving the first dose of investigational medicinal product
13. Prior screen failure, participation, or enrolment in this study

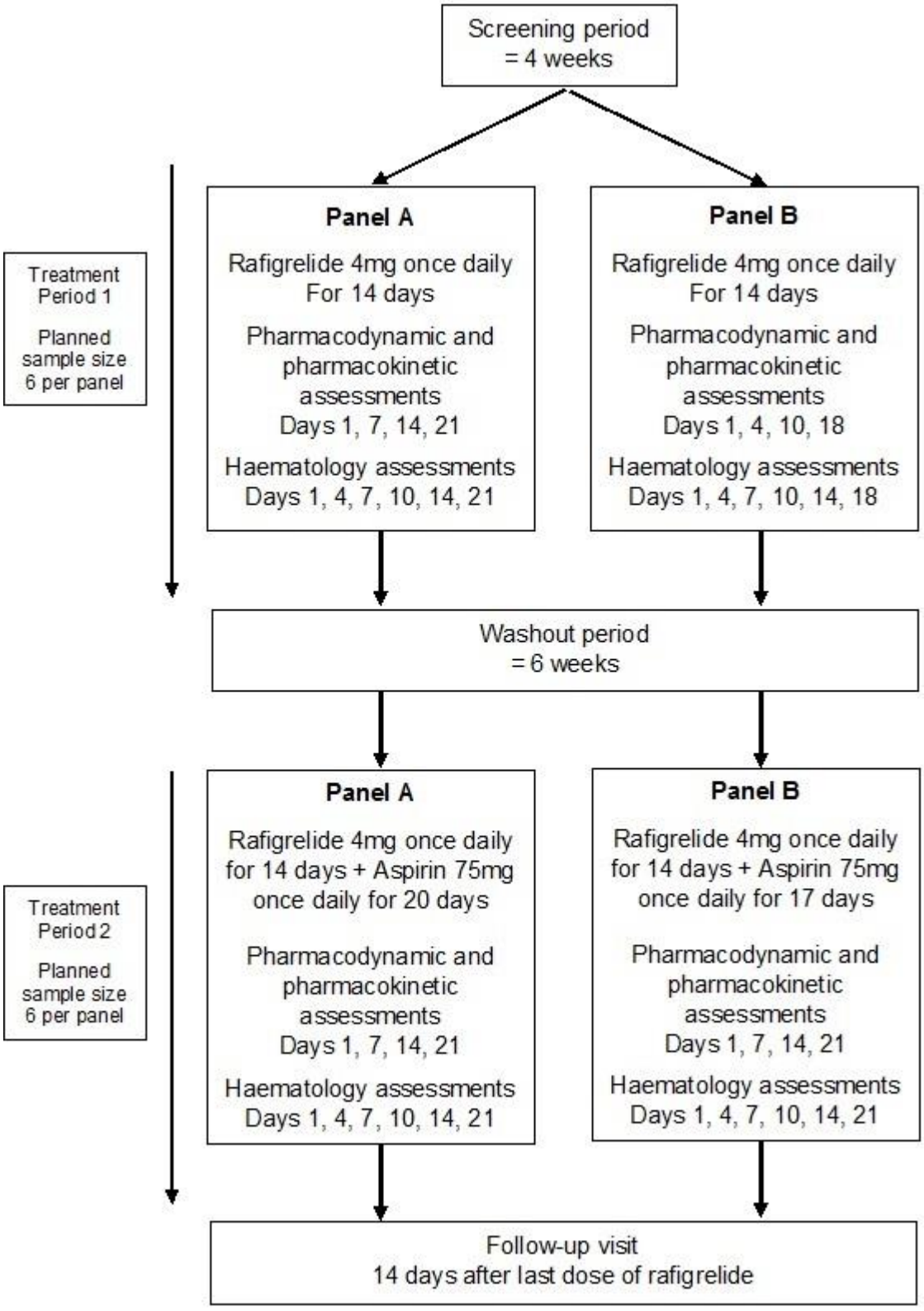


Figure 3.3 Overview of study design

Subject Restrictions

Reproductive Potential

All subjects, including those who were sterilised (vasectomy), were strongly advised to use a condom. I also suggested that their female partners used at least one of the following medically acceptable forms of contraceptives: Intrauterine devices, hormonal contraceptives (oral, depot, patch, injectable, or vaginal ring), double barrier methods (e.g. condoms or diaphragms with spermicidal gel or foam).

Male subjects were not permitted to donate sperm and were advised against unprotected sex during the study or during the 84 days after the last dose of investigational product.

Other Restrictions

During confinement in the Clinical Research Facility, subjects were fasted for at least 2 hours before, until 1 hour after, taking investigational product, and abstained from tobacco use until discharge.

From the first dose of investigational product until the completion of the follow-up visit, subjects were requested not to engage in any activities associated with the potential for bruising or bleeding, and were advised to refrain from taking any prescription or over the counter medication, and alcohol use.

3.3.2 Screening and recruitment

Screening procedures were completed within 28 days prior to receiving the first dose of investigational product. Eligibility criteria were confirmed and informed consent was obtained prior to the initiation of any study-related procedures. Subjects who failed to meet all inclusion/exclusion criteria were not permitted to be rescreened.

Screening procedures included:

- Demographics (including date of birth, gender, ethnicity and race)
- Height and weight
- Medical history and concomitant medications
- Complete physical examination, vital signs and 12-lead electrocardiogram (ECG)

- clinical laboratory assessments including biochemistry, haematology, urinalysis, screen for drugs of abuse and alcohol and virology screen (HIV, HBsAg, and HCV antibody)
- Thromboelastography (TEG)

Treatment Periods 1 and 2

This was a 2-period, single-sequence, crossover study in which each subject was scheduled to receive rafigrelide at a dose of 4mg/day administered alone (Treatment Period 1) and in combination with aspirin at a dose of 75mg/day (Treatment Period 2).

In both treatment periods, eligible subjects returned to the Clinical Research Facility on Day -1. Subjects who completed pre-admission assessments were then admitted to the clinical research facility overnight.

Admission to the Clinical Research Facility (Day –1):

Eligible subjects returned to the Clinical Research Facility on Day –1. The following pre- admission assessments were performed:

- Review/confirm eligibility criteria (Treatment Period 1 only)
- Medical and drug history
- Complete physical examination, vital signs, 12-lead ECG
- Clinical laboratory assessments (biochemistry, haematology, and urinalysis) and Drug and alcohol screen
- Review AEs and concomitant medications since Screening

Subjects who successfully completed the pre-admission assessments and procedures were then admitted to the Clinical Research Facility overnight in order to complete the study related assessments and procedures on Day 1.

Visit 1 (Day 1)

On the morning of Day 1, following at least a 2 hour fast, all subjects underwent the following assessments, prior to dosing:

- Vital signs
- Blood sample for haematology panel
- Blood sample for TEG, PK assessment and Badimon perfusion chamber

- Assessment of AEs and concomitant medications (if applicable)

Treatment Period 1 only: Upon completion and analysis of the TEG assessment, final eligibility was determined. Subjects were then assigned a subject number, prior to the first dose of investigational medicinal product. Investigational medicinal product was administered in the Clinical Research Facility under supervision (following completion of all other assessments).

Prior to discharge from the facility, subjects were given instructions for at-home self-administration of investigational medicinal product and the use of Subject Diary Cards. Subjects returned to the Clinical Research Facility for Visits 2, 3 and 4 according to the schedule of their panel assignment.

Panel A: Visits 3, 5, and 6 / Panel B: Visits 2, 4, and 6

Subjects returned to the Clinical Research Facility in the evening for check-in according to the following schedule:

Panel A: Day 6, Day 13, Day 20, respectively (for testing the following morning on Day 7, Day 14, and Day 21, respectively)

Panel B: Day 3, Day 9, Day 17, respectively (for testing the following morning on Day 4, Day 10, and Day 18, respectively)

Upon admission to the Clinical Research Facility, all subjects underwent the following assessments:

- Returned investigational medicinal product and Subject Diary Cards collected from preceding visit, and compliance determined
- Vital signs
- Assessment of AEs and concomitant medications

The next morning, following at least a 2 hour fast, the following assessments were performed:

- Vital signs
- Blood sample for haematology panel
- Blood sample for TEG, PK assessment, Badimon perfusion chamber
- Physical examination (Visit 4 only)

- Investigational medicinal product administered in the Clinical Research Facility, following completion of all other assessments (does not apply to Visit 6)
- Assessment of AEs and concomitant medications

Prior to discharge from the facility, subjects were given instruction on at-home self-administration of investigational medicinal product and the use of Subject Diary Cards.

Panel A: Visits 2 and 4 / Panel B: Visits 3 and 5

Subjects returned to the Clinical Research Facility as an outpatient and a blood sample for haematology panel was obtained.

Washout Period:

There was a washout period of at least 6 weeks between the last dose in Treatment Period 1 and the first dose in Treatment Period 2. Subjects only received investigational product in Treatment Period 2 if their platelet counts had returned to within their baseline pre-treatment levels.

Follow-up Period:

Approximately 14 days after the last dose of rafigrelide in Treatment Period 2 (which corresponds to 8 days after the last dose of aspirin for subjects in Panel A, and 11 days after the last dose of aspirin for subjects in Panel B), subjects returned for a follow-up visit. During this visit, the following assessments were performed:

- Vital signs and 12-lead ECG
- Clinical laboratory assessments (biochemistry, haematology, and urinalysis)
- Follow-up of any concomitant medications that are new and/or on-going since the last visit
- Follow-up of any AEs and/or SAEs that are new and/or on-going since the last visit

Removal of subjects from study treatment or assessment:

A subject was permitted to stop (in full or in part), taking investigational product or to withdraw from the study at any time for any reason without prejudice to their future medical care by the physician or at the institution. All discontinued subjects underwent the protocol specified follow-up visit.

If platelet count fell below $100x10^9$/L, subjects were to be immediately discontinued from investigational product, but continued to be monitored and to complete all scheduled study assessments for that treatment period. Subjects were also to be removed from treatment (but continued all study procedures) if platelet count fell below $150x10^9$/L and decreased by more than a $20x10^9$/L per day on average (e.g., $60x10^9$/L over 3 days).

If a subject was removed from investigational product in treatment period 1 due to a drop in platelet count, the subject was permitted to complete treatment period 2 receiving rafigrelide at a reduced dose of 3 mg/day.

3.3.3 Study specific procedures:

Safety:

Safety was evaluated by collecting reported AEs at scheduled intervals throughout the study and by the assessment of physical examination findings, vital signs, clinical laboratory parameters, and 12-lead ECGs.

Medical history and concomitant medications

A complete medical and medication history, as well as demographic information were recorded including:

- Date of Birth, Sex, Race and ethnicity
- Recent ingestion of medication (within 30 days prior to Screening)
- History of respiratory, cardiovascular, renal, gastrointestinal (GI), hepatic, endocrine, haematological, neurological, psychiatric and other diseases

Physical Examination

A complete physical examination was performed at screening, Day -1 and the final day after the last dose of investigational product (Day 21 for Panel A and Day 18 for Panel B). This included the following: General appearance, skin, Head, Eyes, Ears, Nose and Throat (HEENT), Spine/Neck/Thyroid, Musculoskeletal, Respiratory, Cardiovascular, Neurological and Abdominal (including liver and kidneys) examination.

Electrocardiogram

A 12-lead ECG was performed at screening, Day -1 and at follow-up. Subjects rested in a supine position for at least 5 minutes prior to collecting the 12-lead ECG. Date, time, assessment of 'normal' or 'abnormal', and the heart rate (RR, PR, QT [corrected values where applicable]) and QRS were collected. The eligibility of the subject was based on 'normal' 12-lead ECG at Screening and Day –1. The determination of normal/abnormal results and the clinical significance was performed by me.

Vital Signs

Measurements of vital signs (blood pressure and pulse) were performed according at screening, on the days of admission to the Clinical Research Facility, and on the inpatients assessment days.

Height and Weight

Height and weight were collected at screening. Height was measured in centimetres (cm) and weight in kilograms (kg). Body Mass Index was calculated at screening in order to determine subject eligibility to enrol in the study.

Clinical Laboratory Measurements

All laboratory assays were performed according to the laboratory's normal procedures. Reference ranges were supplied by the laboratory and used to assess the laboratory data for clinical significance and out-of-range pathological changes. If a subject's laboratory values were out of normal range and deemed to be 'clinically significant', this was recorded as an AE. Clinical laboratory evaluations were performed at Screening, Day -1, and at follow-up.

The following laboratory assessments were performed:

Biochemistry

Patient's blood samples were analysed at nationally accredited clinical biochemistry laboratory at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. Fasting blood samples (6.0mL) for serum biochemistry were collected and the following parameters were assessed:

Sodium Potassium

Calcium	Urea
Creatine kinase (total)	Albumin
Total protein	Troponin I mg/dl
Creatinine µmol/L	

Changes from Screening were recorded as an AE if clinically significant.

Haematology

Patient's blood samples were analysed at nationally accredited general haematology and coagulation laboratories at Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne. A 3.0mL sample of blood was drawn into a tube containing potassium ethylene diamine tetra acetic acid (EDTA) anticoagulant at screening, at Day 1, at all inpatient and outpatient visits (as outlined in Table 1 for each panel), and at follow-up. The following parameters were assessed:

Haemoglobin , g/L	Haematocrit, %
Red blood cells (RBC), 10^{12}/L	Mean corpuscular volume
Platelet volume	Platelet count
Mean corpuscular haemoglobin	Mean corpuscular haemoglobin concentration

White blood cell (WBC) count – total and differential

Neutrophils	Lymphocytes
Monocytes	Eosinophils
Basophils	

Changes from Screening were recorded as an AE if clinically significant.

Thromboelastography

A 4.0mL sample of blood was drawn into a syringe for performing thromboelastography (TEG) at screening and on the days when subjects were inpatients at the Clinical Research Facility. TEG was used to assess blood coagulation, clot kinetics and platelet function. This was performed by me.

Urinalysis

A 10mL midstream urine sample was collected at screening, Day -1, and at follow-up. The following parameters were assessed: Specific Gravity, Protein, Ketones, Bilirubin, pH, Glucose, Blood. Microscopic examination was conducted if protein and/or blood

is/are detected during urinalysis. This consisted of RBC, WBC, casts and bacteria. Changes from screening were recorded as an AE if clinically significant.

Drug and Alcohol Screen

A urine drug screen was performed at Screening and Day –1 of each treatment period. Urine sample was collected in a polystyrene (10mL) tube.

Urine samples were tested for the following: Methadone, Benzodiazepine, Cocaine, Amphetamine/Methamphetamine, Barbiturates, Tricyclic Antidepressants (TCA), Opiates, Tetrahydrocannabinol (THC)

An alcohol breath test was performed at Screening and on Day –1 of each treatment period.

Virology Screen

At Screening, a blood sample of approximately 15mL was drawn into a serum separator tube to test for the presence of HIV, HBsAg, and HCV antibody. The test results were confirmed negative prior to enrolment in the study.

3.3.4 Investigational Medicinal Product(s)

- Rafigrelide: supplied as 0.5mg oral capsules (open-label)
- Aspirin: supplied as 75mg oral enteric-coated tablets (open-label)

Labelling

Rafigrelide was provided by Shire Pharmaceuticals Development Ltd., Basingstoke, UK, to the pharmacy production unit at Royal Victoria Infirmary, Newcastle upon Tyne (MHRA Unit IMP license number 17736), as labelled individual subject dose bottles. These supplies were open-labelled and presented as 0.5mg Swedish orange size 0 capsules packaged in 30ml high-density polyethylene (HDPE) bottles. The batch number of Rafigrelide was PR100103-001.

Aspirin (75mg enteric-coated tablets) was provided as individual subject dose packs. These supplies were also open-label, packaged as a commercial pack containing 28 blistered tablets. The batch number of Aspirin was PR100103-002. Labels for both rafigrelide and aspirin were multi-part computer-generated labels.

One part adhered to the supplies container and the other tear-off part was removed at the time the investigational medicinal product was dispensed to the subject and adhered to the drug accountability form. All supplies were labelled with the protocol number, subject number, medication identification number, dosage form (including product name and quantity in pack), directions for use, storage conditions, expiry date, batch number or packing reference number, the statements ‘For clinical trial use only’, “’Keep out of reach of children’, and the Sponsor's name and address.

Storage

As the Investigator, I had the overall responsibility for ensuring that investigational medicinal product is stored in a secure, limited-access location. Limited responsibility was delegated to the pharmacy or member of the study team, but this delegation was documented. Investigational medicinal products were distributed by the pharmacy or nominated member of the study team.

Investigational medicinal products were stored in accordance with labelled storage conditions. Temperature monitoring was strictly adhered to at the site’s storage location to ensure that the investigational medicinal product was maintained within an

established temperature range. I was responsible for ensuring that the temperature was monitored throughout the total duration of the study and that records were maintained; the temperature was monitored continuously by using a calibrated mechanical recording device and minimum and maximum thermometric values over a specific time period were recorded and retrieved as required. The device was manually reset upon each recording.

Allocation of Subjects to Treatment

Screening numbers were assigned sequentially to all subjects as they consent to take part in the study. This was a 4-digit number starting at 0001. This number was assigned to subjects according to the sequence of presentation for study participation. For screen failures, the Screening Number was the identifying number used throughout the CRF.

A 4-digit subject number was allocated to the subject on Day 1 of Treatment Period 1, after reconfirming eligibility. Subjects were assigned subject numbers starting at 1001. This number was the identifying number used throughout the CRF. All odd-numbered subjects (e.g. 1001, 1003, 1005, etc.) who were eligible for dosing on Day 1 of Treatment Period 1 were assigned to Panel A. All even-numbered subjects (e.g. 1002, 1004, 1006, etc.) who were eligible for dosing on Day 1 of Treatment Period 1 were assigned to Panel B.

Replacement subjects were assigned a subject number that corresponded to the same panel assignment (Panel A or Panel B) of the subject that they replaced.

3.3.5 Pharmacovigilance and data monitoring

All AEs, including those associated with the protocol, were collected from the time written informed consent was obtained until the defined follow-up period and were recorded on the appropriate AE pages in the CRF and in source documents. All AEs were followed to closure (the subject's health had returned to his baseline status or all variables had returned to normal), an outcome was reached, stabilisation (the Investigator does not expect any further improvement or worsening of the event), or the event was otherwise explained regardless of whether the subject continued to participate in the study. The intensity (mild, moderate, severe), relationship to investigational product, and outcome were recorded for all AEs. All AEs were followed to closure, and reviewed, confirmed, and classified by myself. When appropriate,

medical tests and examinations were performed so that resolution of event(s) could be documented.

All initial and follow-up SAE reports were reported by myself to the sponsors within 1 business day of the first awareness of the event. I completed, verified the accuracy of information recorded with the corresponding source documents, signed, and dated the Clinical Trial Serious Adverse Event Form, and sent the form to the sponsor's Pharmacovigilance Department.

The Sponsor took the responsibility to notify the relevant regulatory authorities, European Union (EU) central IECs of related and unexpected SAEs. The principal investigator and I were responsible for notifying the local Institutional Review Board (IRB), local Independent Ethics Committee (IEC), and the relevant local regulatory authority of all SAEs that occurred.

An electronic case report form (eCRF) was used to permit consistent collection of data; source-verified data were subjected to both manual and electronic checks to ensure data integrity and accuracy. Quality control and data validation procedures were applied to ensure the validity and accuracy of the clinical database.

3.3.6 Statistical methods

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc, NY, USA) and Graphpad prism version 6 (Graphpad software, CA, USA). Graphs were constructed after data analyses using Graphpad software. Baseline characteristics were summarised using descriptive statistics. Platelet count, mean platelet volume, thrombus area, standard TEG® parameters and Platelet mapping™ was compared between and within the treatment panel using repeated measures analysis of variance (ANOVA) with time and panel as fixed factors. Separate analysis was performed for Panel A and Panel B in both the treatment periods. All results are expressed as mean±SD unless specified otherwise. A p value of less than 0.05 with two tailed measurement was considered statistically significant.

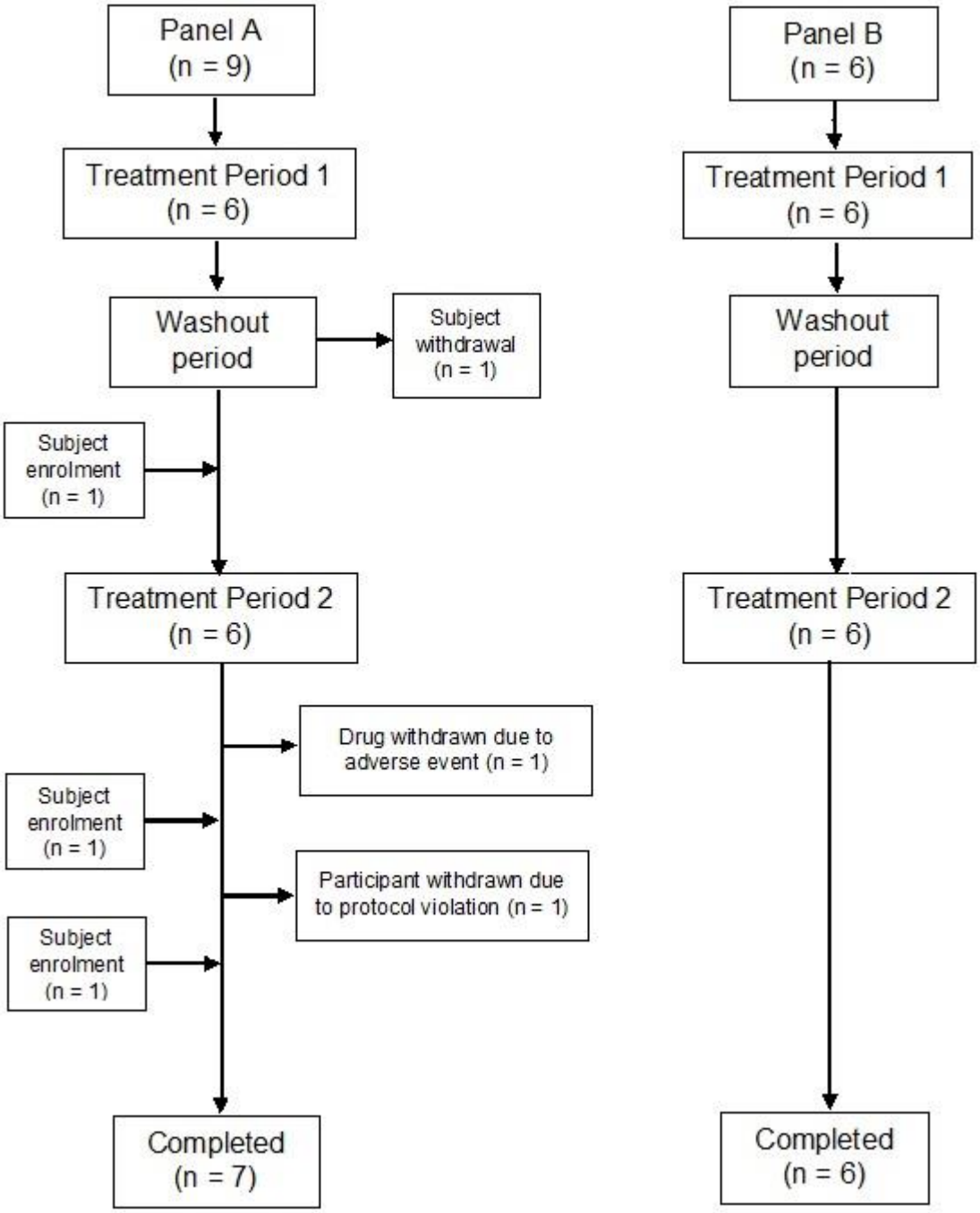


Figure 3.4 Rafigrelide study – Flow chart

3.4 Special methods

3.4.1 Badimon perfusion chamber – platelet dependent thrombus formation

Principles of the Badimon perfusion chamber

The Badimon perfusion chamber system is a well validated model of deep arterial injury and thrombosis (Napoli *et al.*, 2006), and has previously been used to evaluate the effects of novel antithrombotic agents in human participants in different disease states for over fifteen years (Lev *et al.*, 2001; Osende *et al.*, 2001; Sarich *et al.*, 2003). This model enables the measurement of thrombus formation in native (non-anticoagulated) whole blood triggered by exposure to physiologically relevant substrate (e.g. collagen in tunica media), and under different rheological conditions mimicking flow in stenosed coronary arteries. The chamber has been designed to "retain the cylindrical shape typical of the vasculature, to be flexible enough to accept a variety of biologic and prosthetic materials, and to simulate a broad range of physiologic flow conditions" in either an ex vivo or in vitro perfusion system (Badimon *et al.*, 1987). The consistency and reproducibility of the chamber system has been re-validated again recently in a cohort of 24 healthy volunteers (Lucking *et al.*, 2010).

The Badimon chamber in our study is an ex vivo model that mimics:

i. the flow conditions in normal and moderately stenosed arteries
ii. atherothrombotic plaque rupture

Platelet dependent thrombus (PDT) formation in Badimon chamber experiments involves two basic physiological principles of arterial thrombosis. These include:

i. collagen mediated thrombus generation
ii. shear induced platelet activation

Freshly slaughtered pig's aorta was used as a source of collagen in our experiments. The tunica intima (endothelial layer) was surgically dissected and the tunica media, which is rich in collagen, was used as the substrate (simulating an atherothrombotic plaque rupture) in the Badimon chamber. This layer was exposed to the flowing blood.

Platelet activation occurs in the coronary circulation at the site of luminal narrowing produced by atheromatous plaques. At these sites the laminar flow is disrupted and

converted to turbulent flow, thereby increasing the shear rate of flowing blood. In the Badimon chamber system, reduction of luminal diameter increases the velocity of flowing blood within the chamber. This also disrupts the laminar flow of blood. As a result there is re-distribution of platelets in a moving column of blood, with more platelets accumulating in the periphery than in the centre of the flow. This enables activation of the platelets when they come into contact with the tunica media and aggregation of platelets when they come into contact with each other (Ahlqvist, 2001).

Standardisation of the Badimon perfusion chamber

Our reference laboratory at Mount Sinai Medical School, New York, USA, performed a series of experiments to assess thrombus generation at varying shear rates, flow rates and duration of blood flow through the Badimon chamber. Iridium 111 labelled platelets were used to assess PDT burden at time intervals from 1 minute to 20 minutes; flow rates at 5 to 20 ml/minute (blood velocity of 2.65 to 42.3 cm/sec) and shear rates from 106 per sec to 3380 per sec (Badimon *et al.*, 1986).

This experiment demonstrated a steep increase in thrombus generation from 1 to 5 minutes but reached a plateau after 10 minutes. Thrombus generation increased with increase in flow rates from 5ml/minute to 10 ml/minute but not at higher rates. Effects of shear rates on thrombus generation were studied both by radio labelled platelets and by image analysis. At low shear states (inner luminal diameter: 2.0mm; vessel wall shear rate: 100-200s-1, mean blood velocity: 5.3 cm/sec; Reynolds number: 30), fibrin rich thrombus was formed and at high shear rates (inner luminal diameter: 1.0mm; vessel wall shear rate: 1500-3000s-1, mean blood velocity: 21.2 cm/sec; Reynolds number: 60) platelet rich thrombus was formed. Assessment of thrombus quantity, as measured by total thrombus area (platelet and fibrin content combined), showed higher thrombus generation at high shear states compared to low shear states.

In our laboratory, we standardised our chamber experiments to obtain adequate amount of thrombus but using the lowest possible volume of blood. Hence, I used two chambers with inner diameter of 1.0 mm (high shear chambers) and one chamber with inner diameter of 2.0 mm (low shear chamber) to generate high shear rates [Reynolds number 60 (measure of non-laminar flow), shear rate of 1920 per sec] and low shear rates [Reynolds number 30, shear rate of 500 per sec] respectively. I used a flow rate of 10 ml/min for a duration of 5 minutes (total of 50 mls of blood).

An 18 G cannula (inner diameter 1.2mm, maximum flow rates 90-100 ml/min) and Tygon tube (number 14.0, inner diameter 1.6mm) were used in all subjects in order to maintain the same shear force within the Chamber. The same peristaltic pump (Masterflex® pump, Thermo-Fisher Scientific Inc, MA, USA) was used in all the experiments at a fixed flow rate of 10ml/min. Quality control experiments were performed at fixed time intervals to check for consistency as detailed elsewhere.

Technique of Badimon perfusion chamber system

The system consisted of:

i. three small plexi glass chambers – one low shear and two high shear chambers
ii. three plexi glass over-chambers with a screw to secure the substrate inside the chambers
iii. a plexi glass container designed to accommodate the chambers connected in series and which easily sits in a water bath
iv. four plastic connectors – connects the chambers in series

Each of the chambers contain two split individual units, called the upper lid unit and the lower core unit. The lower core unit has cylindrical channels carved out in the rectangular plexi glass block – dimension of 1.0mm×25.0mm and 2.0mm×25.0mm for high shear and low shear chamber respectively. The upper lid unit has a rectangular raised central projection which is 25.0 mm long with a thickness of 1.0mm and 2.0mm for high shear and low shear respectively.

A piece of porcine aorta stripped of its intimal layer to expose the underlying thrombogenic tunica media (substrate for thrombus formation) was placed between the upper lid and lower core unit with the exposed medial layer forming the roof of the cylindrical flow channel in the chambers. The chambers were then placed in the over chamber and were held in position by the screw that opposed the upper lid and lower core units together, sandwiching the substrate between them (Badimon *et al.*, 1986). This enabled the flowing blood to come in contact with the exposed media, thereby initiating platelet activation and thrombus formation. The flow channels of the chamber were then connected in series by plastic connectors with matching diameters. For all my chamber studies, I arranged the chambers in the following order, proximal to distal, low shear unit followed by first high shear unit followed by second high shear unit. This whole assembly was then placed inside the plexi glass container which was in turn

placed in a circulating water bath maintained at 37 degrees Celsius (body temperature) (Figure 3.5).

18 G cannula in participant's antecubital fossa was connected to a three way tap. The other end of the tap was connected to the proximal end of the chamber system using 14.0 Tygon® tubing (Cole Palmer, IL, USA). The third end of the tap was connected to a container that contained 0.01M phosphate buffer solution (PBS) using the same Tygon tubing.

The output of the chamber system was connected to a peristaltic pump (Masterflex, model 7013) using similar Tygon tubing. Initially, to flush and prime the perfusion system, 0.01 M PBS was drawn through the system at a flow rate of 150mL/min for about 10 seconds. Then, the peristaltic pump drew blood (native, non-anticoagulated) directly from the intravenous cannula over the substrate at a constant rate of 10 mL/min for 5 min. The effluent blood was collected between the 3rd and the 5th minute into plain and 3.2N sodium citrate tubes. After 5 minutes, PBS was again drawn through the chamber for 30 seconds by turning the three way connector (flow rate 10mL/min). This was to remove all the unattached cells and blood overlying the formed thrombus on the substrate. The switch from buffer to blood then to buffer were achieved manually by turning the three way valve without any stasis of flow within the chambers. Connectors and Tygon tubing used in the chamber system are non thrombogenic, fits air tight in to the peristaltic pump and maintains constant flow and shear rate during the study.

The aortic tissues with formed thrombi were then removed from the chamber units, with extreme care to prevent any dislodgement of fresh thrombus. Individual aortic segments were then fixed in 10% buffered formalin. After a minimum of 72 hours of fixing, the aorta segments (one low shear and two high shear) were sectioned into eight pieces, each approximately 1mm in width (Natarajan *et al.*, 2008a) and placed in pre-labelled histocassettes, one for each aorta segment. The segments were labelled as below:

i. Low shear aorta segment: A1, A2, A3, A4, B1, B2, B3, B4
ii. High shear aorta segment 1: C1, C2, C3, C4, D1, D2, D3, D4
iii. High shear aorta segment 2: E1, E2, E3, E4, F1, F2, F3, F4

The histocassettes were placed in formalin and taken to Cellular Pathology department, Royal Victoria Infirmary where sectioning, mounting and staining processes were carried out. The aorta segments were embedded in liquefied wax. The tissue blocks in the wax were then sectioned using a microtome to smaller sections of 0.4µm width. These were then mounted on glass slides and stained using modified Masson trichrome stain (described below).

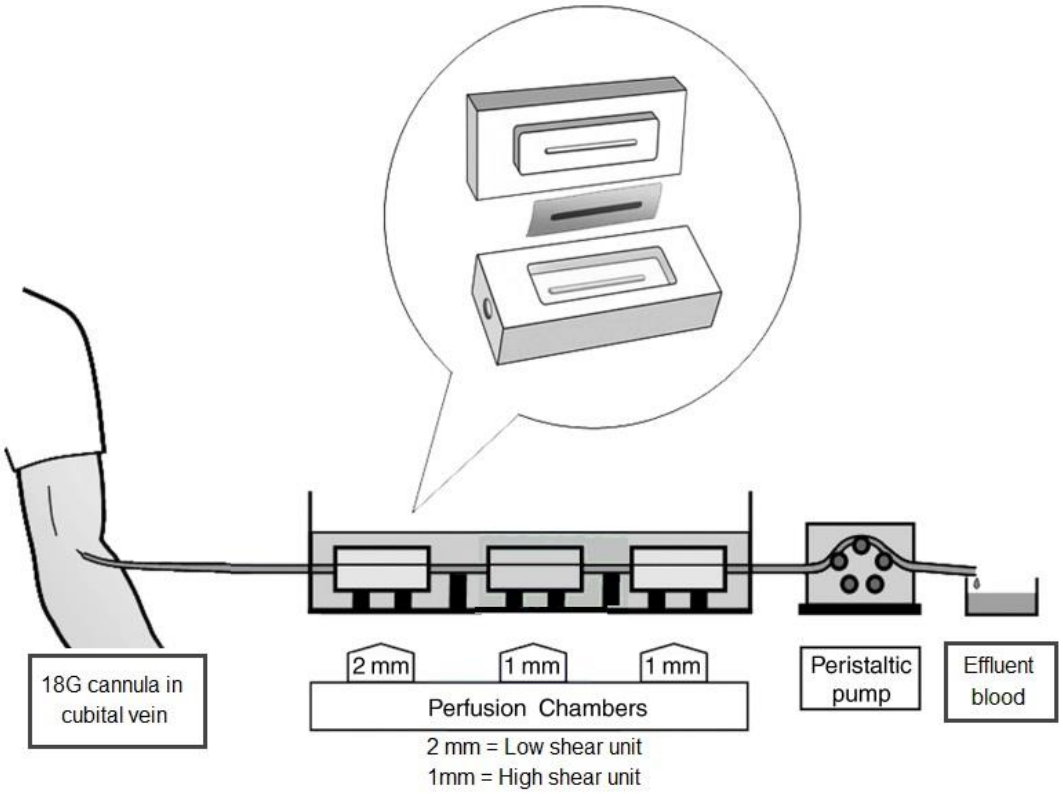


Figure 3.5 Set up of the Badimon chamber

Blood from patients' antecubital fossa is drawn directly and allowed to flow through the Badimon chamber using Tygon tubing. Aorta with stripped intima is used as the substrate in Badimon chamber. The chamber is in a circulating water bath maintained at body temperature. The three chamber units are connected in series – low shear unit then high shear unit 1 followed by high shear unit 2. Uniform flow condition was maintained during the study.

High shear chamber has an inner diameter of 1.0 mm and generates a shear rate of 1920 per sec and Reynold number of 60.

Low shear chamber has an inner diameter of 2.0 mm and generates a shear rate of 500 per sec and Reynold number of 30.

Effluent blood sample was collected from minute 3 to minute 5 and the serum and plasma samples were stored for coagulation and inflammatory biomarkers analysis. Rest of the effluent blood was safely discarded.

Staining of the thrombus

The sections of aorta segment on glass slides were stained using modified Masson trichrome method. This is a three colour stain protocol where after staining, the thrombus with fibrin appears red, the elastin of the aorta substrate appears green and the aortic smooth muscle appears pink. The original Masson trichrome staining was first described in 1929 (Bancroft and M, 2002). The technique was modified to enhance the thrombus staining over the aortic substrate and also to clearly delineate the stained thrombus and tunica media. This modification has been validated by Thrombosis Research Laboratory at Mount Sinai Hospital, New York (Osende *et al.*, 2001). Step by step staining protocol is described below.

Image acquisition

The stained thrombi were viewed using a Leica DM2000 microscope (Leica Mikrosysteme Vertrieb GmbH, Weltzler, Germany). The microscope was calibrated according to manufacturer's recommendations. It was cleaned and quality control checked twice a year. The thrombus was identified (pink to red) on the top of the raised section of tunica media of the aortic substrate (green) under 10X magnification. A high definition digital camera with resolution of 1360x1024 (KY-F1030, JVC, Japan) was connected to the microscope and a computer using an IEEE1396 electronic link and an inbuilt TWAIN driver. The captured image of thrombus and substrate was digitalised and stored without losing its precision using special software (What You See Is What You Get Software (WYSIWYG) ®, JVC, Japan).

Modified Mason trichrome staining protocol

1. 150 ml of Weigart's haematoxylin solutions A and B are mixed together.
2. Slides were taken and serially exposed to solutions – Xylene x2, 99% alcohol x2, 95% alcohol and 75% alcohol.
3. The slides were then left in Weigart's solution (A and B) for 10 minutes.
4. Following this the slides were run in tap water.
5. The slides were dipped in acid alcohol and then run in tap water again.
6. The slides were then dipped in Scotts tap water and then run in tap water again.
7. Then dipped in 2% Poncaeu Red in acetic acid for 10 minutes.
8. The slides were then washed in distilled water.
9. The slides were then left in 2% light green for 40 seconds.
10. Following this the slides were dipped in distilled water then in 2% acetic acid.
11. The slides were then run in tap water and then dehydrated quickly, 70% alcohol, 95% alcohol, 99% alcohol x2, Xylene x2.
12. The slides were then placed in holder and cover slips are pasted.
13. The slides were then blot with tissue and are arranged in boxes safely.

Table 3.1 Stepwise protocol for modified Mason trichrome staining.

Thrombus area measurement

Image ProPlus®, Version 4.0 software (Media Cybernetics, Bethesda, MD, USA) was used for image analysis and total thrombus area measurement. Standard micron calibration scale supplied by the manufacturer was used for calibration prior to image analysis. Acquired images that were stored in .jpeg format were opened using this software. Automated best fit algorithm was used to adjust the brightness and contrast of the images. The thrombus outline was precisely traced with the cursor using 'irregular area of interest (AOI)' in the software. Utmost care was taken to make sure tunica media was not included in the area of interest. Once the area of interest was delineated from the rest of the aortic tissue, the thrombus area was measured using the dark object identification algorithm. This algorithm identifies thrombus as multiple individual dark objects separating it from the background image. The area of the thrombus was quantified as the sum of area of the multiple dark objects. Thrombus areas of each of the 24 sections of aorta (8 low shear and 16 high shear) were counted. Total thrombus area (TTA) was calculated as mean of individual thrombus areas for high shear and low shear sections and expressed as micrometres squared per millimetre ($\mu m^2/mm$) of aorta.

Images were checked by two observers, myself and the senior experienced investigator Prof Azfar Zaman. After reviewing all the images we excluded the aorta sections that were deemed unsuitable for analysis as listed below:

i. Sections with flaps: may lead to sluggish flow and increased red cell thrombus may overestimate the thrombus area
ii. Sections which were damaged by microtome: may underestimate thrombus area
iii. Sections with artefacts from dye or coverslip: may overestimate thrombus area
iv. Sections with negligible thrombus formation (<500 $\mu m^2/mm$): falsely low thrombus area due to wash artefact
v. Sections with red cell thrombi: due to sluggish flow may overestimates thrombus area

We excluded 4.9% of thrombus images from final analysis (538/10880 images) due to one of the above listed reasons.

Quality Control

Before every chamber study, I ensured the integrity of the chamber circuit by priming with 0.01 M PBS at 10mL/min (standard laminar flow) and 150mL/min (high shear, non-laminar flow).

I performed quality control chamber study once every four months. I used 30g of coffee powder mixed with 50 ml of 4% cream and 50ml of water (1:1) to mimic the consistency of viscous blood. Deposition of the coffee powder particles was examined using a magnifying glass (X4). Presence of coffee powder in places other than the central three fourths of aorta was considered non uniform flow. A full chamber study with a volunteer (myself) was also performed every year, exactly similar to a patient study. Mean thrombus area was similar in all the three studies (total thrombus area μ^2/mm, 12453, 13124, 12221).

Quality control for image analysis was also performed. Two independent observers (myself and Prof Azfar Zaman) analysed 5% of randomly chosen images in a blinded fashion. Inter-observer co-efficient of variation was 6.2%. I also analysed the same set of images on three different occasions on three consecutive days, with an intra-observer co-efficient of variation of 4.2%.

3.4.2 Scanning electron microscopy (SEM) – Ultrastructural assessment of thrombus

Introduction

Studying the architecture of platelets and fibrin fibres is done using scanning electron microscopy. Fibrin fibre structural arrangement in thrombus was first demonstrated in 1981 using electron microscopy (Weisel *et al.*, 1981). Thrombus structure was later described using iridium labelled platelets in a small cohort of patients (Badimon *et al.*, 1987). Microscopic mechanical properties of thrombus were first described by Collet et al using electron microscopy (Collet *et al.*, 2005). This was found to be time consuming and error prone, hence the current focus is mainly on fibrin structure derived from platelet poor plasma. However, the quantitative assessment of thrombus using SEM is still in early stages of development and is not yet formally standardised. From our laboratory, we have recently published ultrastructural differences in fibrin fibres of formed thrombus using scanning electron microscopy comparing T2DM and non-DM patients following NSTE-ACS (Viswanathan *et al.*, 2014).

Principles and technique of scanning electron microscopy

Light microscopes cannot generate images of magnification higher than 1000x. Hence scanning electron microscopy (SEM) was used. SEM is a type of electron microscope that produces images of a sample by scanning it with a focused bean of electrons. SEM uses the principles of signal generation when high voltage electron beams are focused on a smaller area on the surface of the solid biological sample. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography / external morphology, composition, crystalline structure and orientation of individual subunits of the sample. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. Specimens can be observed in high vacuum, in low vacuum, in wet conditions (in environmental SEM), and at a wide range of cryogenic or elevated temperatures. The electron beam receiver collects data over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from 1 cm to 5 microns in width can be imaged using conventional SEM techniques (magnification ranging from 20x to approximately 30,000x, spatial resolution of 50 to 100 nm).

Components of SEM include:

i) electron optical column - source to produce electrons
ii) electromagnetic coils to control and modify the beam
iii) vacuum systems - “holds” vacuum to minimise artefacts
iv) Signal detection & display unit - consists of detectors that collect the signal and electronics to produce an image from the signal.

Detailed principles and capacities involved in the image acquisition and analysis are beyond the scope of this thesis. Basically, in SEM accelerated electrons carry significant amounts of kinetic energy, and when these focused electrons hit and decelerate from the surface of the sample, the energy is dissipated as various signals. The types of signals include secondary electrons (SE), back-scattered electrons (BSE), characteristic X-rays, cathodoluminescence (CL), specimen current and transmitted electrons. Back-scattered electrons (BSE) are beam electrons that are reflected from the sample by elastic scattering. A backscatter electron detector covers the trajectory of the backscattered electrons. Secondary electrons and backscattered electrons can be efficiently detected separately at a low accelerating voltage and the detector is placed in such a way to avoid the pathway of the primary electron beam. Images from backscattered and secondary electrons can be of high resolution can be obtained.

Transmission EM (TEM) is another high resolution EM and can achieve magnification of up to 450,000x. I decided to use SEM rather than TEM for the following reasons:

i. SEM is better suited to study the thrombus formed in Badimon chamber as TEM may focus only on a very small area of the thrombus
ii. thrombus preparation on a very thin slice of aorta will be technically challenging for TEM
iii. fibrin to fibre interactions are better studied using SEM
iv. cross sectional imaging of thrombus by TEM will be very time consuming and less likely to yield more information

SEM study protocol

With the help of the senior operators at Electron Microscopy services, Newcastle University, I performed a series of pilot image acquisition under supervision as a part of my training. For better reproducibility and to improve processing speed, the pilot

image was standardised at approximately 60x magnification using an 8 kilo Voltage electron beam and the sample kept at 6mm distance from the electron gun. For high power 3,200x magnification and for ultrahigh power 15,000x magnification were chosen as standard for this study.

I decided to analyse 60 random patient samples (20 in each group) on both visits. The samples were identified by the study identification numbers, chosen randomly by an independent member of our team. This kept me blinded to the group the particular study identification number belonged to during the analysis. In order to detect a 10% change in fibrin diameter from baseline 1 week after clopidogrel therapy with 80% power, we estimated that a sample size of 17 patients per group was required. Hence, I chose my sample size as 20 per group. Each patient had two high shear aorta specimens.

Thrombus laden aorta samples from the Badimon chambers were fixed in 2% glutaraldehyde. A sample of 1mm width was taken from each chamber (1x low shear and 2x high shear) at the median point of the aorta. Only high shear thrombus specimens were used for analysis. After 72 hours of fixing, the thrombus specimens were serially treated with varied concentration of ethyl alcohol. After critical point drying, the samples were mounted using silver adhesive. They were then gold coated in a specialised chamber and the samples were loaded to SEM. Standard pilot images were taken at 60x magnification. Six images were taken at high power $3.2x10^3$ magnification and further six images were taken at $15x10^3$ magnification. The areas of interest were identified using a validated random grid model using 2x3 squares (Silvain *et al.*, 2011). Total numbers of fibres were counted in a square grid model with area of 36sq.microns. All the images were analysed by me and I was blinded from which group the study identification number belonged to.

The images were analysed using Image ProPlus® software 4.0.1 (Media Cybernetics Inc, MD, USA). I first focused on the standard pilot image (60x magnification) and identified platelet rich areas and fibrin areas using a simple grid analysis model and calculated proportional content of each of them. This did not provide the absolute value of platelet or fibrin content of thrombus but percentage area occupied by each of the components. Internal validation was performed and intra observer CV (KB) was 12.4% and inter-observer CV (KB vs KW) was 16.5% for platelet-fibrin content of thrombus.

I then proceeded with high power images of $3.2x10^3$ magnification and focused on two distinct structures, platelets (disc shaped) and fibrin (filament shaped)

Platelets in the sample were identified by the following validated structural features, Figure 3.6 (Williams text book of Haematology, 8th edition, McGraw Hill, New York, USA):

i. disc shaped structure
ii. presence of pseudopodia
iii. Presence of filaments
iv. Presence of exosomal globular structures (exocytosis of golgi apparatus and granules)
v. Presence of clumping

Fibrin fibres were identified by the following validated features, Figure 3.7:

i. Long filamentous architecture
ii. Presence of interlinkage between filaments
iii. Twisting of filaments
iv. Attachment to platelets
v. Entrapment of platelets

The “hub and spokes” model of thrombus assessment was created following the discussion with the senior laboratory technicians. It was hypothesised in our previous work that the presence of higher number of “hub and spokes” would limit the energy to be used in individual fibrin fibre polymerisation and thus make them more susceptible for autolysis.

Dendrite identification algorithm of Image Proplus® version 4.0 was used to analyse the fibrin to fibrin interlinkage. This software identified fibrin filaments in the thrombus and was used to count the numbers and measure the size of individual fibres. The dendrite function algorithm specifically identifies fibrin-fibrin interlinkage in a predefined area of thrombus. The “hub and spokes model” was applied on the image to study individual fibrin to fibrin interaction.

“Hub” was defined as presence of three or more individual fibrin fibres intersecting each other at three different angles with at least 20 degrees in between. Those with angles less than 20 were defined as parallel fibrin fibres. This model helped us to study

the compactness of the thrombus. Individual fibrin fibres were known as “spokes”. As the thrombus was heterogeneous in any given field in SEM, I also measured clusters of hub and spokes per square micron of the field. This provided a quantitative assessment of compactness of the thrombus.

For quality control, every fourth sample was re-analysed and if the differences in fibrin diameter or platelet diameter were more than 10%, analysis was performed for the third time and the results of the third analysis was included in the analysis.

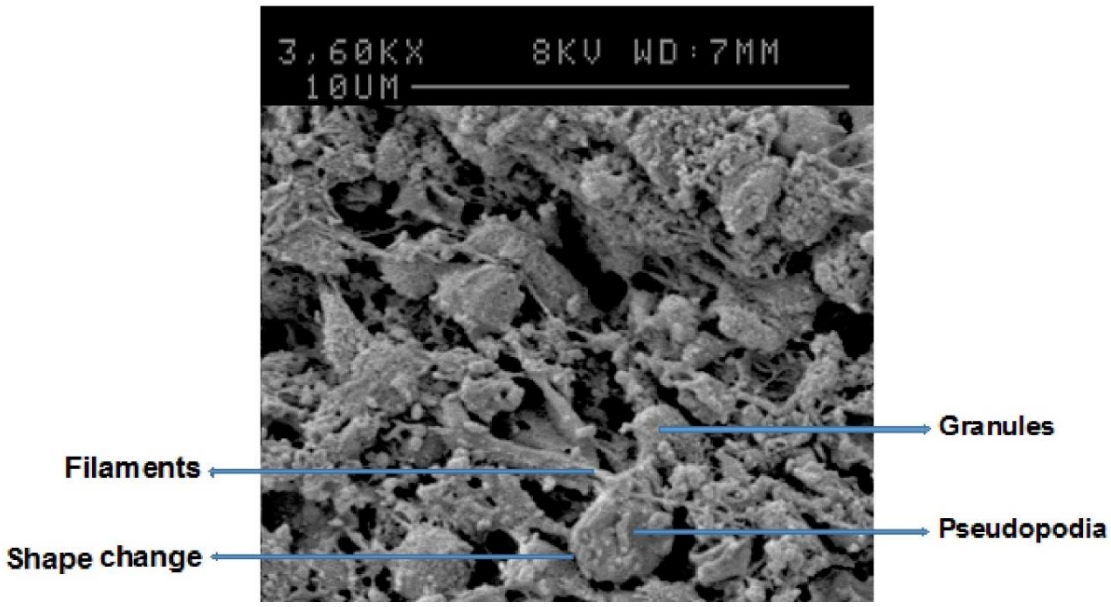


Figure 3.6 Electron microscopy appearance of platelets

Electron microscopy appearance of platelets in various stages of activation was seen at high power magnification (x3600) and at 8kV energy. Activated platelets were characterised by the presence of pseudopodia seen as an “end on” appearance. Granules were seen as small projections on the surface of the platelets. The platelets were seen here at various stages of changes in shape. The fibrin filaments are seen bridging the adjacent platelets, thereby resulting in platelet clumps.

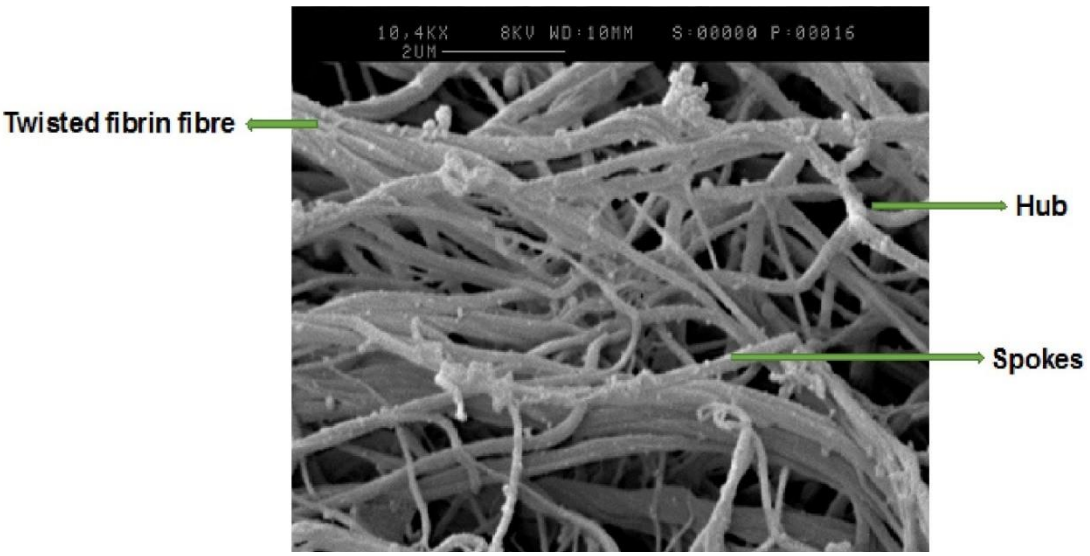


Figure 3.7 Electron microscopic appearance of fibrin fibres

Fibrin fibres viewed at ultra-high power magnification by scanning electron microscopy. Fibrin fibres were arranged in longitudinal pleated structure with trapped RBC's and platelets. The presence of hub and spoke appearance is shown in this section with lateral fibrin fibre interlinkages.

Step by step SEM analysis protocol

SEM analysis of the thrombus consisted of three distinct stages (sample preparation, image acquisition and image analysis), as describe below:

Sample preparation

1. After completion of Badimon chamber study, the aorta substrate was carefully removed from the chamber
2. The middle 1mm width of aorta laden with thrombus was dissected using a precision blade/scalpel. Care was taken not to disturb freshly formed thrombus. The same was repeated for the two other chambers, so that three specimens were obtained (1x low shear, 2x high shear). The samples were immediately fixed in 2% glutaraldehyde in Sorensons phosphate buffer
3. The specimens were then stored at 5°C for at least 72 hours
4. The fixed thrombus samples were then rinsed twice for 15 minutes using 0.01M phosphate buffer solution
5. This was followed by step by step dehydration using various strengths of ethanol as follows: (gradual increase in concentration to minimise artefacts):
    a) 25% ethanol for 30 minutes
    b) 50% ethanol for 30 minutes
    c) 75% ethanol for 30 minutes
    d) 100% ethanol one hour
    e) 100% ethanol for one hour
6. This was followed by critical point drying using Baltec critical point dryer.
7. Samples were then mounted on an aluminium stub with sample number written on the bottom surface and Acheson silver Electrodag was used as an adhesive on the adventitial side of the aorta
8. Using Poloron SEM coating unit, with argon vapour, colloid gold was spray coated (up to 15nm thickness) on the surface of the thrombus

Image acquisition

1. Maximum of six gold coated samples in the aluminium stubs were loaded in the SEM tray
2. One tray was then loaded in to the holder and the door is closed
3. Electron detector was then adjusted
4. SEM machine was then switched on to generate vacuum
5. The green flicker from tungsten filament was adjusted to sharpen the image
6. Image detector was switched on and the sample image appeared on the screen
7. Recommended settings used to obtain a magnification of 60-72x times as a basal image
8. Image was acquired to the folder in the PC using Olympus software
9. This image was the pilot image and used to check patients ID number
10. Major grid was used and the image was divided to 6 squares. One image was obtained from each major grid
11. Image was refocused to the square of interest starting with left top square
12. This focused square was then divided to 4 squares using a high power grid. One square was again chosen at random and focused at 3,200 to 3,600x magnification. Further fine tuning of the image was performed to obtain better contrast. Image was then acquired and stored in the PC
13. The high power image was further divided into 4 squares using ultra power grid. One square was chosen at random and focused at approximately 15,000x magnification. The image was fine-tuned, acquired and stored in PC as in step 8
14. Then returned back to low power at 60-70x magnification and moved to the next square in pilot grid, bottom left corner. Steps repeated as above
15. Image acquisition was repeated from middle top, middle bottom, right top and right bottom squares. One high power and one ultra-high power image for each major square in the pilot grid was available

16. Returned back again to low power at 60-70x magnification and moved to the next square in pilot grid, bottom left corner. Steps repeated as above
17. Image acquisition was repeated from middle top, middle bottom, right top and right bottom squares. One high power and one ultra-high power image for each major square in the pilot grid was available
18. In total each sample had six high power (x3200 magnification) and six ultra-high power (x15000 magnification). There were four samples for each patients (2 from visit 1 and 2 from visit 2) generating 24 samples for each patient
19. Once completed, images were backed up in the portable hard drive
20. Filament was turned down, and SEM left under vacuum

Image analysis

The images were analysed using Image ProPlus® software 4.0.1 (Media Cybernetics Inc, MD, USA) as explained in the previous section.

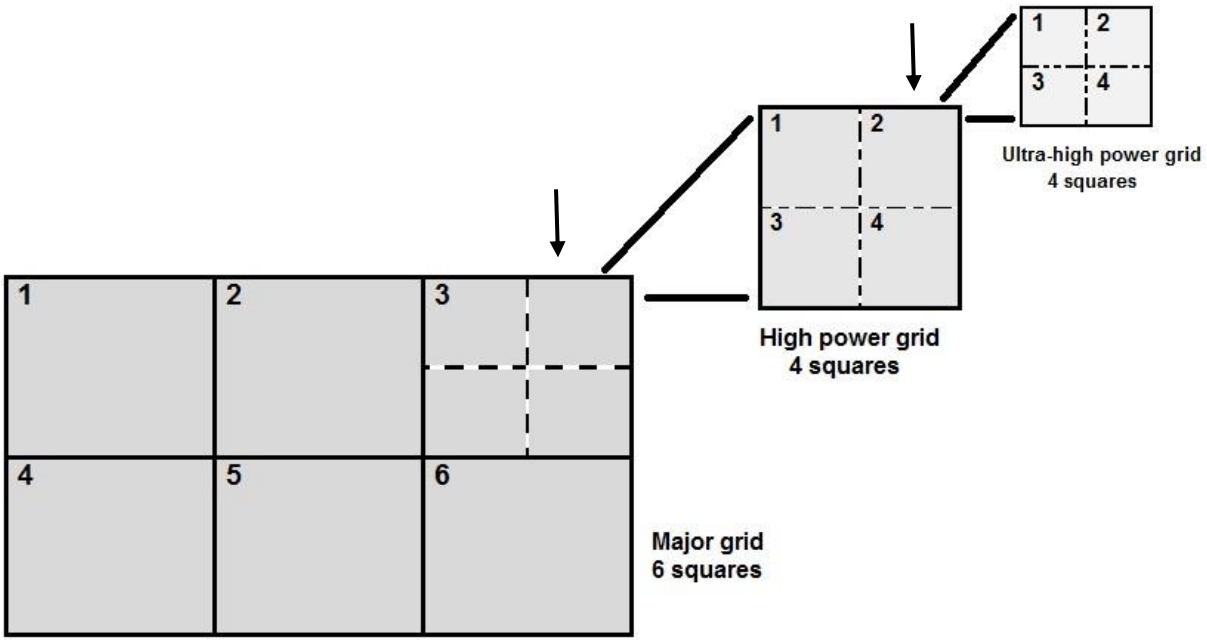


Figure 3.8 Image acquisition in SEM – Major Grid

The sample was divided into 6 squares using major grid. One square was chosen at random and further divided into four high power grid (squares). One square was chosen again at random from the high power grid, for high power image (3200x - 3600x magnification). One high power image per one major grid of the pilot image. Similar steps were repeated for acquiring ultra-power images (15000x magnification) per one major grid of pilot image. Solid arrows indicate the area of interest in the pilot image of high power grid and ultra-high power grid.

3.4.3 Thromboelastography® and Platelet Mapping™ - assessment of viscoelasticity

Principles and technique

Thromboelastography (TEG®) is a non-invasive diagnostic instrument designed to measure the viscoelastic property of the whole blood. The analyser is intended to be used to provide a quantitative and qualitative measurement of haemostatic system and hence can be used to identify defects in thrombus formation and autolysis. TEG® monitors the thrombodynamic properties of blood as it is induced to clot under a low shear environment. The patterns of change in shear-elasticity enable the determination of the kinetics of clot formation and growth as well as the strength and stability of the formed clot. The strength and stability of the clot provide information about the ability of the clot to perform the work of haemostasis, while the kinetics determine the adequacy of quantitative factors available to clot formation. TEG® is used to assess clinical conditions such as post-operative haemorrhage and/or thrombosis during and following cardiovascular surgery, hepatobiliary surgery, organ transplantation, trauma and other cardiology procedures. TEG® provides a dynamic and real time assessment of viscoelastic property of the thrombus (Reikvam *et al.*, 2009).

The TEG® consists of two mechanical parts as demonstrated in Figure 3.9:

i. a heated (37 ºC) oscillating cuvette or cup
ii. a stationary pin which is suspended in the centre of the cup and connected to a torsion wire

Blood is placed in the cuvette and is oscillated through an angle of 4.45 degrees with each rotation cycle lasting for 10 seconds. Whilst the blood remains unclotted the motion of the cuvette does not affect the pin. The torque of the rotating cuvette is transmitted to the immersed pin only after fibrin-platelet bonding has linked the cuvette and pin together. The strength of these fibrin-platelet bonds affects the magnitude of the pin motion, such that strong clots move the pin directly in phase with cuvette motion. Thus, the magnitude of the output is directly related to the strength of the formed clot. As the clot retracts or lyses, these bonds are broken and the transfer of cuvette motion to the pin is diminished. The rotational movement of the pin is converted by a mechanical-electrical transducer to an electrical signal which is monitored by a computer and displayed as a graph. The resulting haemostasis profile is a measure of

the time it takes for the first fibrin strand to be formed, the kinetics of clot formation, the strength of the clot (in shear elasticity units of dyn/sq.cm) and dissolution of clot (Figure 3.10). By measuring the tensile strength of the thrombus continuously, TEG® results reflect the dynamic property of whole blood thrombosis which includes platelet adhesion, aggregation, fibrin-platelet bonds and fibrin polymerization. TEG® also measures the velocity at which the thrombus forms and this is known as clot kinetics.

TEG® measurements are displayed in the form of a graph and various parameters measured are explained in the table below. Initiation of thrombus formation begins with the activation of coagulation cascade and results in formation of fibrin polymers (R time). This is calculated from the graph as the time from initiation of the trace until the amplitude of the clot reaches 2 mm. This is functionally related to plasma clotting and inhibitor activity. The next parameter measures the speed at which the thrombus forms (K time) and is calculated as the time from R until the amplitude of the clot reaches 20 mm. The next parameter α-angle (the slop between R and K valves of the graph), measures the rate of clot formation and this reflects the rapidity of fibrin polymerization. Maximum amplitude of the thrombus (MA) measures the maximum strength of the fibrin clot and represents a direct function of the maximum dynamic properties of fibrin and platelet bonding. This correlates more to platelet function and to a lesser extent to fibrinogen. Early autolysis also known as thrombus retraction is mainly contributed by rearrangement of fibrin to fibrin linkage. This is measured as percentage (%) of lysis at 30 minutes and 60 minutes respectively (CL30 and CL60). This is calculated as percentage decrease in amplitude 30 minutes and 60 minutes post maximum amplitude. The rate of loss of thrombus strength is measured by lysis parameter (L mm/min). L parameter is calculated using a complex first derivative integral calculus equation by continuously measuring loss of maximum amplitude in mm per minute.

Platelet Mapping™, an advancement of TEG® technique, was used to measure platelet inhibitory effect of individual antiplatelet agents such as aspirin, clopidogrel and glycoprotein IIb/IIIa inhibitor. Heparin was used in Platelet Mapping™ to neutralise the effect of thrombin and this enabled us to measure the effects of particular platelet agonists such as arachidonic acid and ADP. Commercially available activator F (A-P1) was used to bypass the effect of heparin. This stimulates conversion of fibrinogen to fibrin and activation of Factor XIIIa to crosslink the fibrin network. Platelet agonists, arachidonic acid (AA 1 mM) and ADP (2 µM) were used alongside A-P1 to study the effect of aspirin and clopidogrel respectively in separate cuvettes. Platelet inhibitory

effect of clopidogrel and aspirin were calculated by measuring the maximum amplitude using the formula:

Percentage reduction in maximum amplitude, MA reduction =

$100 - ((\left[MA_{AA \text{ or } ADP} - MA_{activator}\right] / \left[MA_{thrombin} - MA_{activator}\right)) \times 100)$

There are published data to validate the above formula (Zmuda *et al.*, 2000; Wasowicz *et al.*, 2008).

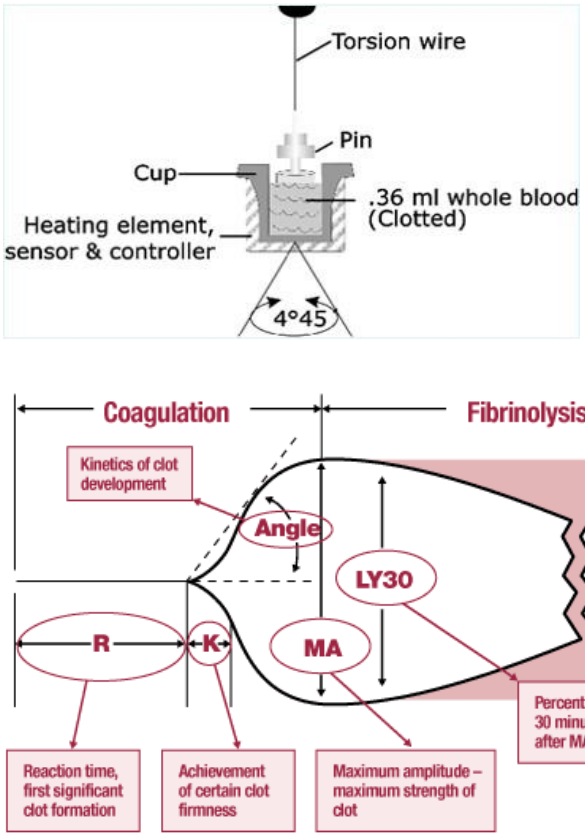


Figure 3.9 Thromboelastography

The first image shows the stationary pin which is suspended in the centre of the cuvette and connected to a torsion wire. Blood is placed in the cuvette and is oscillated through an angle of 4.45 degrees with each rotation cycle lasting for 10 seconds. As blood begins to clot the torque to the inner central pin is transmitted and the elastic force is recorded digitally and displayed as a graph as depicted in the second image. The bottom image shows a 4 channel TEG® analyser and the final image shows close-up of the pins.

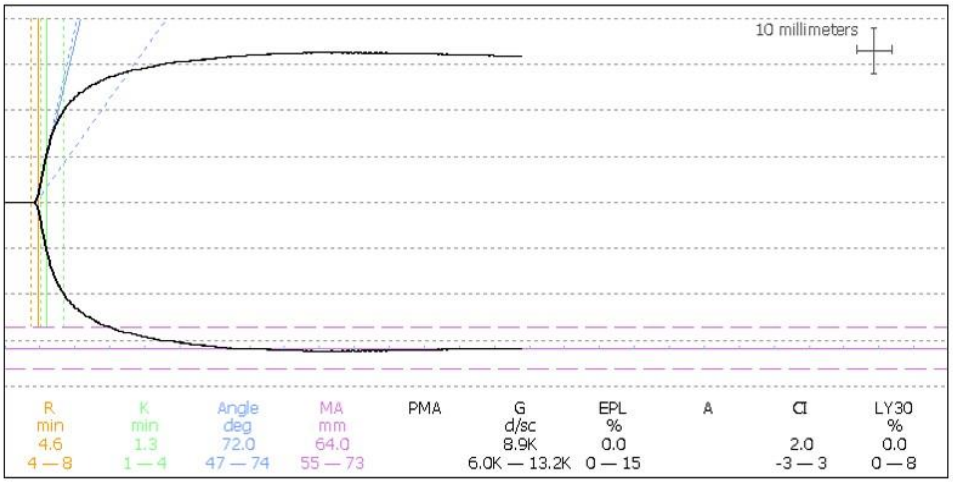


Figure 3.10 Normal TEG® tracing as displayed digitally.
TEG values with normal limits of all the parameters are depicted at the bottom of the graph.

$$\text{Percent MA Aggregation} = \frac{MA_{\text{ADP or AA}} - MA_{\text{Fibrin}}}{MA_{\text{Thrombin}} - MA_{\text{Fibrin}}} \times 100$$

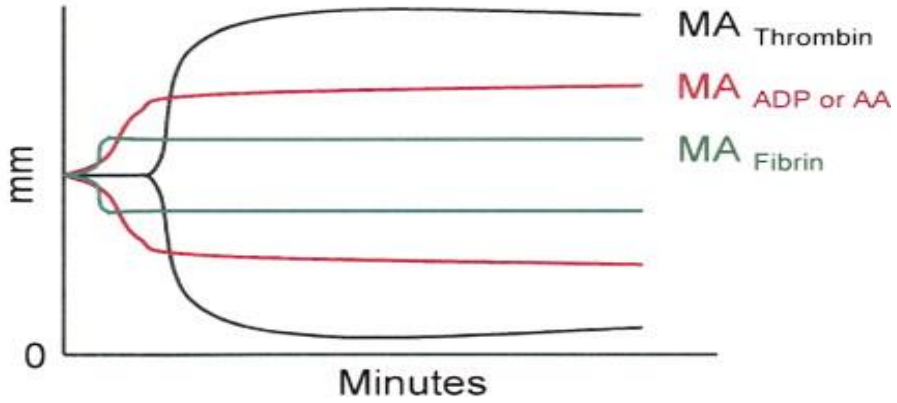


Figure 3.11 TEG® Platelet Mapping™ tracings

Platelet mapping tracings depict the changes in viscoelastic strength of the thrombus upon activation by kaolin (MA thrombin), ADP or arachidonic acid (MA ADP/MA AA) and activator F (MA A). The greater the reduction in MA ADP, the better is the antiplatelet response to clopidogrel.

Name of the parameter	Definition	Clinical relevance	Normal values
R time, min	Time from the beginning of the TEG® trace until an amplitude of 2 mm is reached	This is the time of latency from the time the blood sample was placed in the cuvette until the initial fibrin formation. It shows the rate at which thromboplastin is generated and the function of intrinsic pathway, especially factors XII, XI and VIII	2-8
K time, min	Time from the end of R to the point where TEG amplitude reaches 20 mm	This measures the time taken to form a firm thrombus and is also a measure of intrinsic pathway, platelets and fibrinogen. This is a measure of the rapidity to reach certain level of clot strength	1-3
α- angle (thrombus formation rate)	The angle formed from the horizontal to a point on the trace until amplitude is 20 mm	This measures the rapidity of fibrin polymerisation and fibrin - platelet interaction, thereby assessing the rate at which a solid thrombus is formed. It is an indicator of the quality of platelet and fibrinogen	55-78
MA, maximum amplitude, mm	Measures the maximum distance between the two arms of the graph at their peak amplitude	This measures maximum viscoelastic strength of the formed thrombus. This has been the most studied and consistent measurement among all the TEG® indices. MA is a direct function of the maximum dynamic properties of fibrin and platelet bonding and represents the ultimate strength of the fibrin clot. Using the modification of TEG® assay in Platelet	51-69

Name of the parameter	Definition	Clinical relevance	Normal values
		Mapping™ assay individual effects of aspirin and clopidogrel are studied.	
G, dynes/cm^2	Measure of the shear elastic force of the thrombus, G = 5000 MA / (100-MA)	G value is very sensitive to small changes in MA. G value is an indicator of how firm a formed thrombus is and is reported in absolute numbers.	4,600 – 10,900
CI, Coagulation Index / Clot Index	Dimensionless parameter calculated using the formula, CI **=** - *(0.6516) R - (0.3772) K + (0.1224) MA + (0.0759) α – 7.7922.*	It is a composite measure derived from the R, K, MA and angle (α). It is a dimensionless index. CI measures the combined effect of fibrinogen and platelets on thrombus and	-3 to 3
LY30	Measures the area under the curve 30 minutes after MA has been achieved (rate of amplitude reduction 30 mins after MA)	Indicative of thrombus retraction at 30 mins, signifies early autolysis. This measurement gives an indication of the stability of the clot	0-8
LY60	Measures the area under the curve 60 minutes after MA has been achieved (rate of amplitude reduction 60 mins after MA)	Indicative of thrombus retraction at 60 mins.	0-15
L parameter, mm/min	This is a thrombus lysis parameter, calculated as the average reduction in amplitude per unit of time. This is the first derivative calculus measurement of thrombus strength and is calculated for 90 minutes in standard TEG® tracings.	This represents the changes in viscoelastic property due to ongoing clot retraction or autolysis.	30-60

Table 3.2 Thromboelastography parameters

In addition to standard TEG® parameters, an additional set of parameters is generated from the mathematical first derivative values of TEG® and is displayed as a real time graph known as velocity curve (V curve). V curve provides dynamic information about the velocity of both thrombus formation as well as lysis. The positive curve above the horizontal axis represents thrombus generation. Inherent thrombin activity has a strong influence on clot development. During thrombogenesis, the elastic force of the generated thrombus is directly proportional to the speed of thrombin generation. Thus, measured velocity changes based on the V curve can be used to indirectly quantify thrombin generation (Rivard *et al.*, 2004).

The following parameters are calculated.

i. ***L parameter***, the composite measure of autolysis has been described before.

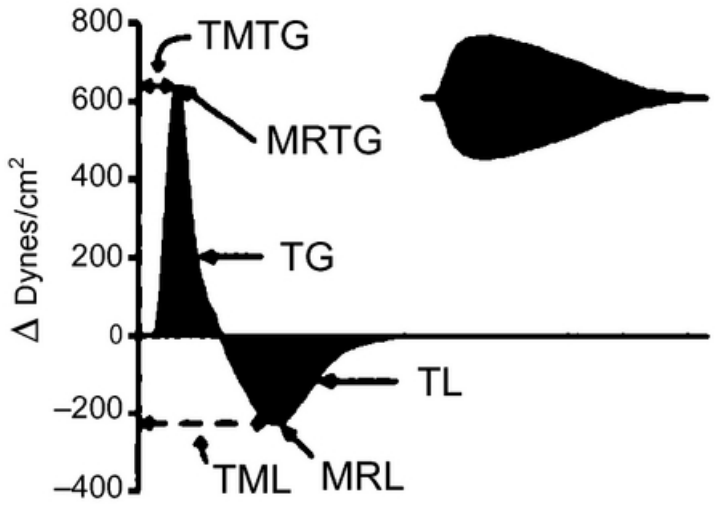
ii. ***Maximum rate of thrombus generation, (MRTG, mm/min)*** measures the maximum velocity reached when the viscoelastic strength develops within a developing thrombus.

iii. ***Time to maximum rate of thrombus generation (TMRTG, seconds)*** measures the time taken from the initiation of thrombus formation to the time it takes for the thrombus to reach its maximum strength.

iv. ***Total thrombus generated (TG, mm/min)*** is measured as the area under the curve (AUC) is an indirect measure of clot strength. Thrombus formation is followed by autolysis.

v. ***Time to maximum rate of clot lysis (TMRL, seconds)*** measures the time from the start of the coagulation cascade until the maximum rate of clot lysis is reached. This indirectly reflects clot stability.

vi. ***Maximum rate of lysis (MRL, mm/min)*** represents the maximum rate of clot breakdown. The standard V curve graph is shown in Figure 3.12



TEG® Velocity Parameters	
TMRTG	Time to maximum rate of thrombus generation
MRTG	Maximum rate of thrombus generation
TG	Total thrombus generation
TMRL	Time to maximum rate of lysis
MRL	Maximum rate of lysis
TL	Total lysis

Figure 3.12 V curve parameters of TEG®

My study protocol

My step by step study protocol, as per manufacturer's guidelines is as described below (Haemoscope, CA, USA). Patients were identified only by their study numbers and the data were stored anonymously in the dedicated computer attached to the TEG® machine.

Quality control for TEG® Platelet Mapping™

I performed electronic quality control every time before starting the test. This is to ensure equilibrium of the central pin and torsion wire. This was recorded and stored automatically. I performed quality control using the control samples provided by the manufacturer once every other week. This was repeated prior to start of each new batch of reagents and for every 30 studies performed (which ever was earlier). These results were also stored.

TEG study protocol

1. After switching on the TEG® analyser, electronic QC was performed and participants' study ID was entered.
2. The plain disposable cups & pins were loaded into all 4 channels of the TEG® analyser.
3. The lyophilised vials containing activator F (A-P1), adenosine diphosphate (ADP-P2) and arachidonic acid (AA-P3) were taken out from storage (6 deg C) and gently brought to room temperature.
4. Blood samples were collected in
   i. sodium citrate tube (1.75 mls of blood into the tube containing 0.25 mls of sodium citrate)
   ii. lithium heparin tube (2 mls of blood into the tube containing 100 units of heparin).
5. After waiting for 30 minutes, 20 µl 0.2M of calcium chloride (supplied by the manufacturer) was pipetted into the plain cup in CHANNEL 1.
6. One mL of citrated blood was pipetted into the kaolin vial (supplied by Haemoscope) by gently letting it run down the side of the vial until it reaches the 1 mL mark. The vial was re-stoppered and mixed by gentle inversion five times without shaking the sample.
7. 340 µl of blood from the kaolin vial was pipetted into the cuvette in CHANNEL 1.
8. Immediately the carrier was slid up, the lever was moved to Test position and the Start button in the software main toolbar was clicked to begin sample analysis.
9. One ml of distilled water (supplied by manufacturer) was added to each vial containing reagents A-P1, ADP-P2 and AA-P3. The vials were gently mixed by rolling between the hands and avoiding foam formation.

10. 10 µL of prepared A-P1 was pipetted out into each plain cup in CHANNELS 2, 3 and 4.
11. 10 µL of ADP-P2 was added to A-P1 in plain cup in CHANNEL3 (for ADP assay).
12. 10 µL of AA-P3 was added to A-P1 in plain cup in CHANNEL 4 (for AA assay).
13. 360 µL of heparinised whole blood was pipetted into A-P1 cup in CHANNEL 2. The blood sample was pipetted up and down three times in cup to mix with the reagent whilst avoiding foam formation. The carrier was slid up, the lever was moved to test position and start button was clicked.
14. 360 µL of heparinised whole blood was pipetted into ADP-P2 cup in CHANNEL 2. The blood sample was pipetted up and down three times in cup to mix with the reagent whilst avoiding foam formation. The carrier was slid up, the lever was moved to test position and start button was clicked.
15. 360 µL of heparinised whole blood was pipetted into AA-P3 cup in CHANNEL 2. The blood sample was pipetted up and down three times in cup to mix with the reagent whilst avoiding foam formation. The carrier was slid up, the lever was moved to test position and start button was clicked.
16. The TEG® analyser now started showing graphs from all the four channels in four different colours.
17. Recording automatically stopped once the graph lines reached a steady state. It usually takes up to 90 minutes for plain kaolin added sample and 60 minutes for other channels. Once the study was completed, a print out of the tracings was taken.
18. The results were digitally stored in the computer that was attached to the TEG® analyser.

19. After the study was over, percentage inhibition was obtained by selecting the Multi button located in the software Main toolbar to select tracings needed for computation of this variable.
20. For the ADP assay, the tracings for A (A-P1), ADP (ADP-P2), and kaolin (K) sample types were selected. The Done button located in the software main toolbar was clicked to display the percentage inhibition.
21. For the AA assay, the tracings for A (A-P1), AA (AA-P3), and kaolin (K) sample types were selected. The Done button located in the software main toolbar was clicked to display the percentage inhibition.
22. After noting the results, the levers were brought back to neutral position and the cups and pins were gently removed and discarded.
23. The device was switched off and the surface was cleaned with 70% alcohol wipes.

Table 3.3 TEG® step by step protocol

3.4.4 VerifyNow® optical aggregometry – Measurement of platelet aggregation

Principles and technique

VerifyNow® (Accumetrics, CA, USA) is a whole blood point of care platelet function assay which measures platelet aggregation as an increase in light transmittance using the principles of optical aggregation and turbimetry. VerifyNow® is a microprocessor-controlled, cartridge-based, software driven automated assay that measures the antiplatelet effect of aspirin, P2Y12 antagonists and glycoprotein IIb/IIIa antagonists in whole blood. The results are displayed in a digitised format. VerifyNow® uses turbimetric principles to measure platelet induced aggregation in response to various agonists such as ADP, prostacyclin E1 and thrombin receptor activating peptide (TRAP). It uses similar principles employed in “gold standard” light transmission aggregometry (LTA).

The VerifyNow® System uses disposable assay devices to conduct the test. There are three types of assay device relating to the appropriate platelet function test: Aspirin, P2Y12 and GP IIb/IIIa inhibitors. Each assay device consists of a sample well, staging well and four detection wells. The instrument automatically draws blood into the assay device from a sample collection tube in the sample well. It then heats the blood to 37 degree Celsius for a period of time specific to each assay, and proceeds with the analysis of the blood in the detection wells. The detection wells of an assay device contains a lyophilized preparation of human fibrinogen-coated beads and a platelet agonist, either arachidonic acid or adenosine di phosphate (ADP). Fibrinogen-coated beads bind to available platelet receptors in the blood sample. When the activated platelets are exposed to fibrinogen-coated beads, agglutination occurs in proportion to the number of available platelet receptors. After activation, the GP IIb/IIIa receptors on platelets will bind to the fibrinogen-coated microbeads and cross link to other microbeads resulting in a clearing of the beads and platelets within the detection well. The instrument uses light transmittance to measure the rate at which this clearing occurs. The instrument measures this agglutination as an increase in light transmittance through the detection wells. The effective platelet inhibition with antiplatelet therapy is measured by the changes in light transmittance using arbitrary (dimensionless) units. The greater the effect of antiplatelet therapy, the greater the platelet inhibition. This will lead on to less platelet aggregation and less light transmittance and thereby will record lower reactive units.

I used two different types of cartridges to measure aspirin induced platelet inhibition and clopidogrel induced platelet inhibition respectively. Cartridges were purchased from the manufacturer and the whole assay has been performed according to the manufacturer's instruction.

In one channel of the assay device, prostaglandin E1 (PGE1) (22 nM) is incorporated with ADP (20 µM). The values measured are reported by the device as P2Y12 Reaction Units – activated (PRUz). In a separate channel iso-thrombin receptor-activating peptide (iso-TRAP) is used as an agonist to measure platelet aggregation independent of the effects of P2Y12 receptors; this value is regarded as baseline or PRUb. The VerifyNow® P2Y12 assay reports the results as P2Y12 Reaction Units ("PRUz"), "% Inhibition" and "baseline PRUb". Percentage inhibition due to clopidogrel (P2Y12 antagonist), "% Inhibition" is calculated using the equation ([PRUb-PRUz] X100)/PRUb. If clopidogrel has produced significant platelet suppression, the PRUz values will be lower and the percentage inhibition will be higher. There is no universal agreement on the cut-offs used to measure platelet inhibition. Hence, I have chosen PRUz of 240 and percentage inhibition of less than 40% as a cut off for high on treatment platelet hyperactivity to clopidogrel ("hypo responders") based on a paper published by Marcucci et al (Marcucci *et al.*, 2009). The sample result for the Aspirin assay is interpreted based on the rate of platelet aggregation measured and is reported in Aspirin Reaction Units (ARU). Patients with ARU ≥495 were classified as high on treatment platelet reactivity to aspirin ("hyporesponders") (Paniccia *et al.*, 2007).

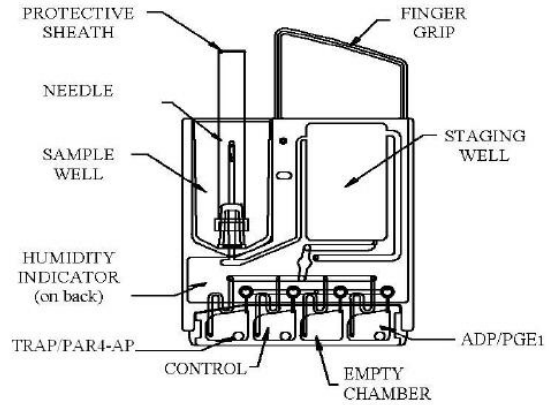
My study protocol

Blood was collected in 2 ml partial-fill sodium citrate blood collection tubes (0.105M, 3.2% sodium citrate + citric acid, pH 5.2, Catalogue number 367691, BD Vacutainer®) and gently mixed by inverting 5 times. After leaving the sample static for 30 minutes, the assay device was taken out of the sealed pouch and inserted into the device port on the VerifyNow® instrument. The citrated blood sample tube was inverted gently again 5 times and was then inserted into the sample well of the assay device with the rubber stopper facing downward so that the needle pierces the stopper. The cover to the assay port was then closed. The instrument automatically draws sample from the vacuum collection tube into the assay device. It then heats the blood to 37 degree Celsius for a time specific to each assay, and proceeds with the analysis. It typically takes 3 to 6 minutes for the assay to complete and the results were displayed as

respective units (PRU or ARU). Two assay device, one for aspirin and other for P2Y12 was used to measure ARU and PRU respectively.

Quality Control for VerifyNow®

VerifyNow® comes with its own manufacturer specified quality control. I performed an electronic quality control (EQC) every day using the standard assay device supplied by the manufacturer. This verifies that instrument components are functioning properly including optics, reagent mixing, and pneumatics. It also confirms correct calibration parameters and checks for correct data acquisition and calculations. An automatic error message would be displayed as “attention” with a diagnostic code, if the results fall outside the wider range for ARU or PRU. I performed a wet quality control assay (supplied by the manufacturer, Level 1 and 2) for both aspirin and clopidogrel assay devices once a month and at every time a new batch of assay devices was used. In addition, I tested reproducibility annually using 10 healthy volunteers measured twice on the same day. The coefficient of variation of ARU was 3.9% and for PRUz 4.1% and the results of ARU were (median, IQR) 606, 599 - 621 and PRUz were 318, 302-334.



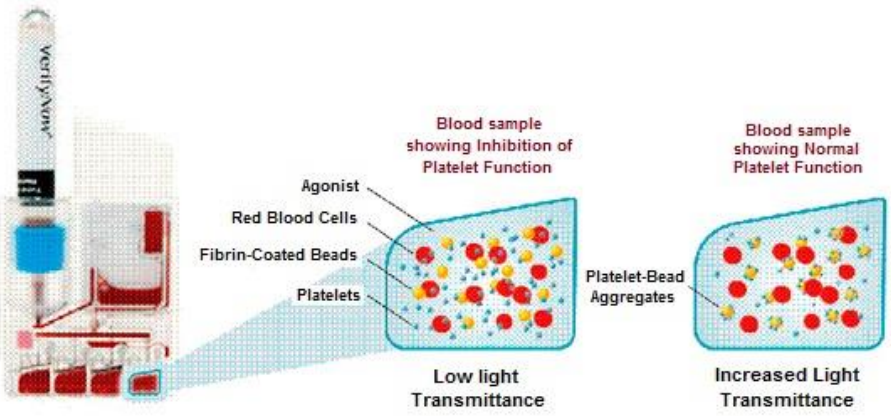


Figure 3.13 VerifyNow® assay

Assay cartridge used in VerifyNow® assay contains fibrinogen coated beads in the four chambers at its bottom. The cartridge is inserted in to its slot as shown in the middle picture and the needle cover is removed. The blood sample vacutainer is gently inverted 5 times and is then inserted into the sample slot and the cover is closed. Blood is drawn into the cartridge and it mixes with these beads and produces platelet aggregation as stimulated by specific agonists like ADP or arachidonic acid. The amount of aggregation depends on the level of residual antiplatelet activity and will result in different light transmittance. The emitted light is read by a sensor and the result will be displayed as a dimensionless unit (ARU, PRU).

3.4.5 Multiplate® impedance aggregometry – Measurement of platelet aggregation

Principles and technique

Multiplate® analyser evaluates whole blood platelet aggregation by measuring the changes in impedance / electrical resistance in the Multiplate sensor wires during thrombogenesis. Impedance aggregometry was developed by Cardinal and Flower (Cardinal and Flower, 1980) and has been used since the 1980's for the assessment of platelet function in whole blood. This is based on the principle that platelets are non-thrombogenic in their resting state, but expose receptors on their surface when they are activated, which allow them to attach to sites of vascular injury and artificial surfaces. The main disadvantages of the earlier devices used in various coagulation laboratories were poor reproducibility due to short life of the electrodes and the presence of residual thrombi attached to the electrode after each test. This problem has been overcome by the Multiplate® analyser by using single use disposable test cells, which incorporate a dual sensor unit and a teflon-coated stirring magnet.

Platelets become sticky upon activation, and therefore have a tendency to adhere and aggregate on metal sensor wires in the Multiplate test cell. When platelets stick on the Multiplate sensor wires they form a sheet over the electrodes and this enhances the electrical resistance between them. With increasing thrombus formation there is a proportionate increase in electrical impedance which indirectly reflects platelet aggregation. This is measured as a continuous variable over time. The maximum increase in the impedance reflects platelet activity and this is plotted as a graph. Adding a specified quantity of platelet agonist to the whole blood from patients taking antiplatelet agents will demonstrate changes in impedance. This change in impedance reflects residual platelet reactivity and this can be used to assess the response of platelets to these agents.

In my study whole blood impedance aggregometry was measured using the novel Multiplate® analyser (Dynabyte medical, Munich, Germany) in accordance with manufacturer's guidelines. Blood samples from patients were collected in hirudin tubes. The test cell was placed into the measurement position and the sensor cable was attached to it. Each test cell has a pipetting inlet and a cup portion with the twin sensor wires which extend into the blood sample. There is a teflon-coated magnet in the cup portion which stirs the blood at 800 rpm. The sensor wires are made of highly

conductive copper which are silver-coated. The test cell is connected to the instrument using a sensor cable and the electrical resistance between the sensor wires is recorded during the test. When activated, platelets adhere to the sensor wires and the electrical resistance between the wires increases**.** The Multiplate® analyser records platelet aggregation at approximately 0.5 second intervals. The study was continued for 6 minutes until steady state electrical impedance was reached. The increase in impedance by the attachment of platelets onto the Multiplate® sensors is transformed to aggregation units (AU) and plotted against time. Results are displayed as a graph. Three parameters that are calculated includes:

i. Platelet aggregation (AU, maximal aggregation)
ii. Area under curve (AUC)
iii. Aggregation velocity (slope of the curve)

AUC is recorded as Units or U. It is affected by the total height of the aggregation curve as well as by its slope and is best suited to express the overall platelet activity. Maximal aggregation (AU) is the maximum height of the curve during the measurement period and the Velocity (AU/min) is the maximum slope of the curve. Maximal aggregation has been used in various clinical contexts as it is the most reproducible parameter and hence I also chose to use the same parameter for analysis (Sibbing *et al.*, 2010a; Kim *et al.*, 2011).

Each test cell incorporates two independent sensor units. The increase of impedance due to the attachment of platelets to the electrodes is detected for each sensor unit separately and transformed to arbitrary aggregation units (AU) that are plotted against time. The duplicate sensors serve as an internal control. The correlation coefficient of the individual measurements is determined and the analysis is accepted when the correlation coefficient is greater than 0.98. Additionally, the difference of each curve from the mean curve is calculated and is accepted when the difference is less than 20%.

Whole blood samples of 1.2 mls from each patient were used to study the effects of four agonists, namely arachidonic acid, ADP, collagen and thrombin in my study.

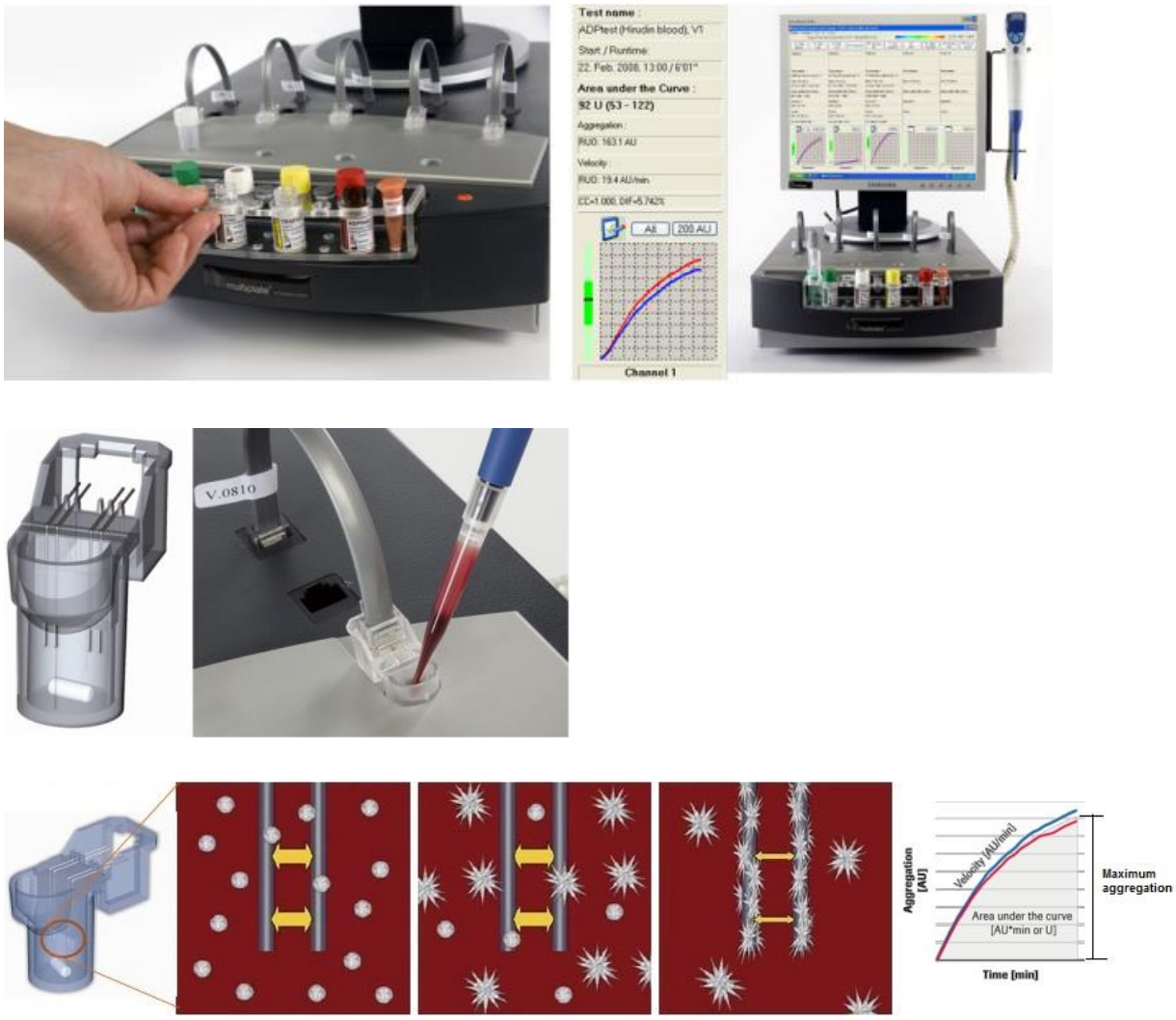


Figure 3.14 Multiplate® assay: Based on impedance aggregometry

Schematic diagram of Multiplate test cell, electrodes, reagents and platelet aggregation inside the cuvette is presented here. Electricity is transmitted across the electrodes which are immersed in the whole blood. When the blood is in fluid state it is a good conductor of electricity and electrical resistance is almost negligible. However, as the thrombus forms, platelet-fibrin interaction forms a sheet over the electrodes, thereby increasing the resistance to the flow of electrons from cathode to anode plates. As thrombus formation increases, the electrical impedance increases proportionately to the platelet aggregation. A change in electrical impedance is a reflection of platelet aggregation and is measured as a continuous variable over time. The maximum increase in electrical impedance represents platelet activity and is displayed as a graph.

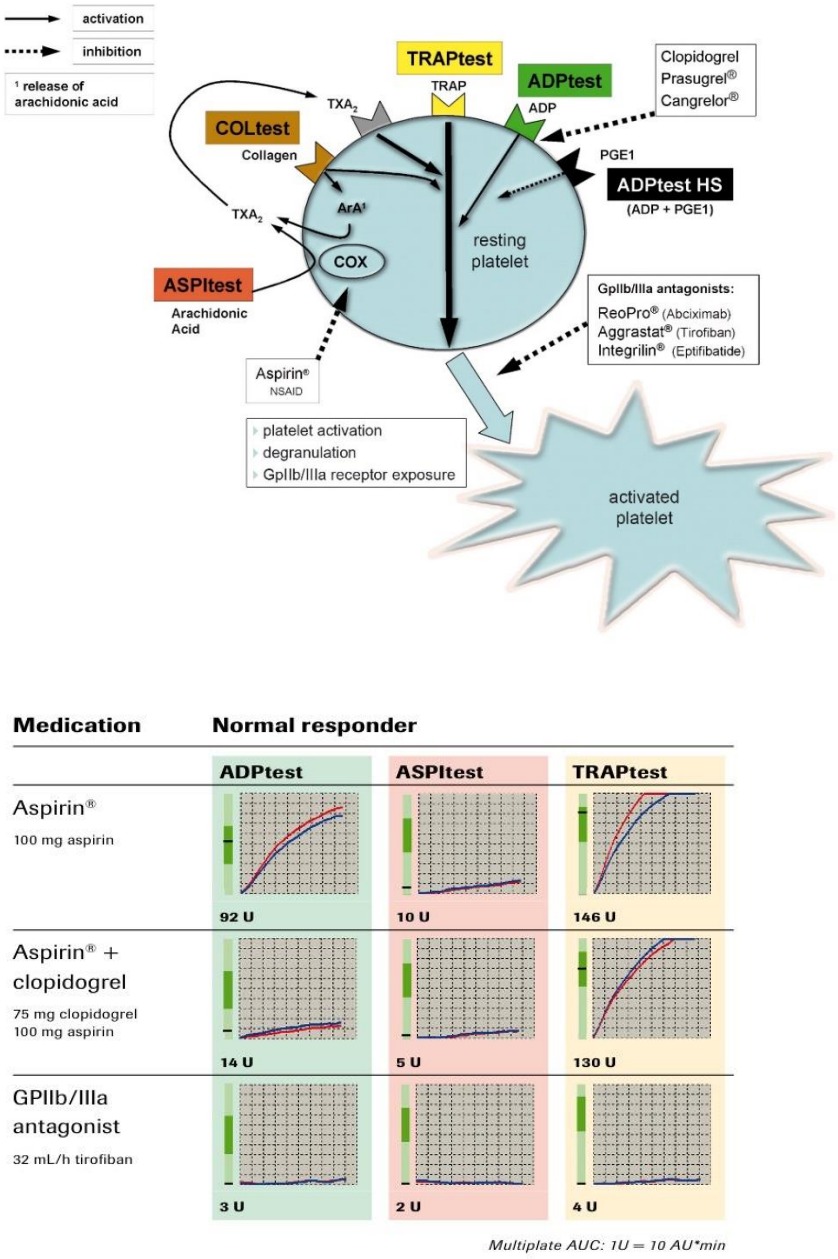


Figure 3.15 Platelet function tests performed by Multiplate® aggregometry

Schematic diagram explaining various platelet function tests that can be performed using Multiplate® aggregometry. Most commonly used test include:

ASPItest – measures aspirin effect using arachidonic acid as a stimulus
ADPtest – measures effects of P2Y12 antagonist such as clopidogrel
COLtest – measures platelet inhibitory effects of either of the agents upon stimulation with collagen
TRAPtest – measures the platelet activity upon stimulation by thrombin receptor activating peptide

Multiplate® study protocol

1. 2mls of blood was drawn in hirudin vacutainer tube supplied by the manufacturer.
2. The blood sample was gently mixed and was left to stand for 35 minutes before analysis.
3. Multiplate® analyser was switched on and allowed 20 minutes to warm up to 37 degree C.
4. Electronic quality control was performed.
5. Patient ID was entered and the following tests were selected - ADPtest, ASPtest, COLtest, TRAPtest.
6. Lyophilised vials of the reagents ADP, ASP (aspirin), COL (collagen) and TRAP (thrombin) are brought to room temperature.
7. Each vial was mixed with 1 ml of distilled water, gently swirled for through mixing and left for 10 minutes.
8. The reagents were then divided into small aliquot tubes each 200 µl, labelled and dated. AA, ADP and thrombin aliquots were stored at -40 deg C and collagen mixture was stored at 5-6 deg C. Expiry date for the reagents stored at -40 deg C and 5-6 deg C were 4 weeks and one week respectively from the date of preparation.
9. Four test cells were inserted into the machine and were connected to the sensor cable firmly.
10. Using autopippette 300 µl of blood from hirudin tube was pipetted in to the test cell cup.
11. Following this 300 µl of normal saline pre-heated to 37 deg C was added.
12. This was followed by 3 minutes of incubation.
13. 20 µl of respective stock reagents were added to the specified test cells.
14. The reaction started and platelet aggregation was displayed as a graph with two lines (from two independent sensor units that act as internal control).

15. The test ran for 6 minutes and final results were printed out.

16. After completion, the test cells were removed after disconnecting the cables and safely disposed in the sharps container.

If the COV between the recorded curves was more than 2% or there was >20% difference in AUC readings, the results were discarded and the test was repeated again.

3.4.6 Coagulation and Inflammatory biomarker assay – serum and plasma samples

Serum and plasma samples were collected and stored for specific biomarker assays at the end of each Badimon chamber study. Both venous blood and effluent blood from chamber were collected and stored for these assays.

Following listed biomarkers were assayed:

Coagulation biomarkers:

i. Soluble P selectin (sP-selectin)
ii. Soluble CD40 ligand (sCD40L)
iii. Plasminogen activating factor inhibitor 1 (PAI-1)

Inflammatory biomarkers (cytokines):

i. Interferon gamma (IFN γ)
ii. Interleukin 1 (IL 1)
iii. Interleukin 6 (IL 6)
iv. Tumour necrosis factor alpha (TNF α)

Sample collection, processing and storage

Serum samples were needed for all biomarker assays except for PAI-1 which was measured using plasma samples. Hence plain glass tubes were used for serum collection and 3.2N sodium citrate blood collection tubes (Grainer-bio one, GmbH) were used for plasma collection.

Pre-chamber venous samples

Venous blood (10mls) was collected from the antecubital vein following Badimon chamber study using the same cannula in plain glass tubes and 3.2 N sodium citrate tube. The samples were allowed to settle for 10 minutes. They were then centrifuged at 1550g (3000 rpm) for 10 minutes. The serum was separated and stored in four aliquots (1ml each) at -80 deg C.

Activated serum samples (post chamber activated effluent blood)

Effluent blood samples were collected between 3rd and 5th minute of the chamber study. Effluent blood samples were collected both in plain glass tubes and 3.2 N sodium citrate tubes. Serum and plasma were separated and stored in exactly similar fashion to pre-chamber venous samples.

The rationale behind doing this is, the chamber experiment simulates a deep arterial injury model and hence there is both shear induced and collagen induced platelet activation. My hypothesis was that the effluent blood consists of coagulation and inflammatory biomarkers that were released from platelet activation and thrombus formation. By measuring the levels of these biomarkers before and after platelet activation we might be able to reflect on their involvement in thrombus generation.

Measurement of soluble P selectin

Soluble P selectin was measured using Human sP-selectin/CD62P, SBBE6, R&D systems, Abington, UK (lot number 300846, part number 890272). The ELISA procedure was followed step by step according to manufacturer's instructions. Quality control for P selectin was performed using the standards supplied by the manufacturer. I used 4 parameter logistic curve fitting method (SoftMax Pro 5.3, Molecular Devices Corporation Sunnyvale, CA, USA) to derive soluble P selectin levels from optical density values. Duplicate samples and samples from normal controls were used in each plate to ensure assay precision. 20 quality control volunteer samples were assayed with a mean Co-efficient of variation (COV) of 5.2%.

Measurement of soluble CD40 ligand

Soluble CD40 ligand was measured using Human CD40 Ligand/TNFSF5 immunoassay, DCDL40, R&D systems, Abington, UK (lot number 298718, part number 890989). I used 4 parameter logistic curve fitting method (SoftMax Pro 5.3, Molecular Devices Corporation Sunnyvale, CA, USA) to derive soluble CD40 ligand levels from optical density values. Quality control was performed using the standards supplied by the manufacturer. Duplicate samples and samples from normal controls were used in each plate to ensure assay precision. 20 quality control volunteer samples were assayed with a mean COV of 6.3%.

Measurement of Plasminogen activator inhibitor – 1 (PAI - 1)

Plasma samples were used to measure PAI-1 levels. I performed PAI – 1 antigen ELISA assay using Technozym® PAI-1 antigen ELISA kit, TC12075, Technoclone GmbH. I performed all the PAI – 1 assay in the nationally accredited laboratory at Freeman Hospital, Newcastle upon Tyne. My performance was supervised by the chief technician. Quality control was performed using the standards provided by the manufacturer. Duplicate samples and samples from normal controls were used in each plate to ensure assay precision. 20 quality control volunteer samples were assayed with a mean COV of 4.2%.

Measurement of inflammatory biomarkers (serum cytokines)

To measure inflammatory cytokines, I used a novel multi array platform which implements electrochemiluminescence (ECL) technology (MesoScale Devices, Gaithersburg, MD, USA). Electrochemiluminescence (ECL) has a lot of advantages over conventional ELISA, a few are as listed below:

i. High sensitivity: Multiple excitation cycles can amplify signals to enhance light levels

ii. Broad dynamic range: this helps to measure high and low expression levels without multiple sample dilutions

iii. Low background: the stimulation mechanism (electrical) is decoupled from the detection signal (light) allowing only labels near the electrode surface to be detected

iv. Carbon electrode plate surface has a 10X greater binding capacity than conventional ELISA microplates

v. Multiple analytes can be run in a single well, reducing required sample volume (Toedter *et al.*, 2008; Fu *et al.*, 2010)

vi. Greater flexibility: Labels are stable, non-radioactive, conveniently conjugated to biological molecules and quicker than conventional ELISA

MSD platform that I used was a 96-well plate-based assay that incorporated ECL technology. MSD wells contains spotted / capture antibody, specific cytokines are captured by these antibodies after the study samples are added, labelled detection

antibodies are added and they bind to the cytokines in a sandwich manner. The detection antibodies are attached to electrochemiluminescent labels that emitted light when electrochemically stimulated via carbon-coated electrodes in the bottom of the array wells. A detection buffer is added prior to the reading of the assay to enhance the electrochemical signal. The resulting signal was read using a photo detector in a charge-coupled device (sector reader) available in Institute of Cellular Medicine, Newcastle University.

Duplicates were run as 1:4 and the reproducibility of the results were excellent with a COV of 3.2% in 20 volunteer samples tested in all the six plates. Five volunteer samples were repeated on three different days and the results showed a COV of 3.4%.

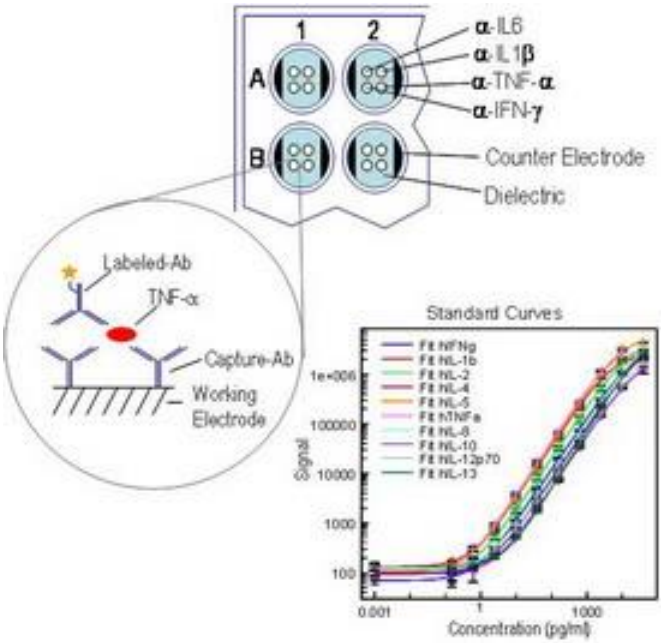


Figure 3.16 MSD Electrochemiluminescence technology

Carbon-coated working electrodes are in the bottom of the array wells. Cytokines from the sample are captured by the spotted antibodies following which the addition of detection antibodies sandwiches the cytokines between these two antibodies. Detection antibodies are attached to electrochemiluminescent labels that emit light when stimulated by the electrodes. Addition of a detection buffer enhanced the electrochemical signal and the resulting signal was read using a photo detector in a charge-coupled device.

Chapter 4 Results and discussion

4.1 Platelet dependant thrombus formation – Elderly stable CAD study

4.1.1 Results

Elderly stable CAD study was a non-randomised, open-labelled study assessing the efficacy of clopidogrel as a second antiplatelet agent alongside aspirin in patients with established CAD, with and without T2DM and over a wide age range. A total of 172 patients were included in this analysis and they were classified into four groups based on age and diabetic status as listed below:

iv. Age < 75 years with type 2 diabetes mellitus (T2DM); n = 41
v. Age < 75 years without diabetes; n = 45
vi. Age ≥ 75 years with T2DM; n = 41
vii. Age ≥ 75 years without diabetes; n = 45

Baseline demographic data comparing all four groups are shown in table 4.1.
Body mass index (BMI) in patients with age < 75+T2DM was significantly higher when compared to elderly patients with and without T2DM. Total cholesterol was significantly lower in the age<75 with T2DM group when compared to the Non DM population. This could be due to effective high dose statin therapy in this group. HDLc levels were lower in T2DM population when compared to the non-diabetic population. Systolic BP as expected was significantly higher in patients with age≥75 compared to the younger population. Haemoglobin levels were significantly lower in the patients with age≥75 when compared to younger population. The lowest haemoglobin was in patients with age≥75 with T2DM.

Thrombus measurements at baseline (aspirin alone, visit 1) and 1 week after 75mg daily clopidogrel therapy as a second antiplatelet agent alongside aspirin (visit 2) are shown in (Table 4.2, Figure 4.1)

At baseline all the four groups had similar high shear thrombus area (thrombus area in μ^2/mm, mean±SD, 14623± 5333 vs. 15633± 5425 vs. 15838± 6383 vs. 17725± 7186, p = 0.143). After treatment with 1 week of clopidogrel all the four groups demonstrated a significant reduction in thrombus area (thrombus area in μ^2/mm, mean±SD; Group 1: 14623±5333 vs. 11615±4313, p=0.002; Group 2: 15633±5425 vs. 12918±4663,

p<0.001; Group 3: 15838± 6383 vs. 13204±4924, p=0.001; Group 4: 17725±7186 vs. 14127±4958, p<0.001). The mean change in thrombus area was similar in all the four groups (μ^2/mm, 95% confidence interval; 3008, 1210-4805 vs. 2716, 1483-3949 vs. 2285, 700-3869 vs. 2903, 2145-3662; p=0.672) Table 4.3.

Similar findings were seen in low shear thrombus measurements as shown in table 4.3, figure 4.1. Low shear thrombus area was similar between the groups at baseline (thrombus area in μ^2/mm, mean±SD, 9204±1802 vs. 9651± 2323 vs. 9839± 2641 vs. 9773± 2273, p=0.671). One week of clopidogrel therapy demonstrated a significant reduction in low shear thrombus area (Group 1: 9204±1802 vs. 7756±1376, p<0.001; Group 2: 9651± 2323 vs. 7573± 2082, p<0.001; Group 3: 9839± 2641 vs. 8717± 1705, p=0.001; Group 4: 9773± 2273 vs. 8483± 1776, p<0.001). The mean change in thrombus area was similar in all the four groups (μ^2/mm, 95% confidence interval; 1806, 1069-2542 vs. 1821, 998-2645 vs. 1485, 776-2193 vs. 1072, 389-1756; p=0.405).

There were moderate but significant correlations between platelet count and high shear thrombus area (rho=0.197, p=0.012) and platelet count and low shear thrombus area (rho=0.264, p=0.001) at baseline (Figure 4.2).

	Age < 75 + T2DM (n = 41)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Demographic data: Mean ± SD or % (n)					
Age, years	63.7 ± 7.9	63 ± 7.3	78.6 ± 3.2	79.0 ± 3.7	
Male gender, % (n)	82.9 (34)	68.9 (31)	80.5 (33)	71.1 (32)	0.346
Body mass index, kg/m^2	31.7 ± 4.4	29.6 ± 3.7	29.1 ± 5.0	27.8 ± 3.6	0.000
Waist to hip ratio	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.090
Heart rate, beats per minute	70 ± 13*	62 ± 11*	68 ± 14	64 ± 10	0.005
Systolic BP, mmHg	143 ± 19.2	137 ± 17	156 ± 22.5	152 ± 22.4	0.000
Diastolic BP, mmHg	77 ± 10.4	77 ± 7.8	76 ± 10	75± 9.0	0.675
Risk profile: % (n)					
Angina	68.3 (28)	77.8 (35)	80.5 (33)	60 (27)	0.131
Previous MI	43.9 (18)	48.9 (22)	46.3 (19)	66.7 (30)	0.130

Previous PCI	29.3 (12)	40 (18)	34.1 (14)	28.9 (13)	0.655
Previous CABG	31.7 (13)	11.1 (5)	39.0 (16)	26.7 (12)	0.026
H/o Hypertension	36.6 (15)	51.1 (21)	68.3 (28)	66.7 (30)	0.610
H/o CKD	9.8 (4)	0 (0)	14.6 (6)	2.2 (1)	0.020
H/o PVD	9.8 (4)	0 (0)	26.8 (11)	11.1 (5)	0.002
H/o CVA	9.8 (4)	4.4 (2)	14.6 (6)	8.9 (4)	0.447
Medications: % (n)					
Sulphonylurea	29.3 (12)		19.5 (8)		
Metformin	63.4 (26)		47.5 (19)		
Insulin	24.4 (10)		48.8 (20)		
Beta-blocker	78 (32)	82.2 (37)	68.3 (28)	66.7 (30)	0.343
ACE inhibitor / ARB	70.7 (29)	73.3 (33)	87.8 (36)	84.4 (38)	0.152
Statin	100 (41)	93.3 (42)	92.7 (38)	95.6 (43)	0.380
Laboratory data: Mean ± SD					
Haemoglobin, g/dl	13.7 ± 1.1	13.6 ± 1.2	12.6 ± 1.3	12.9 ± 1.2	0.000

Platelets x 1000 cells/mm^3	223 ± 53.5	238 ± 63.6	211 ± 48.8	232 ± 66.2	0.182
Fibrinogen, g/ml	3.4 ± 0.6	3.2 ± 0.6	3.4 ± 0.7	3.3 ± 0.6	0.302
HbA1c, mmol/mol	58.8 ± 14.1	39.3 ± 7.0	61.2 ± 15.1	41.3 ± 3.4	
Fasting plasma glucose, mmol/L	8.4 ± 4.6	5.2 ± 0.6	8.3 ± 4.3	5.2 ± 0.7	
Creatinine, micromol/L	100 ± 25.7	83 ± 25.2*	110 ± 53.5*	100 ± 27.7	0.004
Total cholesterol, mmol/L	3.6 ± 0.7	4.2 ± 1.0	4.0 ± 0.9	4.2 ± 0.8	0.005
LDLc, mmol/L	1.9 ± 0.7	2.2 ± 0.9	2.1 ± 0.9	2.1 ± 0.7	0.369
HDLc, mmol/L	1.1 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	0.000
Triglyceride, mmol/L	1.6 ± 0.8	1.4 ± 0.8	1.5 ± 0.7	1.3 ± 0.6	0.303
HsCRP, mg/L	4.1 ± 4.0	1.8 ± 1.8	4.3 ± 6.6	4.4 ± 7.7	0.095

Table 4.1 Baseline characteristics for Elderly stable CAD study.

LDLc – Low density lipoprotein cholesterol; HDLc – High density lipoprotein cholesterol; HsCRP – high-sensitive C-reactive protein; H/o – History of

The p value in the table ANOVA p across all four groups. Significant individual pair differences are as follows:

Body mass index: Age<75+T2DM vs. Age≥75+T2DM, p=0.039; Age<75+T2DM vs. Age≥75+Non DM, p<0.001

Systolic BP: Age<75+T2DM vs. Age≥75+T2DM, p=0.023; Age<75+Non DM vs. Age≥75+T2DM, p<0.001; Age<75+Non DM vs. Age≥75+Non DM, p=0.003

Haemoglobin: Age<75+T2DM vs. Age≥75+T2DM, p<0.001; Age<75+T2DM vs. Age≥75+Non DM, p=0.006; Age<75+Non DM vs. Age≥75+T2DM, p=0.001; Age<75+Non DM vs Age≥75+Non DM, p=0.024

Total cholesterol: Age<75+T2DM vs. Age<75+Non DM, p=0.007, Age<75+T2DM vs. Age≥75+Non DM, p=0.016

HDLc: Age<75+T2DM vs. Age<75+Non DM, p=0.001; Age<75+T2DM vs. Age≥75+Non DM, p<0.001; Age≥75+T2DM vs. Age≥75+Non DM, p=0.002

*p<0.05

Mean thrombus area	**Age < 75 + T2DM (n = 41)**			**Age < 75 + Non T2DM (n = 45)**			**Age ≥ 75 + T2DM (n = 41)**			**Age ≥ 75 + Non T2DM (n = 45)**		
Mean ± SD, μ^2/mm	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear chamber	14623± 5333	11615± 4313	0.002	15633± 5425	12918± 4663	0.000	15838± 6383	13204± 4924	0.001	17725± 7186	14127± 4958	0.000
Low shear chamber	9204± 1802	7756± 1376	0.000	9651± 2323	7573± 2082	0.000	9839± 2641	8717± 1705	0.001	9773± 2273	8483± 1776	0.000

Table 4.2 Mean thrombus area - Elderly stable CAD study.

	Age < 75 + T2DM (n = 41)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean thrombus area: Mean ± SD					
High shear chamber, μ^2/mm	14623± 5333	15633± 5425	15838± 6383	17725± 7186	0.143
Low shear chamber, μ^2/mm	9204± 1802	9651± 2323	9839± 2641	9773± 2273	0.671
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean thrombus area: Mean ± SD					
High shear chamber, μ^2/mm	11615± 4313	12918± 4663	13204± 4924	14127± 4958	0.127
Low shear chamber, μ^2/mm	7756± 1376	7573± 2082*	8717± 1705	8483± 1776*	0.013
Difference in thrombus area between Visit 1 and Visit 2 Mean change in thrombus area, 95% CI					
High shear chamber, μ^2/mm	3008, 1210-4805	2716, 1483-3949	2285, 700-3869	2903, 2145-3662	0.672
Low shear chamber, μ^2/mm	1806, 1069-2542	1821, 998-2645	1485, 776-2193	1072, 389-1756	0.405

Table 4.3 Comparison of mean thrombus area.

Post hoc analysis to compare the mean thrombus area and difference in thrombus area between visit 1 and visit 2 among the four groups. The p value is the ANOVA p across all four groups

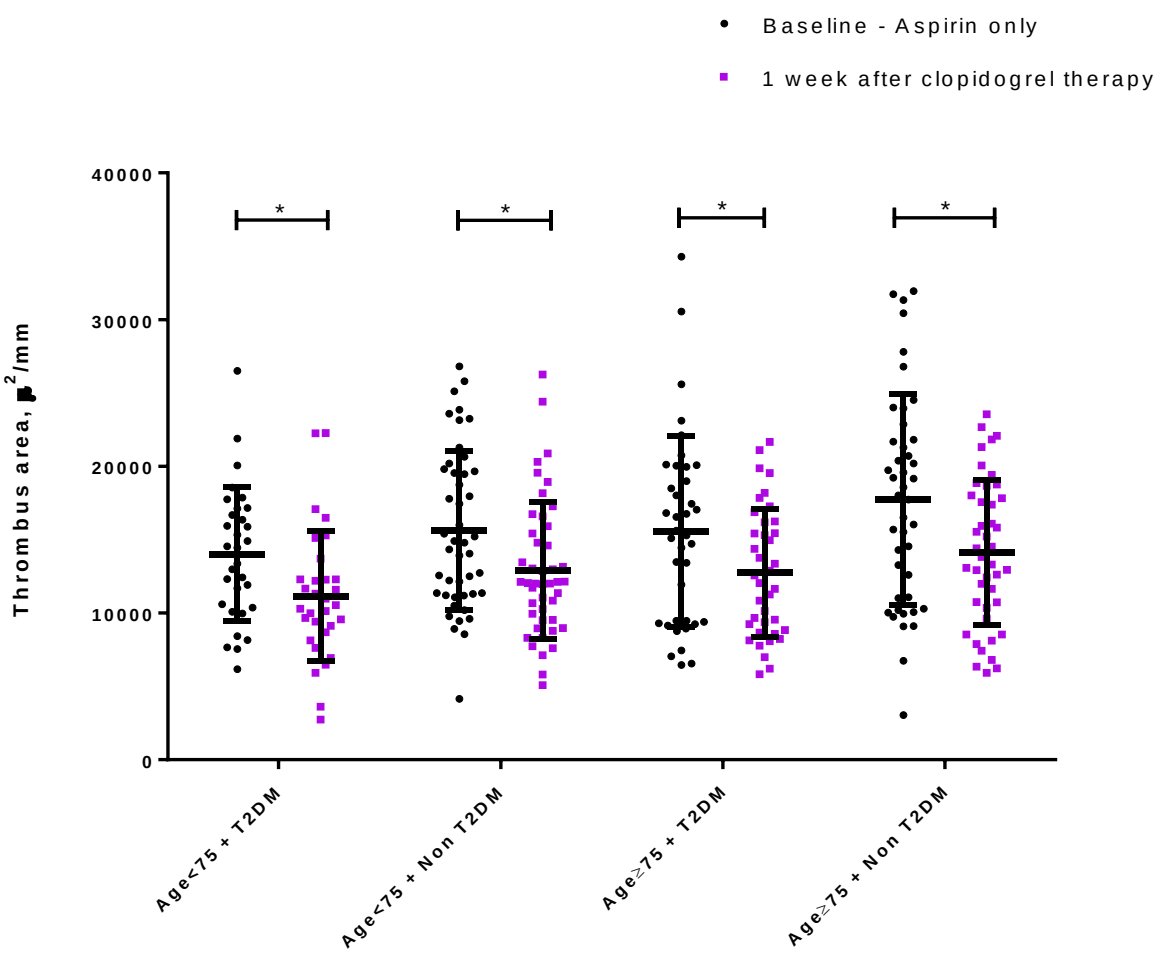


Figure 4.1 High shear thrombus area in elderly stable CAD study.

After treatment with 1 week of clopidogrel all the four groups demonstrated a significant reduction in thrombus area (μ^2/mm, mean±SD; Group 1: 14623±5333 vs. 11615±4313, p=0.002; Group 2: 15633±5425 vs. 12918±4663, p<0.001; Group 3: 15838± 6383 vs. 13204±4924, p=0.001; Group 4: 17725±7186 vs. 14127±4958, p<0.001).

Mean difference in thrombus area was similar in all the four groups (μ^2/mm, 95% confidence interval; 3008, 1210-4805 vs. 2716, 1483-3949 vs. 2285, 700-3869 vs. 2903, 2145-3662; p=0.672).

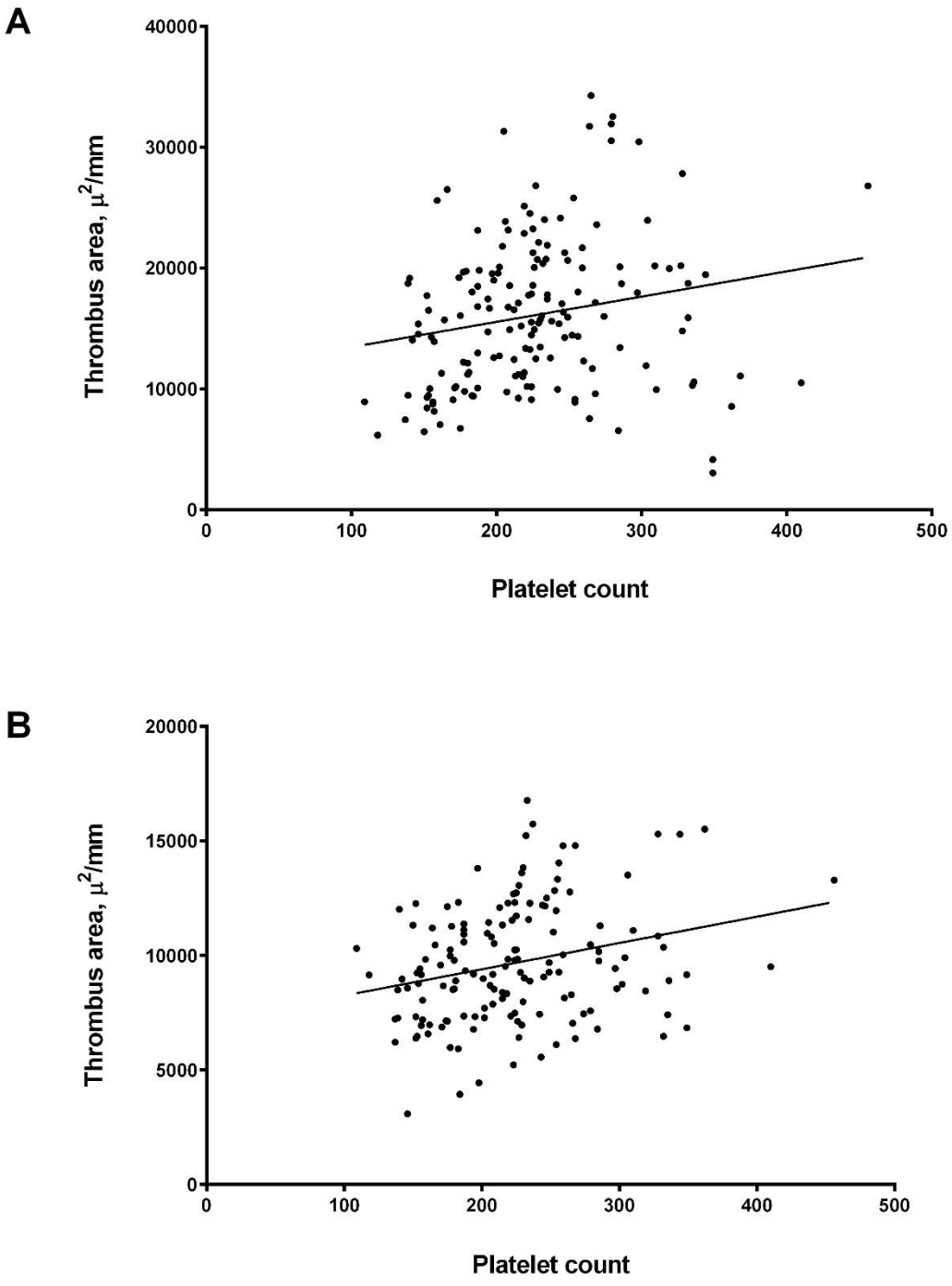


Figure 4.2 Correlation of platelet count with high shear (A) and low shear thrombus (B)

There a significant positive correlation between high shear thrombus area and platelet count (rho=0.197, p=0.012). Low shear thrombus area also demonstrated a significant positive correlation with platelet count (rho=0.264, p=0.001).

Age<75 years vs. Age≥75 years

I then classified the study population as young (age<75 years) and elderly (age≥75 years) to assess the effect of age on clopidogrel response and platelet dependent thrombus formation.

Demographic data between these two groups is shown in Table 4.4. The elderly group had significantly higher number of participants with history of hypertension and peripheral vascular disease (PVD). Mean systolic BP was higher in the elderly population. Laboratory data demonstrated significantly higher serum creatinine and HDLc levels and lower haemoglobin levels in the elderly group. Though the absolute value of mean HsCRP was higher in the elderly group, it was not statistically significant. Significantly higher number of patients in the elderly group were on ACE-inhibitor therapy. Though the number of participants with diabetes was similar in the younger and older groups, a significantly higher number of older individuals were prescribed Insulin therapy (Table 4.4).

At baseline, though the absolute numerical value of mean high thrombus area was slightly higher in the elderly population this was not statistically significant (thrombus area in μ^2/mm, mean±SD, 15171±5374 vs. 16804±6831, p=0.088).

After treatment with 1 week of clopidogrel there was a significant reduction in thrombus area in both the groups (Age<75: 15171± 5374 vs. 12321± 4526, p<0.001; Age≥75: 16804± 6831 vs. 13677± 4934, p<0.001). The mean change in the thrombus area was similar between the groups (μ^2/mm, 95% confidence interval; 2967, 2013-3921 vs. 3168, 2011-4324, p=0.889) (Table 4.5, Figure 4.3, Figure 4.6).

Low shear thrombus area demonstrated a similar response pattern as shown in Table 4.5 and Table 4.6.

	Age < 75 (n = 87)	Age ≥ 75 (n = 86)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	63.4±7.5	78.9±3.5	
Male gender, % (n)	75.9 (66)	75.6 (65)	0.966
Body mass index, kg/m^2	30.8±4.4	28.4±4.3	0.001 *
Waist to hip ratio	0.97±0.10	0.95±0.07	0.079
Heart rate, beats per minute	66±12	66±12	0.959
Systolic BP, mmHg	140±18.2	154±22.4	0.000 *
Diastolic BP, mmHg	77±9	76±9.5	0.296
Risk profile: % (n)			
Angina	73.6 (64)	69.8 (60)	0.580
Previous MI	46 (40)	57 (49)	0.148
Previous PCI	35.6 (31)	31.4 (27)	0.555
Previous CABG	20.7 (18)	32.6 (28)	0.077
Diabetes Mellitus	48.3 (42)	47.7 (41)	0.937
Hypertension	44.8 (39)	67.4 (58)	0.003
CKD	4.6 (4)	8.1 (7)	0.340
PVD	4.6 (4)	18.6 (16)	0.004
CVA	6.9 (6)	11.6 (10)	0.283
Medications: % (n)			
Sulphonylurea	13.8 (12)	9.3 (8)	0.356
Metformin	31 (27)	22.4 (19)	0.198
Insulin	12.6 (11)	24.4 (21)	0.046
Beta-blocker	79.3 (69)	67.4 (58)	0.198

ACE inhibitor / ARB	72.4 (63)	86 (74)	0.027
Statin	96.6 (84)	94.2 (81)	0.459
Laboratory data: Mean ± SD			
Haemoglobin, g/dl	13.6±1.2	12.7±1.2	0.000
Platelets x 1000 cells/mm^3	233±60.5	222±59.2	0.248
Fibrinogen, g/ml	3.3±0.6	3.3±0.6	0.748
HbA1c, mmol/mol	48.6±14.6	50.8±14.6	0.319
Fasting plasma glucose, mmol/L	6.7±3.5	6.7±3.4	0.943
Creatinine, micromol/L	91±26.5	105±42	0.010
Total cholesterol, mmol/L	3.9±0.9	4.1±0.9	0.272
LDLc, mmol/L	2.1±0.8	2.1±0.8	0.890
HDLc, mmol/L	1.2±0.3	1.3±0.3	0.018
Triglyceride, mmol/L	1.5±0.8	1.4±0.7	0.343
HsCRP, mg/L	2.9±3.2	4.3±7.2	0.093

Table 4.4 Baseline characteristics (age<75 years vs. age≥75 years)

Mean thrombus area	**Age < 75 (n = 87)**			**Age ≥ 75 (n = 86)**		
Mean ± SD, μ^2/mm	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear chamber	15171± 5374	12321± 4526	0.000	16804± 6831	13677± 4934	0.000
Low shear chamber	9481± 2114	7666± 1822	0.000	9803± 2411	8589± 1737	0.000

Table 4.5 Mean thrombus area (age<75 years vs. age≥75 years)

	Age < 75 (n = 87)	**Age ≥ 75 (n = 86)**	**P Value**
Stable CAD Patients on Aspirin alone (Visit 1) Mean thrombus area: Mean ± SD			
High shear chamber, μ^2/mm	15171±5374	16804±6831	0.088
Low shear chamber, μ^2/mm	9481± 2114	9803± 2411	0.347
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean thrombus area: Mean ± SD			
High shear chamber, μ^2/mm	12321± 4526	13677± 4934	0.066
Low shear chamber, μ^2/mm	7666± 1822	8589± 1737	0.001*
Change in thrombus area between Visit 1 and Visit 2 Mean change in thrombus area, 95% CI			
High shear chamber, μ^2/mm	2967, 2013-3921	3168, 2011-4324	0.889
Low shear chamber, μ^2/mm	1799, 1229-2369	1227, 734-1719	0.132

Table 4.6 Comparison of thrombus area (age<75 years vs. age≥75 years)

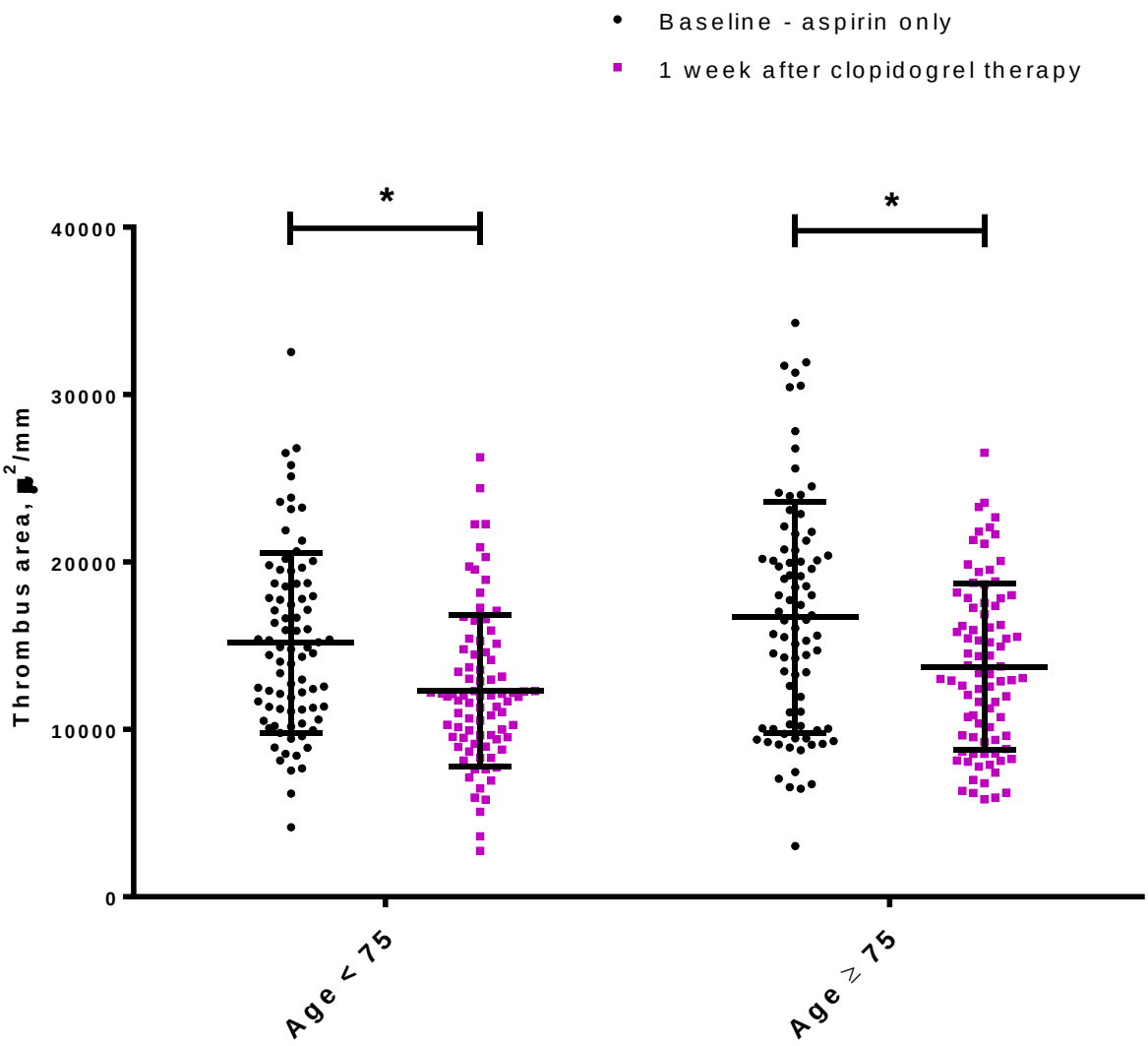


Figure 4.3 High shear thrombus area (age<75 and age≥75)

At baseline, there was no difference in thrombus area between elderly and young (thrombus area in μ2/mm, mean±SD, 15171±5374 vs. 16804±6831, p=0.088).

After treatment with 1 week of clopidogrel there was a significant reduction in thrombus area in both the groups (Age<75: 15171± 5374 vs. 12321± 4526, p<0.001; Age≥75: 16804± 6831 vs. 13677± 4934, p<0.001).

The mean difference in the thrombus area was similar between the groups (μ2/mm, 95% confidence interval; 2967, 2013-3921 vs. 3168, 2011-4324, p=0.889).

T2DM vs. Non DM

I assessed the effect of T2DM on clopidogrel response and platelet dependant thrombus formation. For this, I classified the study population in to patients with T2DM and non-diabetic patients.

Demographic data between the two groups are shown in the Table 4.7. The T2DM group demonstrates significantly higher BMI, waist to hip ratio (WHR), resting heart rate, history of chronic kidney disease (CKD), coronary artery bypass graft (CABG) and PVD. Laboratory data demonstrated significantly higher serum creatinine levels but lower total cholesterol and HDLc levels in T2DM group. Absolute values of fibrinogen and HsCRP were high in the T2DM group but the differences were not statistically significant (Table 4.7).

At baseline, the mean high shear thrombus area was similar in patients with T2DM and non DM group (thrombus area in μ^2/mm, mean±SD, 15254±5895 vs. 16656±6397, p=0.142).

After treatment with 1 week of clopidogrel there was a significant reduction in thrombus area in both the groups (T2DM: 15254± 5895 vs. 12440± 4679, p<0.001; Non DM: 16656± 6397 vs. 13509± 4820, p<0.001). The mean change in the thrombus area was similar between the groups (μ^2/mm, 95% confidence interval; 2771, 1625-3917 vs. 3312, 2305-4318, p=0.508) (Figure 4.4, Table 4.8, Table 4.9).

Low shear thrombus area demonstrated a similar response pattern as shown in Table 4.8 and Table 4.9.

	T2DM (n = 83)	Non DM (n = 90)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	71±9.6	71±9.9	0.985
Male gender, % (n)	81.9 (68)	70 (63)	0.068
Body mass index, kg/m^2	30.1±5.1	28.7±3.7	0.006 *
Waist to hip ratio	0.98±0.07	0.95±0.10	0.006 *
Heart rate, beats per minute	69±13	63±11	0.001 *
Systolic BP, mmHg	150±21.7	145±21.3	0.118
Diastolic BP, mmHg	77±10	76±8	0.579
Risk profile: % (n)			
Angina	74.7 (62)	68.9 (62)	0.397
Previous MI	45 (37)	58 (52)	0.083
Previous PCI	32.5 (27)	34.4 (31)	0.790
Previous CABG	34.9 (29)	18.9 (17)	0.017 *
Hypertension	53 (44)	58.9 (53)	0.437
CKD	12 (10)	1.1 (1)	0.003 *
PVD	18.1 (15)	5.6 (5)	0.010 *
CVA	12 (10)	6.7 (6)	0.222
Medications: % (n)			
Sulphonylurea	24.1 (20)	0	
Metformin	56.1 (46)	0	
Insulin	37.3 (31)	0	
Beta-blocker	72.3 (60)	74.4 (67)	0.154
ACE inhibitor / ARB	79.5 (66)	78.9 (71)	0.919
Statin	96.4 (80)	94.4 (85)	0.544
Laboratory data: Mean ± SD			

Haemoglobin, g/dl	13.1±1.3	13.2±1.2	0.593
Platelets x 1000 cells/mm^3	218±53	235±64.6	0.077
Fibrinogen, g/ml	3.4±0.6	3.2±0.6	0.060
HbA1c, mmol/mol	60.1±14.5	40.3±5.5	
Fasting plasma glucose, mmol/L	8.3±4.4	5.2±0.6	
Creatinine, micromol/L	105±41.9	91±27.7	0.014 *
Total cholesterol, mmol/L	3.8±0.9	4.2±0.9	0.003 *
LDLc, mmol/L	2.0±0.8	2.2±0.8	0.190
HDLc, mmol/L	1.1±0.3	1.4±0.3	0.000 *
Triglyceride, mmol/L	1.6±0.7	1.4±0.7	0.109
HsCRP, mg/L	4.2±5.4	3.1±5.7	0.196

Table 4.7 Baseline characteristics (T2DM vs. Non-DM)

*p<0.05

Mean thrombus area	**T2DM (n = 83)**			**Non DM (n = 90)**		
Mean ± SD, μ^2/mm	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear chamber	15254± 5895	12440± 4679	0.000 *	16656± 6397	13509± 4820	0.000 *
Low shear chamber	9573± 2303	8295± 1641	0.000 *	9714± 2265	8050± 1970	0.000 *

Table 4.8 Mean thrombus area. (T2DM vs. Non-DM)

	T2DM (n = 83)	**Non DM (n = 90)**	**P Value**
Stable CAD Patients on Aspirin alone (Visit 1) Mean thrombus area: Mean ± SD			
High shear chamber, μ^2/mm	15254± 5895	16656± 6397	0.142
Low shear chamber, μ^2/mm	9200± 2476	9939± 2666	0.076
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean thrombus area: Mean ± SD			
High shear chamber, μ^2/mm	12440± 4679	13509± 4820	0.148
Low shear chamber, μ^2/mm	7601± 1880	8505± 2240	0.007 *
Change in thrombus area between Visit 1 and Visit 2 Mean change in thrombus area, 95% CI			
High shear chamber, μ^2/mm	2771, 1625-3917	3312, 2305-4318	0.508
Low shear chamber, μ^2/mm	1613, 1096-2131	1409, 872-1945	0.575

Table 4.9 Comparison of mean thrombus area (T2DM vs. Non-DM)

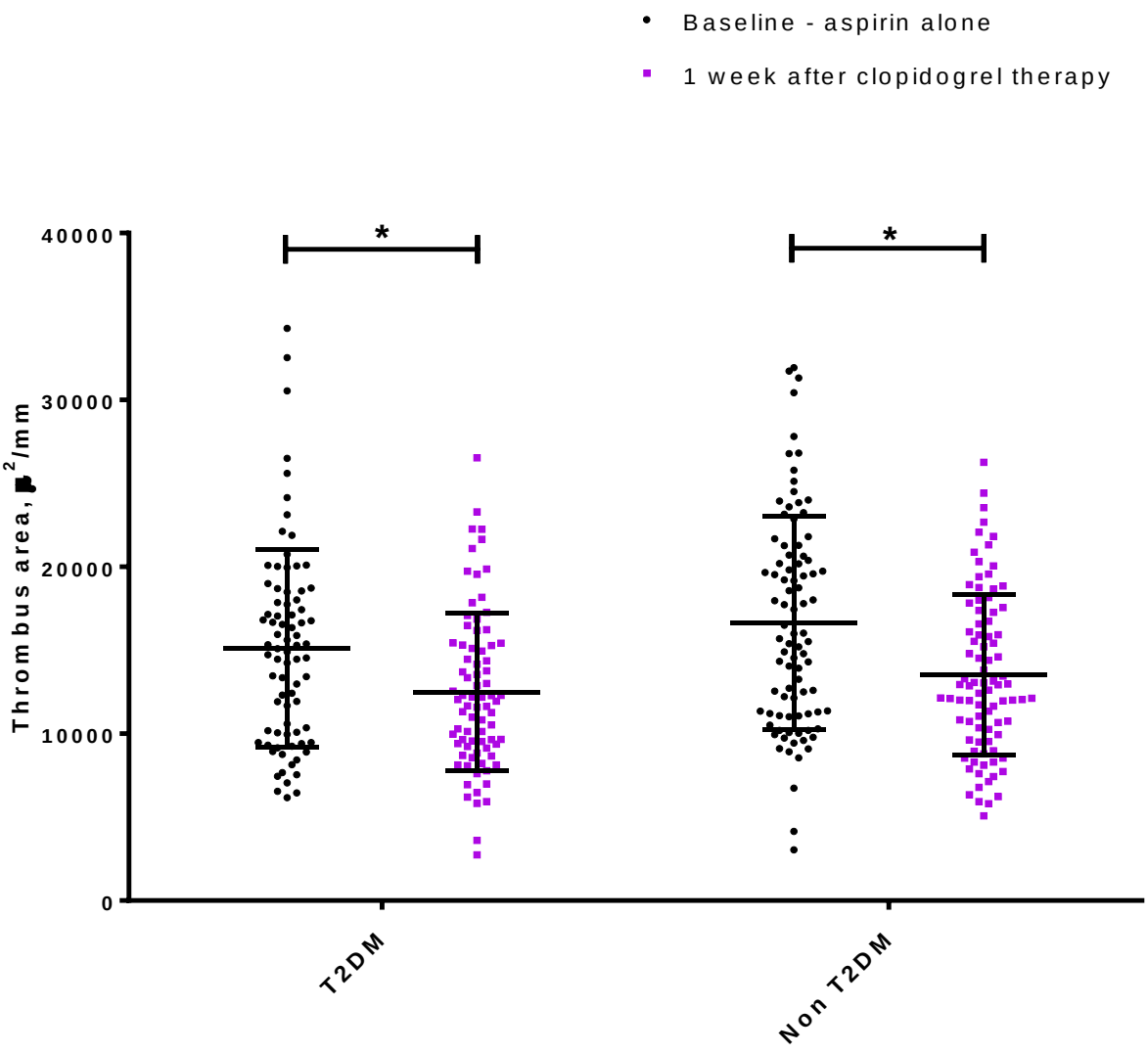


Figure 4.4 High shear thrombus area (T2DM vs. Non-DM)

At baseline, the thrombus area was similar in patients with T2DM and non DM (thrombus area in μ2/mm, mean±SD, 15254±5895 vs. 16656±6397, p=0.142).

After treatment with 1 week of clopidogrel there was a significant reduction in thrombus area in both the groups (T2DM: 15254± 5895 vs. 12440± 4679, p<0.001; Non DM: 16656± 6397 vs. 13509± 4820, p<0.001).

The mean difference in the thrombus area was similar between the groups (μ2/mm, 95% confidence interval; 2771, 1625-3917 vs. 3312, 2305-4318, p=0.508).

4.1.2 Discussion

Elderly patients are at increased risk of atherothrombosis and coronary artery disease. However, these patients also encounter an increased risk of bleeding during antiplatelet therapy. Thus, we face a challenging treatment dilemma in these patients. This is further complicated by frequent co-morbid diseases and polypharmacy. Knowledge about the risk/benefit relationship of dual antiplatelet therapy is poorly understood in the elderly population since they are under-represented in all the major clinical trials (9 – 17%) (Alexander *et al.*, 2007). The current ACS treatment and stable CAD treatment guidelines are based primarily upon these studies largely comprising of younger patients. Newer P2Y12 antagonists such as prasugrel and ticagrelor have demonstrated superior platelet inhibition when compared to clopidogrel, with better ischaemic outcomes but at the expense of increased bleeding risk especially in the elderly study population. Although age may alter the risk benefit balance of dual antiplatelet therapy, data from various trials regarding elderly patients with ACS have usually been limited to subgroup analyses rather than derived from age-specific studies. Hence, despite increased usage of these agents in younger patients with NSTE-ACS / unstable angina, their usage in the elderly population remains controversial.

The elderly population with CAD has never been extensively studied as far as thrombus formation and antiplatelet therapies are concerned.

The findings from this elderly stable CAD study are important as:

i. This is the first study assessing and comparing platelet dependent thrombus formation using the Badimon chamber, in stable CAD patients with age<75 years and age≥75 years, with and without T2DM

ii. All participants were on appropriate secondary prevention therapy including aspirin (75mg daily); clopidogrel (75mg daily) was added for 1 week as dual antiplatelet therapy (DAPT)

iii. The data describe the effect of dual antiplatelet therapy on thrombus formation and platelet function in stable CAD patients with age≥75 years and age<75 years, with and without T2DM

The aim of our study was to compare the effect of clopidogrel on thrombus formation, when added to aspirin 75mg daily, in elderly and young patients with stable CAD, in the presence and absence of T2DM. Our study demonstrated a significant reduction in thrombus area when clopidogrel was added to aspirin 75mg daily. This reduction was consistently seen in both elderly (age≥75 years) and young (age<75 years) patients with and without T2DM. When elderly patients were compared with the young, there was no difference in thrombus area between the groups at baseline. The reduction in thrombus area with clopidogrel were similar between the groups. When T2DM patients were compared with non-diabetic patients, baseline thrombus area and the reduction in thrombus area after a week of clopidogrel therapy were similar between the groups.

This is the first study that evaluates the effect of “age” (an established cardiovascular risk factor) on platelet dependent thrombus formation, platelet reactivity, coagulation and inflammatory biomarkers and response to dual antiplatelet therapy. The findings from this stable CAD study have significant implications in the management of elderly patients with CAD. To achieve a significant reduction of thrombus area with DAPT in all the 4 groups of patients with stable CAD and good secondary prevention therapy (good lipid control, good hypertension control and good glycaemic control) is clinically very important. The reduction in thrombus area was also similar in all the four groups. This reduction in thrombus formation was not associated with any increase in bleeding complications during and one week after the study period. If DAPT is continued beyond the study duration the benefits are likely to be sustained and whether any long term benefits might outweigh the bleeding risk remains to be ascertained.

My findings complement the results from the Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management and Avoidance (CHARISMA) trial and support the use of clopidogrel added to aspirin therapy in stable CAD groups. The CHARISMA trial was designed to examine whether DAPT provided greater vascular protection than aspirin alone in patients with stable CAD. In the final analysis, there was a suggestion of marginally significant reduction of the cumulative risk of myocardial infarction, stroke or cardiovascular death in the subset of patients with symptomatic atherothrombotic disease (6.9% vs. 7.9%; relative risk, 0.88; 95%CI, 0.77 to 0.998; p=0.046). This marginal benefit of DAPT came at the expense of an increased risk of Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (GUSTO) defined moderate bleeding (2.1% vs. 1.3%,

p<0.001) (Bhatt *et al.*, 2006). A separate subgroup analysis of patients with previous myocardial infarction, ischaemic stroke or symptomatic peripheral arterial disease (PAD) also demonstrated similar results. In this cohort, DAPT decreased the rate of cardiovascular death, myocardial infarction or stroke (7.3% vs. 8.8%; HR, 0.83; 95%CI, 0.72 to 0.96; p=0.01), and the risk of hospitalisations for ischaemia (11.4% vs. 13.2%; HR, 0.86; 95%CI, 0.76 to 0.96; p=0.008) when compared with aspirin and placebo. This benefit was associated with increased risk of GUSTO defined moderate (2.0% vs. 1.3%; HR, 1.60; 95%CI, 1.16 to 2.20; p=0.004), but not severe bleeding (1.7% vs. 1.5%; HR, 1.12; 95%CI 0.81 to 1.53; p=0.50). This analysis demonstrated the potential benefit from intensification of antithrombotic therapy beyond aspirin alone, especially in patients with risk factors such as documented prior MI, ischaemic stroke or symptomatic PAD. A subgroup analysis comparing patients with age<75 years and age≥75 years did not demonstrate any significant difference in clopidogrel response both in terms of primary outcomes and bleeding complications. Another subgroup analysis of the CHARISMA trial also showed that the benefits of clopidogrel are greater in the population with T2DM and a previous cardiovascular event (relative risk reduction: 17.1%; 95% CI: 4.4 – 28.1). Overall, the CHARISMA trial demonstrated a small but significant benefit in the subgroup of patients with known atherothrombosis and a potential role for intensification of antithrombotic therapy beyond aspirin alone (long-term dual antiplatelet therapy) in certain high-risk groups. My study has demonstrated a significant reduction in overall thrombus formation in both the younger and older cohorts, in both patients with and without T2DM, highlighting the fact that clopidogrel might still have a significant role in selected groups of patients, despite the availability of prasugrel and ticagrelor. Though the current study has been performed in patients with stable coronary artery disease, the results from this study will certainly help in assessing the way patients might respond to dual antiplatelet therapy in the setting of an acute coronary event.

In the management of acute coronary syndrome, current cardiovascular society guidelines (AHA/ACC/ESC) do not recommend prasugrel in elderly patients (age≥75 years) and recommend ticagrelor over clopidogrel even in elderly population unless there is a specific contraindication to ticagrelor (Amsterdam *et al.*, 2014; Roffi *et al.*, 2015).

In TRITON–TIMI 38 (TRial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet InhibitioN with Prasugrel–Thrombolysis In Myocardial Infarction 38)

study, Prasugrel has been shown to reduce the risk of ischaemic events when compared with clopidogrel in ACS patients undergoing PCI with a 60mg loading dose followed by 10mg daily maintenance dose (Wiviott *et al.*, 2007a). However, no net benefit was observed in elderly patients (≥75 years) due to increased occurrence of intracranial and fatal bleeding. Hence prasugrel 10mg daily is not recommended in patients with age ≥ 75 years. GENERATIONS (comparison of prasugrel and clopidogrel in very elderly and non-elderly patients with stable coronary artery disease) trial recruited aspirin treated patients with stable CAD, to compare pharmacodynamic (PD) and pharmacokinetic effects of adding prasugrel 5mg in patients aged ≥ 75 years with prasugrel 10mg in patients aged ≥ 45 and < 65 years. Prasugrel 5mg attenuated platelet inhibition while meeting pre-specified non inferiority criteria versus prasugrel 10mg in younger patients. Prasugrel 5mg also demonstrated significantly better PD response with fewer poor responders compared to clopidogrel 75mg in patients aged ≥ 75 years (Erlinge *et al.*, 2012).

The Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes (TRILOGY ACS) study demonstrated a steep increase in risks of ischaemic and bleeding events with increasing age among ACS patients managed medically without revascularisation. Prasugrel was not shown to be superior to clopidogrel in reducing the primary end point during 2.5 years of follow-up, even though signs of intensified platelet inhibition were observed in the prasugrel group. There was no difference in the risks of both ischaemic and bleeding outcomes with reduced-dose prasugrel (5mg daily) versus clopidogrel in elderly patients (age ≥ 75 years) (Gurbel *et al.*, 2012).

The PLATelet inhibition and patient Outcomes (PLATO) trial demonstrated the superiority of ticagrelor as compared with clopidogrel in 18,624 patients with STEMI or NSTE-ACS. The trial showed a significant reduction in the primary composite end point (death from vascular causes, myocardial infarction, or stroke) with ticagrelor compared to clopidogrel (9.8% vs. 11.7%, HR: 0.84, 95% CI: 0.77–0.92, $p < 0.001$). There were also significant reductions of the individual endpoints of total death, cardiovascular death, myocardial infarction and stent thrombosis. There were no differences in overall major bleeding but a significant increase in non-procedure related major bleeding (James *et al.*, 2009; Wallentin *et al.*, 2009). A substudy from PLATO trial investigated the effect and treatment related complications of ticagrelor versus clopidogrel in elderly patients (age ≥ 75 years) with ACS compared with those < 75 years of age. The clinical

benefit of ticagrelor over clopidogrel was not significantly different between patients aged ≥ 75 years (n=2878) and those aged < 75 years (n=15744) with respect to composite endpoint of CV death, myocardial infarction, or stroke (interaction p=0.56), MI (p=0.33), CV death (P=0.47), definite stent thrombosis (p=0.81) or all-cause mortality (p=0.76). No increase in overall major bleeding with ticagrelor versus clopidogrel was observed in patients aged ≥ 75 years (HR: 1.02, 95% CI: 0.82-1.27) or patients aged < 75 years (HR: 1.04, 95% CI: 0.94-1.15) (Husted *et al.*, 2012). Dyspnoea and ventricular pauses were more common with ticagrelor with no evidence of an age-by-treatment interaction. Over all, ticagrelor is preferred over clopidogrel in ACS setting with or without coronary revascularisation in elderly and young patients.

The optimal treatment duration and intensity of P2Y12 inhibition after a coronary event in patients who do not undergo revascularization remain uncertain. In a recent study conducted by Mauri et al, a total of 9961 patients who underwent coronary revascularisation with drug-eluting stent were randomly assigned to continue DAPT or to receive placebo alongside aspirin after 1 year of DAPT and were followed up to 30 months. 65 percent of patients received clopidogrel and 35% received prasugrel. The patients in DAPT group were continued on the same P2Y12 inhibitor that they were on initially. This study demonstrated that DAPT as compared to aspirin alone, beyond 1 year reduced the risks of stent thrombosis and further ischaemic events. Although longer duration of DAPT was associated with greater risk of bleeding, severe or fatal bleeding was uncommon and the rate did not differ significantly between the groups (Mauri *et al.*, 2014).

Our findings in stable CAD patients highlight the fact that clopidogrel is effective in reducing platelet dependent thrombus in older and younger patients, in individuals with and without diabetes. Clopidogrel has the least bleeding risk when compared to prasugrel or ticagrelor and is much cheaper. With more and more data being published about the need for extended DAPT following an acute coronary event and revascularisation, this study reinforces the potential usage of clopidogrel in certain high risk group especially in the elderly.

Despite the currently recommended aggressive secondary prevention therapy, patients with T2DM experience more thrombotic events and have poorer prognosis compared to people without diabetes, both in NSTE-ACS setting and stable CAD setting (Bartnik *et al.*, 2005). In my study, platelet dependent thrombus (measured as

total thrombus area) was similar in patients with and without T2DM. Addition of clopidogrel to aspirin resulted in a significant reduction in thrombus formation in stable CAD patients with and without T2DM. This reduction in thrombogenicity was not associated with any increase in bleeding complications during and one week after the study period.

Our research group has previously demonstrated higher thrombus area in patients with T2DM and stable CAD compared to people without diabetes and without CAD. Thrombus area was similar in patients with T2DM without CAD and patients with CAD without T2DM suggesting T2DM as “Coronary artery disease equivalent” (Natarajan *et al.*, 2007; Natarajan *et al.*, 2008a). We have also demonstrated that in NSTE-ACS setting, patients with T2DM demonstrated significantly higher blood thrombogenicity despite the current recommended antithrombotic therapy (Viswanathan *et al.*, 2012a). A subgroup analysis of the CHARISMA study demonstrated greater beneficial effects of clopidogrel in patients with T2DM and a previous cardiovascular event (relative risk reduction: 17.1%; 95% CI: 4.4-28.1) (Bhatt *et al.*, 2007). Despite the availability of newer antiplatelet agents, outcomes in patients with T2DM have not improved to the levels seen in non-diabetic patients. In PLATO and TRITON-TIMI 38 trials in which ticagrelor and prasugrel were compared with clopidogrel, one year mortality rate in the diabetic cohort still remained at 7.0% and 12.2% respectively (Wiviott *et al.*, 2008; James *et al.*, 2010).

My findings strengthen the argument that dual antiplatelet therapy might be able to provide better platelet inhibition without overtly increasing the bleeding risk in stable CAD patients with T2DM and/or elderly who are at high risk of future thrombotic events. Large scale studies are needed to further validate the role of long term dual antiplatelet therapy in providing clinical benefits with reduction in future thrombotic events without significant increase in bleeding in this population.

4.2 Thromboelastography® (TEG) and Platelet Mapping™ - Elderly stable CAD study

4.2.1 Results – Viscoelastic properties of thrombus

I undertook TEG® and platelet mapping in the same cohort in whom thrombus area was measured. However, in the young T2DM cohort TEG and Platelet Mapping were performed in only 33 participants due to non-availability of equipment at the beginning of the previous study. The demographic and biochemical data characteristics are similar to the whole cohort (Table 4.10). Baseline TEG® parameters (e.g. R, K, MA to kaolin, G, clot index) were similar across all the four groups.

After one week of clopidogrel therapy, there was a significant reduction in high shear thrombus area (age<75+T2DM: 14020± 4580 vs. 11151± 4445, p=0.029; age<75+non DM: 15633± 5425 vs. 12918± 4663, p<0.001; age≥75+T2DM: 15577± 6511 vs. 12733± 4387, p<0.001; age≥75+non DM: 17725± 7186 vs. 14127± 4958, p<0.001). A similar response was seen in low shear thrombus area as well (Table 4.12).

In thromboelastography parameters, elderly non diabetic patients demonstrated a reduction in clot index (CI) and prolongation of R time. All the other parameters remained unchanged. All the standard TEG® parameters (e.g. R, K, MA to kaolin, G, clot index) did not change after one week of clopidogrel therapy in the other groups (Table 4.11, Table 4.13Figure 4.5).

Baseline platelet mapping™ tests were compared between the groups. Maximum viscoelastic strength of thrombus as measured by maximum amplitude upon stimulation with 10µl of arachidonic acid (MA-AA, in mm) was significantly lower in the non-diabetic elderly group compared to the non-diabetic younger population (29.6±17.2 vs. 40.4±18.6, p=0.025). Percentage aggregation to arachidonic acid (% aggregation to AA, %) was also significantly lower in the elderly nondiabetic group when compared to the young diabetic population (32.7±31.3 vs. 55.0±37.7, p=0.007). This demonstrates good platelet inhibition with aspirin in the elderly non-diabetic group (Table 4.11).

After one week of clopidogrel therapy, there was a significant reduction in the maximum viscoelastic strength of thrombus (MA, mm) upon stimulation by 10µl of ADP. This reduction was consistently seen across all the four groups (MA, mm; age<75+T2DM:

59.9±7.4 vs. 54.0±10.8, p=0.007; age<75+non DM: 60.8±10.2 vs. 55.8±9.4, p=0.004; age≥75+T2DM: 61.5±6.3 vs. 54.5±11.2, p<0.001; age≥75+non DM: 59.8±12.7 vs. 55.6±11.7, p<0.001). Percentage aggregation to ADP demonstrated similar reduction in all the four groups (Figure 4.6).

An interesting finding was that even though participants had been on long term aspirin therapy, there was a significant improvement in aspirin induced platelet inhibition as measured by MA-AA in 3 out of 4 groups after the addition of clopidogrel (MA-AA, mm; age<75+T2DM: 34.7±19.2 vs. 25.9±15.7, p=0.002; age<75+non DM: 40.4±18.6 vs. 26.1±17.1, p<0.001; age≥75+T2DM: 29.6±17.2 vs. 20.9±12.1, p=0.001). This improvement was not demonstrated in the elderly T2DM group (31.4±14.9 vs. 27.4±16.4, p=0.125). A similar reduction was seen in the percentage aggregation to AA, further establishing the fact that clopidogrel therapy potentiated the platelet inhibitory effect of aspirin. This potentiation effect was maximum in the young non-diabetic group and the change in viscoelastic strength was significantly higher when compared to the elderly T2DM group as measured by MA-AA, in mm (mean, 95% CI; 14.3, 9.6-18.9 vs. 4.1, -1.2-9.3, p=0.033) (Table 4.13).

Moderate but statistically significant correlations were demonstrated between thrombus area and various TEG® parameters on aspirin alone, as shown in Table and figure. After one week of clopidogrel therapy, MA-ADP demonstrated a significant positive correlation with both high shear thrombus area (rho=0.219, p=0.007) and low shear thrombus area (rho=0.176, p=0.034) (Figure 4.7).

	Age < 75 + T2DM (n = 33)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Demographic data: Mean ± SD or % (n)					
Age, years	63.6 ± 7.2	63 ± 7.3	78.9 ± 3.1	79.0 ± 3.7	
Male gender, % (n)	84.8 (28)	68.9 (31)	81.6 (31)	71.1 (32)	0.275
Body mass index, kg/m^2	32.5 ± 4.9	29.6 ± 3.7	29.1 ± 5.1	27.8 ± 3.6	0.000
Waist to hip ratio	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	0.079
Heart rate, beats per minute	71 ± 12	62 ± 11	68 ± 14	64 ± 10	0.003
Systolic BP, mmHg	142 ± 19.9	137 ± 17	158 ± 22.9	152 ± 22.4	0.000
Diastolic BP, mmHg	77 ± 10.5	77 ± 7.8	76 ± 10.1	75± 9.0	0.679
Risk profile: % (n)					
Angina	75.8 (25)	77.8 (35)	81.6 (31)	60 (27)	0.114
Previous MI	48.5 (16)	48.9 (22)	50 (19)	66.7 (30)	0.258

PCI	30.3 (10)	40 (18)	36.8 (14)	28.9 (13)	0.664
CABG	27.3 (9)	11.1 (5)	42.1 (16)	26.7 (12)	0.016
Hypertension	36.4 (12)	51.1 (23)	73.7 (28)	66.7 (30)	0.006
Heart failure	9.1 (3)	0 (0)	13.2 (5)	8.9 (4)	0.128
CKD	6.1 (2)	0 (0)	13.2 (5)	2.2 (1)	0.036
PVD	12.1 (4)	0 (0)	28.9 (11)	11.1 (5)	0.001
CVA	12.1 (4)	4.4 (2)	13.2 (5)	8.9 (4)	0.523
Medications: % (n)					
Sulphonylurea	24.2 (8)		21.1 (8)		
Metformin	60.6 (20)		44.7 (17)		
Insulin	33.3 (11)		52.6 (20)		
Beta-blocker	75.8 (25)	82.2 (37)	71.1 (27)	66.7 (30)	0.401
ACE inhibitor / ARB	75.8 (25)	73.3 (33)	89.5 (34)	84.4 (38)	0.224
Statin	100 (33)	93.3 (42)	92.1 (35)	95.6 (43)	0.438
Laboratory data: Mean ± SD					

Haemoglobin, g/dl	13.7 ± 1.1	13.6 ± 1.2	12.6 ± 1.3	12.9 ± 1.2	0.000
Platelets x 1000 cells/mm^3	223 ± 53.5	238 ± 63.6	211 ± 48.8	232 ± 66.2	0.171
Fibrinogen, g/ml	3.4 ± 0.6	3.2 ± 0.6	3.4 ± 0.7	3.3 ± 0.6	0.466
HbA1c, mmol/mol	58.8 ± 14.1	39.3 ± 7.0	61.2 ± 15.1	41.3 ± 3.4	
Fasting plasma glucose, mmol/L	8.4 ± 4.6	5.2 ± 0.6	8.3 ± 4.3	5.2 ± 0.7	
Creatinine, micromol/L	100 ± 25.7	83 ± 25.2*	110 ± 53.5*	100 ± 27.7	0.005
Total cholesterol, mmol/L	3.6 ± 0.7	4.2 ± 1.0	4.0 ± 0.9	4.2 ± 0.8	0.039
LDLc, mmol/L	1.9 ± 0.6	2.2 ± 0.9	2.0 ± 0.9	2.1 ± 0.7	0.459
HDLc, mmol/L	1.1 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	0.000
Triglyceride, mmol/L	1.6 ± 0.8	1.4 ± 0.8	1.5 ± 0.7	1.3 ± 0.6	0.306
HsCRP, mg/L	4.1 ± 4.0	1.8 ± 1.8	4.3 ± 6.6	4.4 ± 7.7	0.114

Table 4.10 Baseline characteristics of participants who underwent TEG® and platelet mapping™

LDLc – Low density lipoprotein cholesterol; HDLc – High density lipoprotein cholesterol; HsCRP – high-sensitive C-reactive protein

Body mass index: Age<75+T2DM vs. Age<75+Non DM, p=0.026; Age<75+T2DM vs. Age≥75+T2DM, p=0.007; Age<75+T2DM vs. Age≥75+Non DM, p<0.001

Heart rate: Age<75+T2DM vs. Age<75+Non DM, p=0.004; Age<75+T2DM vs. Age≥75+Non DM, p=0.047

Systolic BP: Age<75+T2DM vs. Age≥75+T2DM, p=0.009; Age<75+Non DM vs. Age≥75+T2DM, p<0.001; Age<75+Non DM vs. Age≥75+Non DM, p=0.004

Haemoglobin: Age<75+T2DM vs. Age≥75+T2DM, p=0.003; Age<75+T2DM vs. Age≥75+Non DM, p=0.034; Age<75+Non DM vs. Age≥75+T2DM, p=0.001; Age<75+Non DM vs. Age≥75+Non DM, p=0.022

Total cholesterol: Age<75+T2DM vs. Age<75+Non DM, p=0.007, Age<75+T2DM vs. Age≥75+Non DM, p=0.016

HDLc: Age<75+T2DM vs. Age<75+Non DM, p=0.004; Age<75+T2DM vs. Age≥75+Non DM, p<0.001; Age≥75+T2DM vs. Age≥75+Non DM, p=0.001

	Age < 75 + T2DM (n = 33)			Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
R, min	6.1±1.5	6.4±1.5	0.442	6.5±1.7	6.6±1.5	0.721	6.6±1.9	6.0±1.4	0.013*	6.0±1.6	6.5±1.7	0.033*
K, min	1.8±0.5	1.8±0.5	0.646	1.9±0.6	1.8±0.4	0.076	1.8±0.4	1.7±0.4	0.048*	1.7±0.5	1.8±0.5	0.434
MA, mm	65.2±4.0	64.3±5.7	0.226	62.9±4.6	63.0±4.9	0.828	63.6±3.6	64.1±5.0	0.468	64.3±4.3	63.6±4.9	0.157
G Kdynes/sec	9.6±1.7	9.3±2.0	0.283	8.7±1.6	8.8±1.8	0.722	8.9±1.4	9.2±2.1	0.222	9.2±1.8	9.0±2.0	0.240
CI	0.5±2.0	0.1±2.0	0.377	-0.2±2.1	-0.1±1.7	0.788	0±1.8	0.6±1.4	0.016*	0.5±1.8	0.1±2.0	0.039*
MA-A, mm	11.4±6.6	11.8±4.1	0.703	15.9±14.7	16.1±14.2	0.892	14.7±7.5	16.4±10.2	0.151	13.8±8.1	12.1±4.5	0.074
MA-AA, mm	34.7±19.2	25.9±15.7	0.002*	40.4±18.6	26.1±17.1	0.000*	31.4±14.9	27.4±16.4	0.125	29.6±17.2	20.9±12.1	0.001*
MA-ADP, mm	59.9±7.4	54.0±10.8	0.007*	60.8±10.2	55.8±9.4	0.004*	61.5±6.3	54.5±11.2	0.000*	59.8±12.7	55.6±11.7	0.000*
% aggregation to AA	44.7±35.5	26.9±32.1	0.000*	55.0±37.7	23.8±38.1	0.000*	32.5±30.0	23.7±32.6	0.124	32.7±31.3	16.9±24.1	0.001*
% aggregation to ADP	90.0±14.0	80.7±20.1	0.002*	97.7±21.0	85.0±23.5	0.002*	96.9±14.2	81.1±22.9	0.000*	92.1±21.1	84.3±20.2	0.000*

Thrombin generation	739±295.2	741±269.3	0.954	763±55.7	764±59.7	0.778	772±42.5	777±58.4	0.476	781±54.4	771±57.3	0.066
Maximum rate of thrombin generation, min	16.8±27.3	12.7±3.9	0.391	11.5±2.9	11.8±2.5	0.419	12.3±2.5	12.9±2.5	0.065	12.9±3.6	12.8±3.8	0.814
Thrombus retraction, L parameter mm/min	74.2±32.8	78.4±17.7	0.521	84.8±26.4	83.7±21.8	0.746	87.5±22.9	88.6±36.9	0.119	83.1±16.5	80.4±23.2	0.511
Maximum rate of thrombus retraction, min	0.26±0.17	0.44±0.58	0.868	0.27±0.16	0.29±0.17	0.429	0.34±0.26	0.32±0.19	0.920	0.25±0.13	0.24±0.11	0.627

Table 4.11 Standard TEG® parameters, Platelet Mapping™ and V-curve data

Mean thrombus area	**Age < 75 + T2DM (n = 33)**			**Age < 75 + Non T2DM (n = 45)**			**Age ≥ 75 + T2DM (n = 38)**			**Age ≥ 75 + Non T2DM (n = 45)**		
Mean ± SD, μ^2/mm	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear chamber	14020± 4580	11151± 4445	0.029	15633± 5425	12918± 4663	0.000	15577± 6511	12733± 4387	0.000	17725± 7186	14127± 4958	0.000
Low shear chamber	9173± 1624	7660± 1242	0.046	9651± 2323	7573± 2082	0.000	9771± 2648	8710± 1729	0.000	9773± 2273	8483± 1776	0.000

Table 4.12 Thrombus area in patients who underwent TEG® measurements

*p<0.05

	Age < 75 + T2DM (n = 33)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 38)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD					
R, min	6.1±1.5	6.5±1.7	6.6±1.9	6.0±1.6	0.264
K, min	1.8±0.5	1.9±0.6	1.8±0.4	1.7±0.5	0.279
MA, mm	65.2±4.0	62.9±4.6	63.6±3.6	64.3±4.3	0.102
G dynes/sec	9.6±1.7	8.7±1.6	8.9±1.4	9.2±1.8	0.101
CI	0.5±2.0	-0.2±2.1	0±1.8	0.5±1.8	0.205
MA-A, mm	11.4±6.6	15.9±14.7	14.7±7.5	13.8±8.1	0.308
MA-AA, mm	34.7±19.2	40.4±18.6 *	31.4±14.9	29.6±17.2 *	0.025 *
MA-ADP, mm	59.9±7.4	60.8±10.2	61.5±6.3	59.8±12.7	0.854
% aggregation to AA	43.2±35.9	55.0±37.7 *	33.1±29.8	32.7±31.3 *	0.007 *
% aggregation to ADP	90.0±13.8	95.0±27.1	96.9±14.2	92.1±21.1	0.503
Thrombin generation	739±295.2	763±55.7	772±42.5	781±54.4	0.605

Maximum rate of thrombin generation, min	16.8±27.3	11.5±2.9	12.3±2.5	12.9±3.6	0.291
Thrombus retraction, L parameter mm/min	74.2±32.8	84.8±26.4	87.5±22.9	83.1±16.5	0.388
Maximum rate of thrombus retraction, min	0.26±0.17	0.27±0.16	0.34±0.26	0.25±0.13	0.238
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD					
R, min	6.4±1.5	6.6±1.5	6.0±1.4	6.5±1.7	0.244
K, min	1.8±0.5	1.8±0.4	1.7±0.4	1.8±0.5	0.372
MA, mm	64.3±5.7	63.0±4.9	64.1±5.0	63.6±4.9	0.709
G dynes/sec	9.3±2.0	8.8±1.8	9.2±2.1	9.0±2.0	0.639
CI	0.1±2.0	-0.1±1.7	0.6±1.4	0.1±2.0	0.272
MA-A, mm	11.8±4.1	16.1±14.2	16.4±10.2	12.1±4.5	0.054
MA-AA, mm	25.9±15.7	26.1±17.1	27.4±16.4	20.9±12.1	0.228
MA-ADP, mm	54.0±10.8	55.8±9.4	54.5±11.2	55.6±11.7	0.873
% aggregation to AA	26.9±32.1	23.8±38.1	19.2±42.5	16.9±24.1	0.610
% aggregation to ADP	80.7±20.1	91.1±47.1	88.1±22.9	84.3±20.2	0.405

Thrombin generation	741±269.3	764±59.7	777±58.4	771±57.3	0.689
Maximum rate of thrombin generation, min	12.7±3.9	11.8±2.5	12.9±2.5	12.8±3.8	0.333
Thrombus retraction, L parameter mm/min	78.4±17.7	83.7±21.8	88.6±36.9	80.4±23.2	0.530
Maximum rate of thrombus retraction, min	0.44±0.58	0.29±0.17	0.32±0.19	0.24±0.11	0.079
Difference in variables between Visit 1 and Visit 2 Mean difference, 95% CI					
Delta MA-AA, mm	9.5, 3.8-15.2	14.3, 9.6-18.9 *	4.1, -1.2-9.3 *	8.7, 4.0-13.4	0.033 *
Delta MA-ADP, mm	5.9, 1.8-10.0	5.0, 1.7-8.3	6.9, 3.7-10.1	4.2, 2.3-6.2	0.614
Delta % aggregation to AA	17.9, 7.4-28.3	31.2, 19.3-43.1 *	8.8, -2.5-20.0 *	15.7, 6.5-25.0	0.023 *
Delta % aggregation to ADP	9.3, 1.5-17.0	12.6, 4.9-20.3	15.8, 8.7-22.8	7.8, 4.0-11.5	0.307

Table 4.13 Comparison of TEG® parameters, Platelet Mapping™ and V-curve data

Post hoc analysis (One way ANOVA)

*p<0.05

Visit 1 % aggregation to AA: Age<75+Non DM vs. Age≥75+T2DM, p=0.022; Age<75+Non DM vs. Age≥75+Non DM, p=0.012

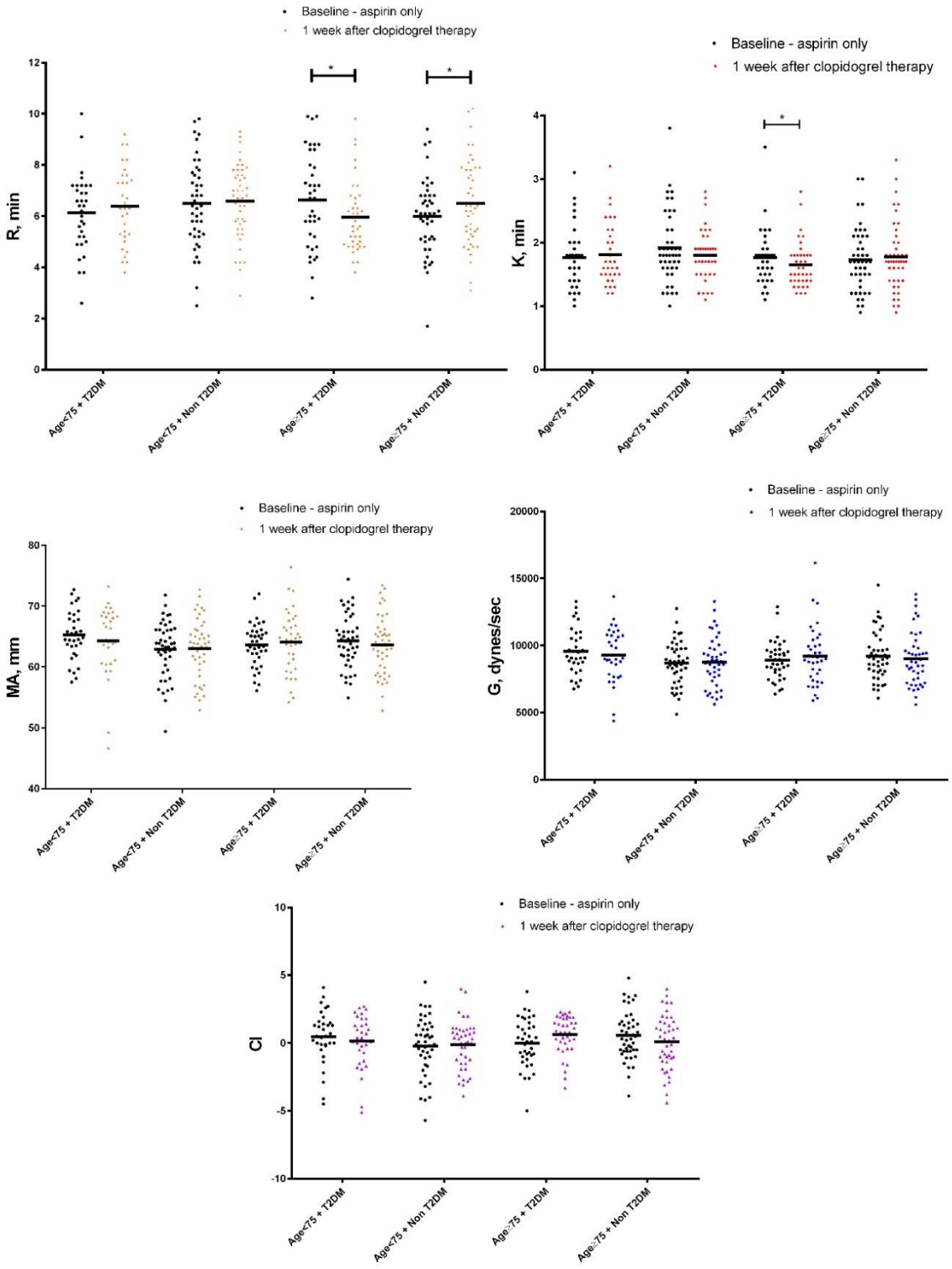


Figure 4.5 Changes in TEG® parameters after clopidogrel therapy

*p<0.05

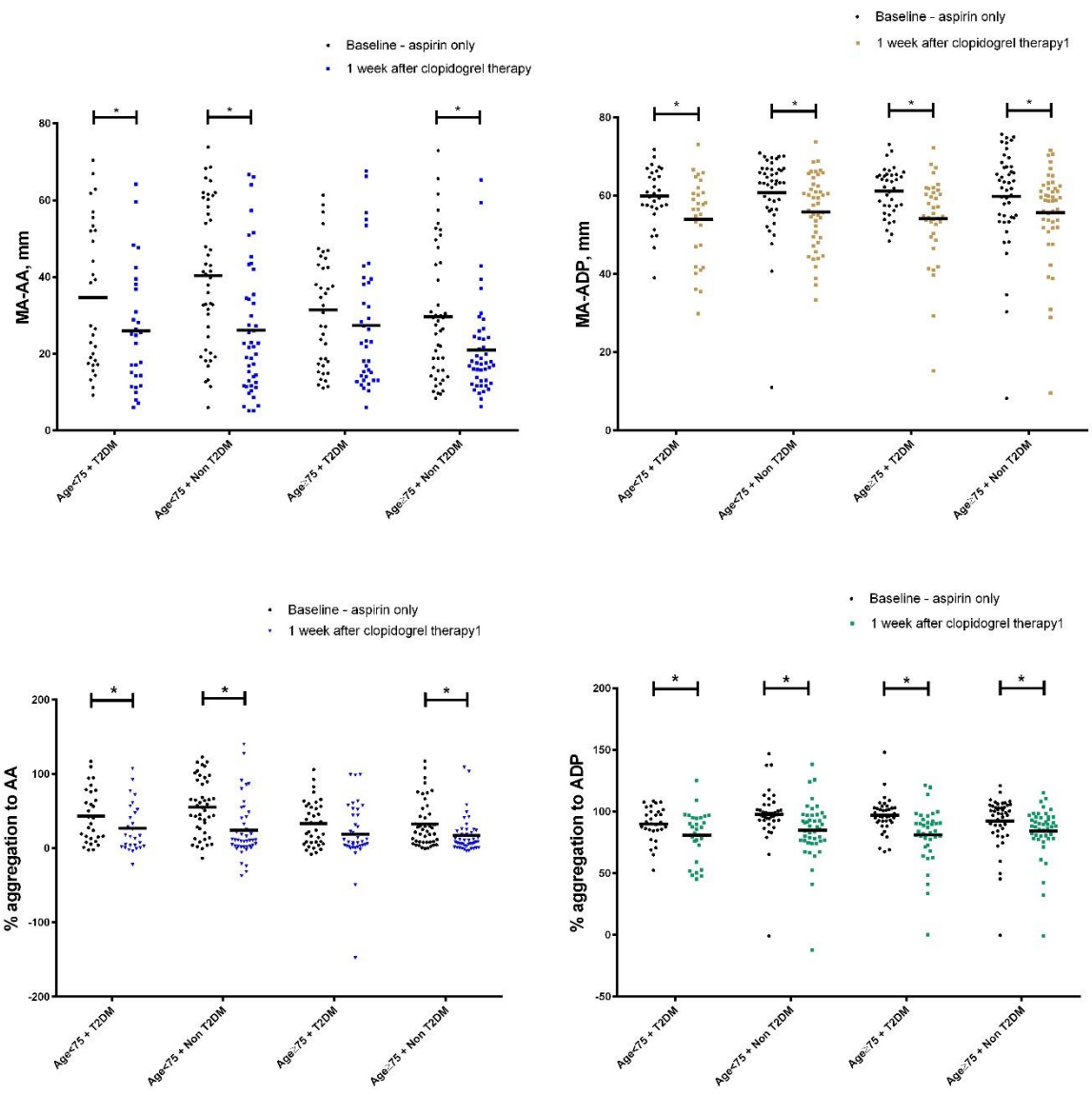


Figure 4.6 Changes in Platelet Mapping™ after clopidogrel therapy

*p<0.05

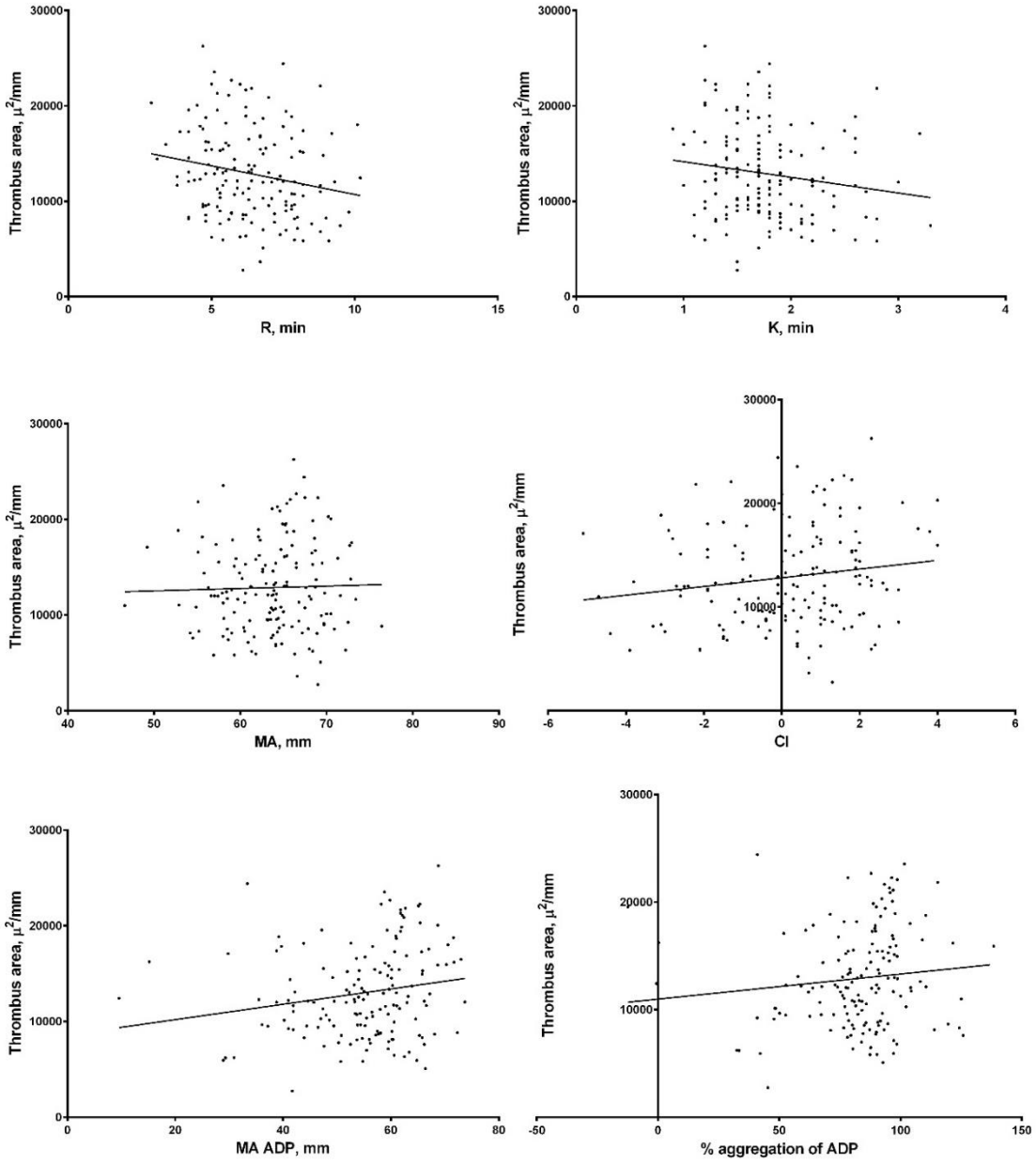


Figure 4.7 Correlation between TEG®-Platelet mapping™ and thrombus area

R time negatively correlated with high shear thrombus area (rho= -0.212, p=0.008). K time again demonstrated negative correlation with high shear (rho= -0.182, p=0.023). Standard Maximum amplitude of the thrombus using Kaolin activated citrated sample did not correlate with thrombus area (rho=0.049, p=0.541). Clot index (CI) also demonstrated positive correlation with high shear (rho=0.169, p=0.035). Maximum amplitude of the thrombus formed in TEG®- Platelet mapping™ upon stimulation by ADP (MA-ADP, mm) demonstrated positive correlation with high shear thrombus (rho=0.219, p=0.007). Percentage aggregation of ADP demonstrated a similar correlation with high shear thrombus.

Age<75 years vs. Age≥75 years

The baseline characteristics and demographic details between these groups have already been described earlier (Table 4.4).

Standard TEG® parameters (R, K, MA, G and CI) were similar between the groups. After one week of clopidogrel therapy, all the standard TEG® parameters remained unchanged

Baseline platelet mapping™ tests were compared between the groups. Maximum viscoelastic strength of thrombus as measured by maximum amplitude upon stimulation with 10µl of arachidonic acid (MA-AA, in mm) was significantly lower in the elderly group compared to the younger population in the study (30.5±16.2 vs. 38.4±18.9, p=0.007). Percentage aggregation to arachidonic acid (% aggregation to AA, %) was also significantly lower in the elderly when compared to the young (32.6±30.5 vs. 51.0±37.0, p=0.002). This demonstrates good platelet inhibition with aspirin in elderly when compared to the young.

After one week of clopidogrel, there was a significant reduction in the maximum viscoelastic strength of thrombus upon stimulation by 10µl of ADP. This reduction was consistently seen in both the groups (Age<75: 60.5±9.2 vs. 55.1±10.0, p<0.001; Age≥75: 60.6±10.3 vs. 55.1±11.4, p<0.001). Percentage aggregation to ADP also demonstrated similar reduction in both the groups (Age<75: 94.6±18.8 vs. 83.3±22.2, p<0.001; Age≥75: 94.3±18.3 vs. 82.9±21.4, p<0.001) (Figure 4.8, Table 4.15).

Despite being on long term aspirin, there was a significant improvement in aspirin induced platelet inhibition as measured by MA-AA in both the groups after the addition of clopidogrel (Age<75: 38.4±18.9 vs. 26.0±16.5, p<0.001; Age≥75: 30.5±16.2 vs. 23.9±14.5, p<0.001). Similar reduction was seen in percentage aggregation to AA establishing the fact that clopidogrel potentiated the platelet inhibitory effect of aspirin. Though this potentiation was seen in both the groups, it was significantly greater in the young when compared to the elderly. This was demonstrated by comparing the change in viscoelastic strength as measured by MA-AA, in mm (mean, 95% CI; 12.6, 8.9-16.2 vs. 6.5, 3.0-10.1, p=0.021) between the groups (Table 4.15, Table 4.16).

	Age < 75 (n = 78)	Age ≥ 75 (n = 83)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	63.3±7.2	79.0±3.4	
Male gender, % (n)	75.6 (59)	75.9 (63)	0.969
Body mass index, kg/m^2	30.8±4.5	28.4±4.4	0.001 *
Waist to hip ratio	0.97±0.13	0.95±0.07	0.085
Heart rate, beats per minute	66±12	66±12	0.949
Systolic BP, mmHg	139±18.4	155±22.6	0.000 *
Diastolic BP, mmHg	77±9	75±9.5	0.264
Risk profile: % (n)			
Angina	76.9 (60)	69.9 (58)	0.313
Previous MI	48.7 (38)	59 (49)	0.189
Previous PCI	35.9 (28)	32.5 (27)	0.653
Previous CABG	20.7 (18)	33.7 (28)	0.017 *
Diabetes Mellitus	42.3 (33)	45.8 (38)	0.657
Hypertension	44.9 (35)	69.9 (58)	0.001 *
CKD	2.6 (2)	7.2 (6)	0.173
PVD	5.1 (4)	19.3 (16)	0.007 *
CVA	7.7 (6)	10.8 (9)	0.492
Medications: % (n)			
Sulphonylurea	10.3 (8)	9.6 (8)	0.896
Metformin	25.6 (20)	20.5 (17)	0.437
Insulin	14.1 (11)	25.3 (21)	0.075
Beta-blocker	79.5 (62)	68.7 (57)	0.219

ACE inhibitor / ARB	74.4 (58)	86.7 (72)	0.046
Statin	96.2 (75)	94.0 (78)	0.525
Laboratory data: Mean ± SD			
Haemoglobin, g/dl	13.6±1.2	12.7±1.2	0.000 *
Platelets x 1000 cells/mm^3	234±61.0	222±59.8	0.191
Fibrinogen, g/ml	3.3±0.6	3.3±0.6	0.710
HbA1c, mmol/mol	47.1±13.9	50.7±14.8	0.122
Fasting plasma glucose, mmol/L	6.6±3.7	6.7±3.4	0.958
Creatinine, micromol/L	91±26.6	105±42.6	0.008 *
Total cholesterol, mmol/L	4.0±0.9	4.0±0.9	0.631
LDLc, mmol/L	2.1±0.8	2.1±0.8	0.698
HDLc, mmol/L	1.2±0.3	1.3±0.3	0.061
Triglyceride, mmol/L	1.5±0.8	1.4±0.7	0.406
HsCRP, mg/L	2.9±3.3	4.2±7.2	0.112

Table 4.14 Baseline characteristics - TEG® substudy (Age<75 vs. Age≥75)

*p<0.05

	Age < 75 (n = 78)			Age ≥ 75 (n = 83)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
R, min	6.3±1.6	6.5±1.5	0.428	6.3±1.7	6.2±1.6	0.844
K, min	1.9±0.5	1.8±0.4	0.406	1.7±0.5	1.7±0.5	0.540
MA, mm	64.0±7.2	64.8±5.2	0.499	64.0±4.0	63.9±4.9	0.742
G Kdynes/sec	9.1±1.7	9.0±1.9	0.696	9.1±1.6	9.1±2.0	0.776
CI	0.1±2.1	0±1.8	0.655	0.3±1.8	0.3±1.8	0.848
MA-A, mm	14.1±12.4	14.4±11.5	0.771	14.2±7.8	14.1±7.9	0.842
MA-AA, mm	38.4±18.9	26.0±16.5	0.000 *	30.5±16.2	23.9±14.5	0.000 *
MA-ADP, mm	60.5±9.2	55.1±10.0	0.000 *	60.6±10.3	55.1±11.4	0.000 *
% aggregation to AA	51.0±37.0	25.0±35.6	0.000 *	32.6±30.5	20.0±28.3	0.001 *
% aggregation to ADP	94.6±18.8	83.3±22.2	0.000 *	94.3±18.3	82.9±21.4	0.000 *
Thrombin generation	752±196.5	755±178.3	0.947	777±49.3	774±57.5	0.505
Maximum rate of thrombin generation, min	13.8±18.0	12.2±3.2	0.511	12.6±3.2	12.8±3.3	0.299
Thrombus retraction, L parameter mm/min	84.4±26.0	84.9±19.6	0.031	88.2±19.9	95.1±30.0	0.088
Maximum rate of thrombus retraction, min	0.25±0.14	0.26±0.13	0.240	0.27±0.15	0.27±0.17	0.892

Table 4.15 Changes in TEG®, Platelet Mapping™ and V-curve data (Age<75 vs. Age≥75)

*p<0.05

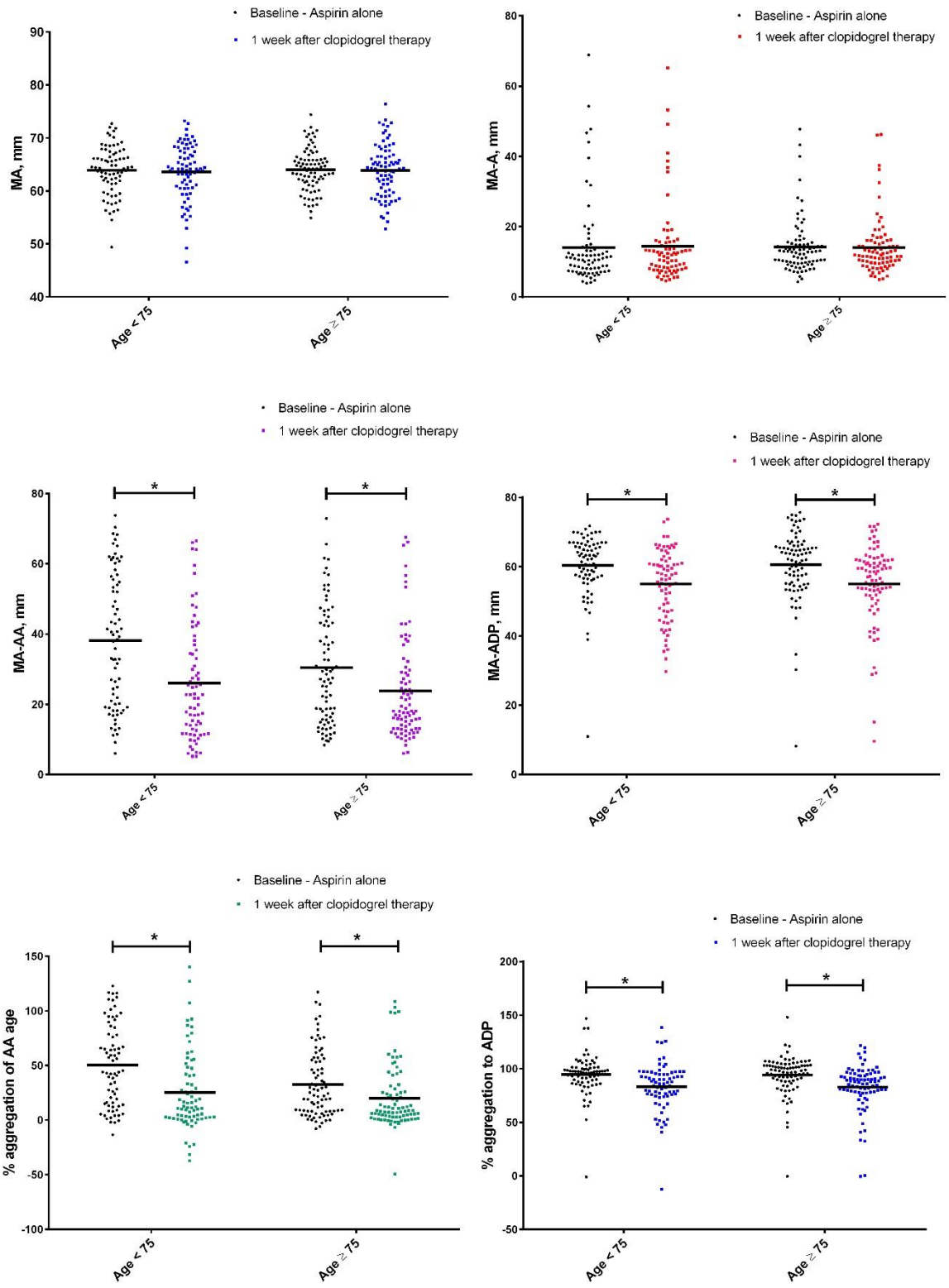


Figure 4.8 Changes in Platelet Mapping™ (Age<75 vs. Age≥75)

*p<0.05

	Age < 75 (n = 78)	Age ≥ 75 (n = 83)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
R, min	6.3±1.6	6.3±1.7	0.830
K, min	1.9±0.5	1.7±0.5	0.157
MA, mm	64.0±7.2	64.0±4.0	0.895
G Kdynes/sec	9.1±1.7	9.1±1.6	0.997
CI	0.1±2.1	0.3±1.8	0.486
MA-A, mm	14.1±12.4	14.2±7.8	0.925
MA-AA, mm	38.4±18.9	30.5±16.2	0.007 *
MA-ADP, mm	60.5±9.2	60.6±10.3	0.938
% aggregation to AA	51.0±37.0	32.6±30.5	0.002 *
% aggregation to ADP	94.6±18.8	94.3±18.3	0.930
Thrombin generation	752±196.5	777±49.3	0.281
Maximum rate of thrombin generation, min	13.8±18.0	12.6±3.2	0.590
Thrombus retraction, L parameter mm/min	84.4±26.0	88.2±19.9	0.426
Maximum rate of thrombus retraction, min	0.25±0.14	0.27±0.15	0.477
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
R, min	6.5±1.5	6.2±1.6	0.289
K, min	1.8±0.4	1.7±0.5	0.202
MA, mm	64.8±5.2	63.9±4.9	0.710
G Kdynes/sec	9.0±1.9	9.1±2.0	0.722
CI	0±1.8	0.3±1.8	0.219
MA-A, mm	14.4±11.5	14.1±7.9	0.841
MA-AA, mm	26.0±16.5	23.9±14.5	0.389
MA-ADP, mm	55.1±10.0	55.1±11.4	0.996

% aggregation to AA	25.0±35.6	20.0±28.3	0.335
% aggregation to ADP	83.3±22.2	82.9±21.4	0.894
Thrombin generation	755±178.3	774±57.5	0.368
Maximum rate of thrombin generation, min	12.2±3.2	12.8±3.3	0.172
Thrombus retraction, L parameter mm/min	84.9±19.6	95.1±30.0	0.582
Maximum rate of thrombus retraction, min	0.26±0.13	0.27±0.17	0.228
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta MA-AA, mm	12.6, 8.9-16.2	6.5, 3.0-10.1	0.021 *
Delta MA-ADP, mm	5.4, 2.9-8.0	5.3, 3.5-7.1	0.942
Delta % aggregation to AA	28.2, 21.1-35.3	12.6, 5.5-19.6	0.015 *
Delta % aggregation to ADP	11.3, 5.8-16.8	11.2, 7.4-15.1	0.962

Table 4.16 Comparison of TEG®, Platelet Mapping™ and V-curve data (age<75 vs. age≥75)

*p<0.05

T2DM vs. Non DM

The baseline characteristics and demographic details between T2DM and Non DM groups have already been described earlier (Table 4.17).

Standard TEG® and Platelet mapping™ parameters were similar between the groups. After one week of clopidogrel therapy, all the standard TEG® parameters remained unchanged

After one week of clopidogrel therapy, there was a significant reduction in the maximum viscoelastic strength of thrombus upon stimulation by 10µl of ADP. This reduction was consistently seen in both the groups (T2DM: 60.8±6.8 vs. 54.3±10.9, p<0.001; Non DM: 60.3±11.5 vs. 55.7±10.6, p<0.001). Percentage aggregation to ADP also demonstrated similar reduction in both the groups (T2DM: 93.9±14.5 vs. 81.0±21.6, p<0.001; Non DM: 43.8±36.3 vs. 20.3±31.9, p<0.001) (Table 4.18, Figure 4.9).

Treatment with clopidogrel for one week also demonstrated significant improvement in aspirin induced platelet inhibition as measured by MA-AA in both the groups (T2DM: 33.1±16.8 vs. 26.7±16.0, p<0.001; Non DM: 35.0±18.7 vs. 23.5±15.0, p<0.001). Similar reduction was seen in percentage aggregation to AA further establishing the fact that clopidogrel therapy potentiated platelet inhibitory effect of aspirin. Though this potentiation effect was demonstrated in both the groups, it was significantly lower in T2DM group when compared to non-diabetic group. This was demonstrated by comparing the change in viscoelastic strength as measured by MA-AA, in mm (mean, 95% CI; 6.4, 2.5-10.3 vs. 11.6, 8.3-14.9, p=0.048) and change in percentage aggregation to AA between the groups (Table 4.18, Table 4.19).

	T2DM (n = 71)	Non DM (n = 90)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	72±9.4	71±9.9	0.625
Male gender, % (n)	83.1 (59)	70 (63)	0.054
Body mass index, kg/m^2	30.7±5.3	28.7±3.7	0.008 *
Waist to hip ratio	0.98±0.07	0.95±0.12	0.046
Heart rate, beats per minute	69±13	63±11	0.001 *
Systolic BP, mmHg	150±22.9	145±21.3	0.121
Diastolic BP, mmHg	76±10	76±8	0.757
Risk profile: % (n)			
Angina	78.9 (56)	68.9 (62)	0.155
Previous MI	49.3 (35)	57.8 (52)	0.284
Previous PCI	33.8 (24)	34.4 (31)	0.932
Previous CABG	35.2 (25)	18.9 (17)	0.019
Hypertension	56.3 (40)	58.9 (53)	0.745
CKD	9.9 (7)	1.1 (1)	0.011
PVD	21.1 (15)	5.6 (5)	0.003
CVA	12.7 (9)	6.7 (6)	0.193
Medications: % (n)			
Sulphonylurea	22.5 (16)	0	
Metformin	52.1 (37)	0	
Insulin	43.7 (31)	0	
Beta-blocker	73.2 (52)	74.4 (67)	0.528
ACE inhibitor / ARB	83.1 (59)	78.9 (71)	0.501
Statin	95.8 (68)	94.4 (85)	0.700
Laboratory data: Mean ± SD			
Haemoglobin, g/dl	13.0±1.3	13.2±1.2	0.409

Platelets x 1000 cells/mm^3	217±53	235±64.6	0.066
Fibrinogen, g/ml	3.4±0.6	3.2±0.6	0.119
HbA1c, mmol/mol	60.3±14.7	40.3±5.5	
Fasting plasma glucose, mmol/L	8.5±4.7	5.2±0.6	
Creatinine, micromol/L	105±44.1	91±27.7	0.019 *
Total cholesterol, mmol/L	3.8±0.9	4.2±0.9	0.009 *
LDLc, mmol/L	2.0±0.8	2.2±0.8	0.219
HDLc, mmol/L	1.1±0.3	1.4±0.3	0.000 *
Triglyceride, mmol/L	1.6±0.7	1.4±0.7	0.098
HsCRP, mg/L	4.2±5.6	3.1±5.7	0.232

Table 4.17 Baseline characteristics - TEG® substudy (T2DM vs. Non-DM)

*p<0.05

	T2DM (n = 71)			Non DM (n = 90)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
R, min	6.4±1.7	6.1±1.4	0.288	6.2±1.6	6.5±1.6	0.098
K, min	1.8±0.5	1.7±0.4	0.513	1.8±0.5	1.8±0.5	0.421
MA, mm	64.4±3.9	64.2±5.3	0.690	63.6±4.5	63.4±4.9	0.521
G Kdynes/sec	9.2±1.6	9.3±2.1	0.833	8.9±1.7	8.9±1.9	0.720
CI	0.2±1.9	0.4±1.7	0.488	0.2±2.0	0±1.9	0.298
MA-A, mm	13.2±7.3	14.4±8.4	0.170	14.8±11.9	14.1±10.6	0.433
MA-AA, mm	33.1±16.8	26.7±16.0	0.001 *	35.0±18.7	23.5±15.0	0.000 *
MA-ADP, mm	60.8±6.8	54.3±10.9	0.000 *	60.3±11.5	55.7±10.6	0.000 *
% aggregation to AA	37.9±32.8	25.1±32.2	0.002 *	43.8±36.3	20.3±31.9	0.000 *
% aggregation to ADP	93.9±14.5	81.0±21.6	0.000 *	94.8±21.1	84.7±21.8	0.000 *
Thrombin generation	756±204.1	761±186.4	0.888	772±55.6	768±58.3	0.428
Maximum rate of thrombin generation, min	14.5±18.8	12.8±3.2	0.468	12.2±3.4	12.3±3.3	0.639
Thrombus retraction, L parameter mm/min	84.3±26.0	94.2±33.1	0.087	87.9±20.6	88.1±20.5	0.949
Maximum rate of thrombus retraction, min	0.29±0.19	0.28±0.18	0.858	0.24±0.11	0.26±0.13	0.350

Table 4.18 Changes in TEG®, Platelet Mapping™ and V-curve data (T2DM vs. Non-DM)

*p<0.05

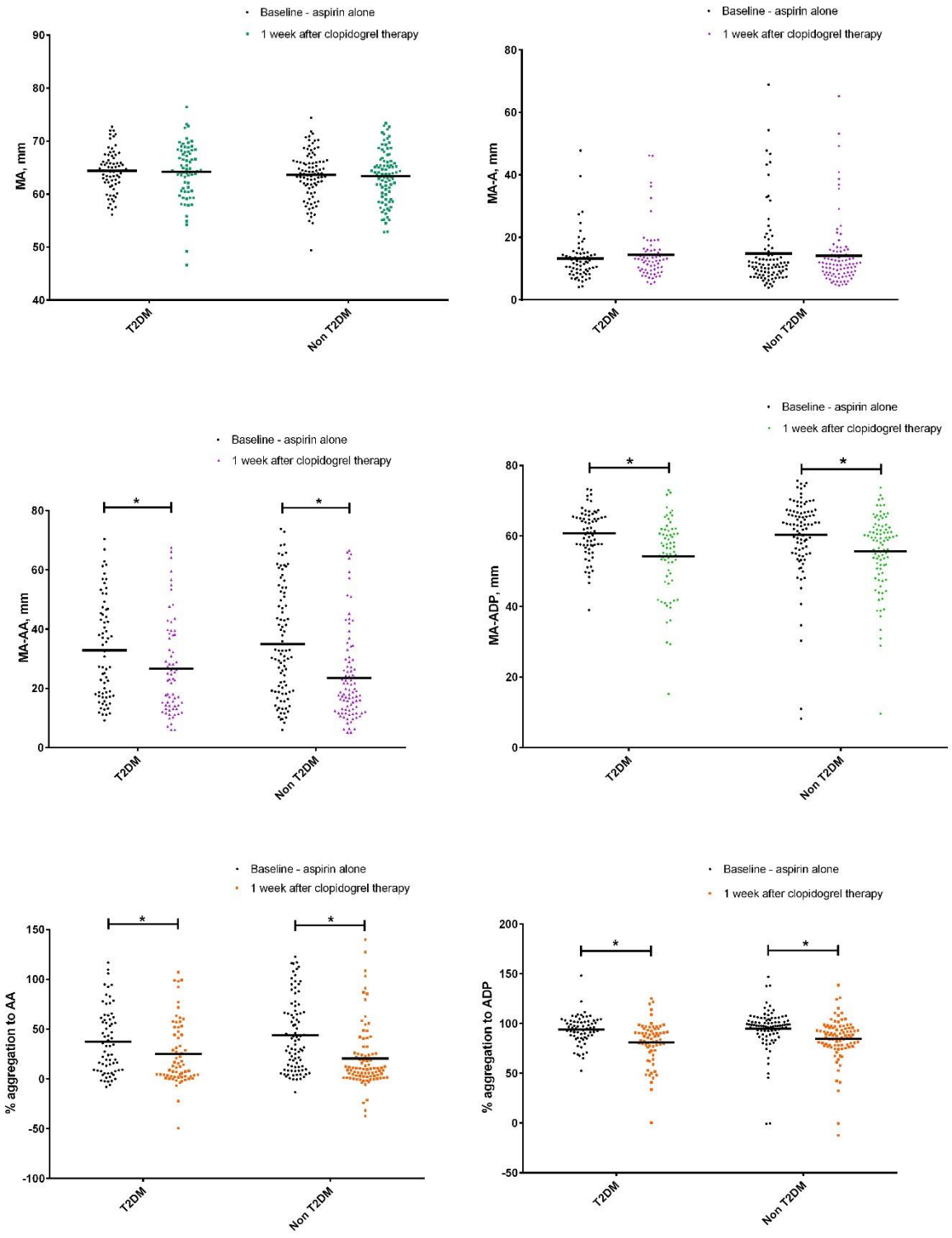


Figure 4.9 Changes in Platelet Mapping™ (T2DM vs. Non-DM)

*p<0.05

	T2DM (n = 71)	Non DM (n = 90)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
R, min	6.4±1.7	6.2±1.6	0.558
K, min	1.8±0.5	1.8±0.5	0.429
MA, mm	64.4±3.9	63.6±4.5	0.239
G Kdynes/sec	9.2±1.6	8.9±1.7	0.321
CI	0.2±1.9	0.2±2.0	0.850
MA-A, mm	13.2±7.3	14.8±11.9	0.301
MA-AA, mm	33.1±16.8	35.0±18.7	0.448
MA-ADP, mm	60.8±6.8	60.3±11.5	0.768
% aggregation to AA	37.9±32.8	43.8±36.3	0.254
% aggregation to ADP	93.9±14.5	94.8±21.1	0.736
Thrombin generation	756±204.1	772±55.6	0.541
Maximum rate of thrombin generation, min	14.5±18.8	12.2±3.4	0.323
Thrombus retraction, L parameter mm/min	84.3±26.0	87.9±20.6	0.749
Maximum rate of thrombus retraction, min	0.29±0.19	0.24±0.11	0.212
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
R, min	6.1±1.4	6.5±1.6	0.098
K, min	1.7±0.4	1.8±0.5	0.371
MA, mm	64.2±5.3	63.4±4.9	0.311
G Kdynes/sec	9.3±2.1	8.9±1.9	0.257
CI	0.4±1.7	0±1.9	0.127
MA-A, mm	14.4±8.4	14.1±10.6	0.826
MA-AA, mm	26.7±16.0	23.5±15.0	0.205
MA-ADP, mm	54.3±10.9	55.7±10.6	0.420

% aggregation to AA	25.1±32.2	20.3±31.9	0.365
% aggregation to ADP	81.0±21.6	84.7±21.8	0.293
Thrombin generation	761±186.4	768±58.3	0.754
Maximum rate of thrombin generation, min	12.8±3.2	12.3±3.3	0.315
Thrombus retraction, L parameter mm/min	94.2±33.1	88.1±20.5	0.592
Maximum rate of thrombus retraction, min	0.28±0.18	0.26±0.13	0.073
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta MA-AA, mm	6.4, 2.5-10.3	11.6, 8.3-14.9	0.048 *
Delta MA-ADP, mm	6.3, 3.8-8.8	4.7, 2.8-6.6	0.236
Delta % aggregation to AA	12.8, 5.1-20.4	25.2, 18.5-32.0	0.050 *
Delta % aggregation to ADP	12.7, 7.5-17.9	10.2, 6.0-14.4	0.406

Table 4.19 Comparison of TEG®, Platelet Mapping™ and V-curve data (T2DM vs. Non-DM)

*p<0.05

4.2.2 Discussion - viscoelastic properties of thrombus

In this study at baseline, kaolin stimulated TEG® parameters (standard TEG®) were similar in elderly and young patients, with and without diabetes. The conventional TEG® parameters remained unaltered in elderly T2DM, young T2DM and young non DM group, after addition of clopidogrel 75mg OD for a week. In elderly non DM group, there was a reduction in clot index (CI) and prolongation of R time, after clopidogrel therapy. When standard TEG® parameters where compared between young and older patients, there was no difference in values both at baseline and after one week of clopidogrel therapy. These findings probably demonstrate a stronger response to clopidogrel among elderly non-diabetic patients when compare to younger patients and patients with T2DM.

It is not surprising that standard TEG® parameters remained unaltered after addition of clopidogrel in all the groups except for elderly non-DM group. Kaolin stimulates thrombus formation by activating thrombin which acts in the final common pathway of coagulation cascade, thereby bypassing the effect of ADP inhibition. Clopidogrel therapy has been shown to reduce thrombin generation in small studies, but no study has been performed using kaolin stimulated TEG assay to support this finding (Wegert *et al.*, 2002).

In CLEAR PLATELET-2 study (Clopidogrel With Eptifibatide to Arrest the Reactivity of Platelets), TEG was used to assess platelet reactivity in patients who received clopidogrel, bivalirudin and eptifibatide. There was an immediate and marked reduction in maximum amplitude as measured by TEG, following the addition of eptifibatide to bivalirudin therapy. The values correlated with peri-procedural myonecrosis. This study demonstrated the usefulness of TEG in assessing platelet reactivity (Gurbel *et al.*, 2009). Various studies have demonstrated the usefulness of TEG parameters post cardiac surgery, as a guide to decide on blood products transfusion (Hertfelder *et al.*, 2005; Ak *et al.*, 2009). Even these studies did not report any difference in TEG parameters in patients who were on clopidogrel. Gurbel et al in PREPARE POST-STENTING study measured standard TEG parameters in patients treated with dual antiplatelet therapy following elective coronary stenting. Patients with higher MA and shorter R time had more ischaemic events (Gurbel *et al.*, 2005). Unfortunately this study did not report any pre and post clopidogrel TEG values. It is possible that DAPT with aspirin and clopidogrel is not powerful enough to demonstrate changes in kaolin

stimulated standard TEG parameters. The other explanation could be that the sample size in my study was too small to demonstrate any significant changes in TEG parameters. Despite that, significant difference in CI and R time was demonstrated in elderly non-diabetic patients. Effect of prasugrel and ticagrelor on standard TEG parameters remains to be seen.

The results from modified TEG assay namely platelet mapping (PM), demonstrated a significantly lower maximum viscoelastic strength of thrombus upon stimulation with 10µl of arachidonic acid (MA-AA) in elderly patients (age≥75) when compared to the young (age<75), thereby demonstrating better platelet inhibition with aspirin in the elderly group. There was a significant reduction in maximum viscoelastic strength of thrombus upon stimulation by ADP (MA-ADP), in elderly and young patients, with and without diabetes. The other interesting finding was a significant reduction in MA-AA in young people with and without diabetes, and in the elderly non-diabetic participants, after the addition of clopidogrel to aspirin. This suggests the possibility of a potentiating effect of clopidogrel on the antiplatelet effect of aspirin. This reduction was not demonstrated in elderly patients with T2DM.

Previous studies which employed modified TEG percentage inhibition to measure the response to aspirin and clopidogrel have used cut-off values of <50% and <30% to define hypo-responsiveness to aspirin and clopidogrel respectively. Using the same cut-off values, results from my PM assay showed inadequate response to clopidogrel (ADP inhibition) in the following percentage of patients: 76% (young T2DM), 84% (young non DM), 79% (elderly T2DM) and 89% (elderly non DM). Percentage of patients with inadequate response to aspirin (AA inhibition) at baseline and after one week of clopidogrel treatment: 43% vs. 24% (age<75+T2DM), 53% vs. 20% (age<75+non DM), 32% vs. 24% (age≥75+T2DM) and 24% vs. 7% (age≥75+non DM). There was a significant variability in individual clopidogrel responses at all ages and with and without diabetes, ranging from 0% to 95%.

Roeloffzen et al demonstrated a tendency towards hypercoagulability in both the sexes with increasing age using standard TEG parameters. Cut off age 50 was used to divide the study population but in my study, I used 75 as the cut off age to divide patients as young and elderly. Weak to moderate correlations between age and most TEG variables were observed but the strongest correlation was between age and MA (in normal controls thromboelastography) (Roeloffzen *et al.*, 2010).

When comparing elderly and young stable CAD patients, standard TEG® parameters were similar between the groups. After one week of clopidogrel therapy, all the standard TEG® parameters remained unchanged. From TEG-PM data, elderly patients demonstrated better platelet inhibition with aspirin, compared to the young. After one week of clopidogrel therapy, there was a significant and similar reduction in the maximum viscoelastic strength of thrombus upon stimulation by 10µl of ADP (MA-ADP) suggesting good response to clopidogrel in elderly and young. Despite being on long term aspirin therapy, addition of clopidogrel enhanced aspirin induced platelet inhibition in both the groups, but this potentiation effect was significantly greater in the young compared to the elderly group.

Standard TEG® parameters were similar between T2DM and non-DM patients at baseline (on aspirin alone). All the standard TEG® parameters remained unchanged after addition of clopidogrel for one week. From TEG-PM data, T2DM and non-DM patients demonstrated similar antiplatelet effect with aspirin. After one week of clopidogrel therapy, there was a significant and similar reduction in the maximum viscoelastic strength of thrombus upon stimulation by 10µl of ADP (MA-ADP) suggesting good response to clopidogrel in both T2DM and non-DM patients. Despite being on long term aspirin therapy, addition of clopidogrel enhanced aspirin induced platelet inhibition in both diabetic and non-diabetic participants, but this potentiation effect was significantly greater in the non-diabetic group.

Swallow et al demonstrated that TEG-PM (MA-AA and MA-ADP) could be used as a reliable bedside test to measure the effects of aspirin and clopidogrel therapy, alone or in combination, both in an individual and between different groups (Swallow *et al.*, 2006). Bliden et al measured platelet reactivity and percentage aggregation in patients who were on prescribed DAPT with aspirin and clopidogrel and who underwent elective coronary stent insertion. In this study 87% of the patients displayed high on-treatment platelet reactivity and this was the only variable that significantly predicted future ischaemic events (Bliden *et al.*, 2007). Collyer et al performed a prospective observational study in three groups of preoperative acute surgical patients to assess the ability of TEG-PM in detecting platelet inhibition. This study demonstrated that TEG-PM was able to identify statistically significant inhibition of platelet ADP and TXA_2 receptor after clopidogrel and aspirin therapy respectively (Collyer *et al.*, 2009).

In a 3 year follow up study of patients who underwent coronary stent insertion and were treated with DAPT, Gurbel et al identified MA-ADP of >47 mm to be the best predictor of long-term ischaemic events and MA-ADP <31 mm to be the predictor of bleeding (Gurbel *et al.*, 2010b). In another study, Hobson and colleagues demonstrated greater baseline MA-ADP, reduced response to clopidogrel and higher post treatment platelet reactivity while on aspirin and clopidogrel treatment in young women with previous stent thrombosis, when compared to healthy volunteers (Hobson *et al.*, 2009). In a subgroup of patients enrolled in the Optimizing anti-Platelet Therapy In diabetes MellitUS (OPTIMUS) study, Angiolillo and colleagues demonstrated T2DM patients with suboptimal clopidogrel response to have enhanced platelet procoagulant activity compared to patients with optimal response. This procoagulant activity can be down-regulated by more potent platelet P2Y12 inhibition using high clopidogrel maintenance dosing. This study highlighted the usefulness of TEG® in monitoring platelet function from patients who are on long term antiplatelet therapy (Angiolillo *et al.*, 2009).

It is well known that aspirin resistance is rare in compliant patients with established CAD and that the occurrence of aspirin resistance is overestimated in published reports (Tantry *et al.*, 2005). This may explain the higher than expected aspirin resistance at baseline in my cohort of patients. The platelet inhibitory effect was enhanced after the addition of clopidogrel in all the groups but was highest in young non-diabetic group and was lowest in elderly T2DM group. The mechanisms underlying this enhanced antiplatelet effect of aspirin measured using pathway specific agonist arachidonic acid (MA-AA) remains to be explained. There is a possibility that after recruitment in the study, compliance to aspirin improved along with clopidogrel treatment resulting in significantly enhanced antiplatelet effect of aspirin. Alstrom et al demonstrated a similar finding in a small TEG-PM study (Alstrom *et al.*, 2007).

The Badimon chamber is predominantly a measure of platelet dependent thrombus formation in both high shear and low shear conditions and TEG® evaluates the viscoelastic property of clot formation under low shear condition, with fibrinogen levels and platelet counts having a major influence on its parameters. Badimon chamber is used to evaluate the area of formed thrombus when whole blood is exposed to collagen for 5 minutes under different rheological conditions whereas TEG® measures viscoelastic properties of thrombus continuously over 90 minutes.

Time to initial fibrin formation (R time) negatively correlated with high shear thrombus area. Time taken from initial fibrin formation to formation of a firm thrombus (K time) also demonstrated negative correlation with both high shear and low shear thrombus area. Maximum viscoelastic strength of the thrombus (MA mm) and the elastic force of the clot (G dynes/m^2) had a positive correlation with low shear thrombus area. Overall measure of thrombus strength, clot index (CI) also demonstrated positive correlation with high shear and low shear thrombus area. Thrombin generation and maximum rate of thrombin generation also correlated positively with low shear thrombus area. These interesting correlations between TEG® parameters and platelet dependent thrombus formation highlights the potential role of TEG® in evaluation of novel antithrombotic agents that might interact in different stages of coagulation cascade. My findings have also demonstrated that TEG-PM assay can be helpful in assessing response to antiplatelet therapy in CAD patients, both elderly and young and in the presence and absence of T2DM.

4.3 VerifyNow® (Optical aggregometry) - Elderly stable CAD study

4.3.1 Results – Platelet reactivity indices

Baseline characteristics of the patients in all the four groups who had their platelet reactivity indices measured are as in the earlier section (Table 4.1). PRUz cut-off value of ≥240 was used to classify patients as on treatment high platelet reactivity (hyporesponders). Patients with PRUz<240 were classified as good responders to clopidogrel.

Young T2DM group had the least number of good responders to clopidogrel, 12 patients (36.4%) and the young non-diabetic group had the highest number of good responders, 27 patients (67.5%). The elderly T2DM group had 12 good responders (36.4%) compared to 22 patients (52.4%) of good responders in elderly non-diabetic group. The young T2DM group had the least number of hyporesponders to aspirin, 7 patients (17.1%) and elderly non-DM had the highest number of hyporesponders to aspirin, 17 patients (37.8%) (Figure 4.11, Table 4.20).

The absolute mean PRUz value was significantly reduced in all the four groups after one week of clopidogrel therapy (young T2DM: 322±38.5 to 260±59.4, p<0.001; old T2DM: 303±39.4 to 257± 68.8, p<0.001; young non-diabetic: 305± 47.1 to 211± 76.4, p<0.001; old non-diabetic: 313± 58.8 to 224± 68.1, p<0.001). Despite significant reduction in PRUz, the mean PRUz in the T2DM group: young (260±59.4) and old (257± 68.8) remained ≥240, confirming high platelet reactivity in the diabetic population. Similar trends were seen with percentage platelet inhibition to clopidogrel. Mean difference in PRUz from visit 1 and visit 2 was lowest in Age≥75+T2DM group (Mean, 95%CI: 55, 40.4-69.4) and highest in Age<75+Non DM group (105, 82.8-126.8) and this difference was significant (p=0.013) (Figure 4.10, Table 4.21).

Despite being on long term aspirin therapy, there was a numerical reduction in ARU from visit 1 to visit 2 in the younger population both in T2DM and non DM group. This reduction was significant in T2DM group (mean±SD, 458±55.5 to 430±76.5; p=0.027) demonstrating improved platelet inhibition to aspirin after the addition of clopidogrel as a second antiplatelet agent. There was no change in ARU value in the elderly population (both T2DM and non DM group) after the addition of clopidogrel.

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean PRUz < 240) from all the four groups. There was a consistent reduction both in high shear (age<75+T2DM: 15579± 4650 vs. 11338± 4228, p=0.024; age<75+non DM: 15629± 4968 vs. 12422± 4521, p<0.001; age≥75+T2DM: 18217± 6227 vs. 14332± 5073, p=0.027; age≥75+non DM: 17069± 7575 vs. 13537± 4718, p=0.011) and low shear thrombus area following one week of clopidogrel therapy. PRUz was lower in non-diabetic group compared to T2DM group: both in the young (mean±SD; 164±54.4 vs. 202±37.9) and in the elderly (mean±SD; 166± 41.2 vs. 177±30.1). There was no significant change in ARU values at baseline and one week after clopidogrel therapy in all the four groups (Table 4.22, Table 4.23).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean PRUz ≥ 240) were compared, patients with age<75, both the T2DM group and the non-diabetic group demonstrated numerical reduction in high shear thrombus area but this was not statistically significant (age<75+T2DM: 14321± 5899 vs.12269± 4164, p=0.063; age<75+non DM: 16113± 1820 vs. 14809± 1442, p=0.370). In patients with Age≥75, both T2DM and non-diabetic group demonstrated significant reduction in thrombus area after one week of clopidogrel therapy (age≥75+T2DM: 15113± 6561 vs. 12440± 4637, p=0.007; age≥75+non DM: 17596± 6101 vs. 14484± 5024, p=0.012). All the four groups demonstrated significant reduction in PRUz but the mean PRUz remained ≥ 240 (Table 4.24, Table 4.25).

At baseline when patients were on aspirin alone, there was a significant negative correlation between haemoglobin level and PRUz (rho= -0.684; p<0.001). Parameters which measured aspirin induced platelet inhibition using TEG®, MA-AA and percentage aggregation to AA, correlated positively with ARU (aspirin induced platelet inhibition measurement using VerifyNow®) (rho=0.246; p=0.002 and rho=0.248; p=0.002 respectively). After a week of clopidogrel therapy, significant negative correlation between haemoglobin level and PRUz persisted (rho= -0.649; p<0.001). Parameters which measured clopidogrel induced platelet inhibition using TEG®, MA-ADP and percentage aggregation to ADP correlated positively with PRUz (clopidogrel induced platelet inhibition measurement using VerifyNow®) (rho=0.181; p=0.024 and rho=0.225; p=0.005 respectively). Positive correlation between MA-AA and ARU persisted (rho=0.186; p=0.020) (Table 4.26, Table 4.27).

	Age < 75 + T2DM (n = 41)			Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
ARU	458± 55.5	430± 76.5	0.027	483± 78.8	458± 81.1	0.114	476± 62.8	470± 78.0	0.399	475± 58	481± 87.0	0.690
PRUz	322± 38.5	260± 59.4	0.000	305± 47.1	211± 76.4	0.000	303± 39.4	257± 68.8	0.000	313± 58.8	224± 68.1	0.000
Platelet inhibition, %	2.3± 6	19.6± 16.0	0.000	2.3± 4.5	29.6± 23.7	0.000	2.6± 4.2	19.5± 15.6	0.000	3.8± 5.5	29.0± 20.1	0.000

Table 4.20 VerifyNow ® indices in Elderly stable CAD study

*p<0.05

	Age < 75 + T2DM (n = 41)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD					
ARU	458±55.5	483±78.8	476±62.8	475±58	0.371
PRUz	322±38.5	305±47.1	303±39.4	313±58.8	0.249
Platelet inhibition, %	2.3±6	2.3±4.5	2.6±4.2	3.8±5.5	0.493
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD					
ARU	430±76.5 *	458±81.1	470±78.0	481±87 *	0.037 *
PRUz	260±59.4	211±76.4	257±68.8	224±68.1	0.002 *
Platelet inhibition, %	19.6±16.0	29.6±23.7	19.5±15.6	29.0±20.1	0.015 *
Good responders, %	42.1 (16)	67.5 (27) *	36.4 (12) *	52.4 (22)	0.039 *
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI					
Delta ARU	28, 3.4-53.1	25, -6.3-56.2	10, -14.1-34.7	-5.4, -33.0-22.0	0.273
Delta PRUz	69, 52.7-85.6	105, 82.8-126.8	55, 40.4-69.4	96, 71.7-120.2	0.002 *
Delta Platelet inhibition, %	17.8, 12.1-23.5	26.9, 20.2-33.6	14.6, 9.3-19.9	24.4, 18.2-30.7	0.016 *

Table 4.21 Comparison of VerifyNow® platelet reactivity indices

Post hoc analysis (One way ANOVA); *p<0.05

PRUz (Visit 2): Age<75+Non T2DM vs Age<75+T2DM, P=0.008; Age<75+Non T2DM vs Age≥75+T2DM, P=0.013

Platelet inhibition, %: Age<75+T2DM vs Age≥75+Non T2DM, p=0.039; Age≥75+T2DM vs Age≥75+Non T2DM, p=0.026

Delta PRUz: Age<75+Non T2DM vs Age≥75+T2DM, p=0.005; Age≥75+T2DM vs Age≥75+Non T2DM, p=0.029

Delta Platelet inhibition, %: Age<75+Non T2DM vs Age≥75+T2DM, p=0.028

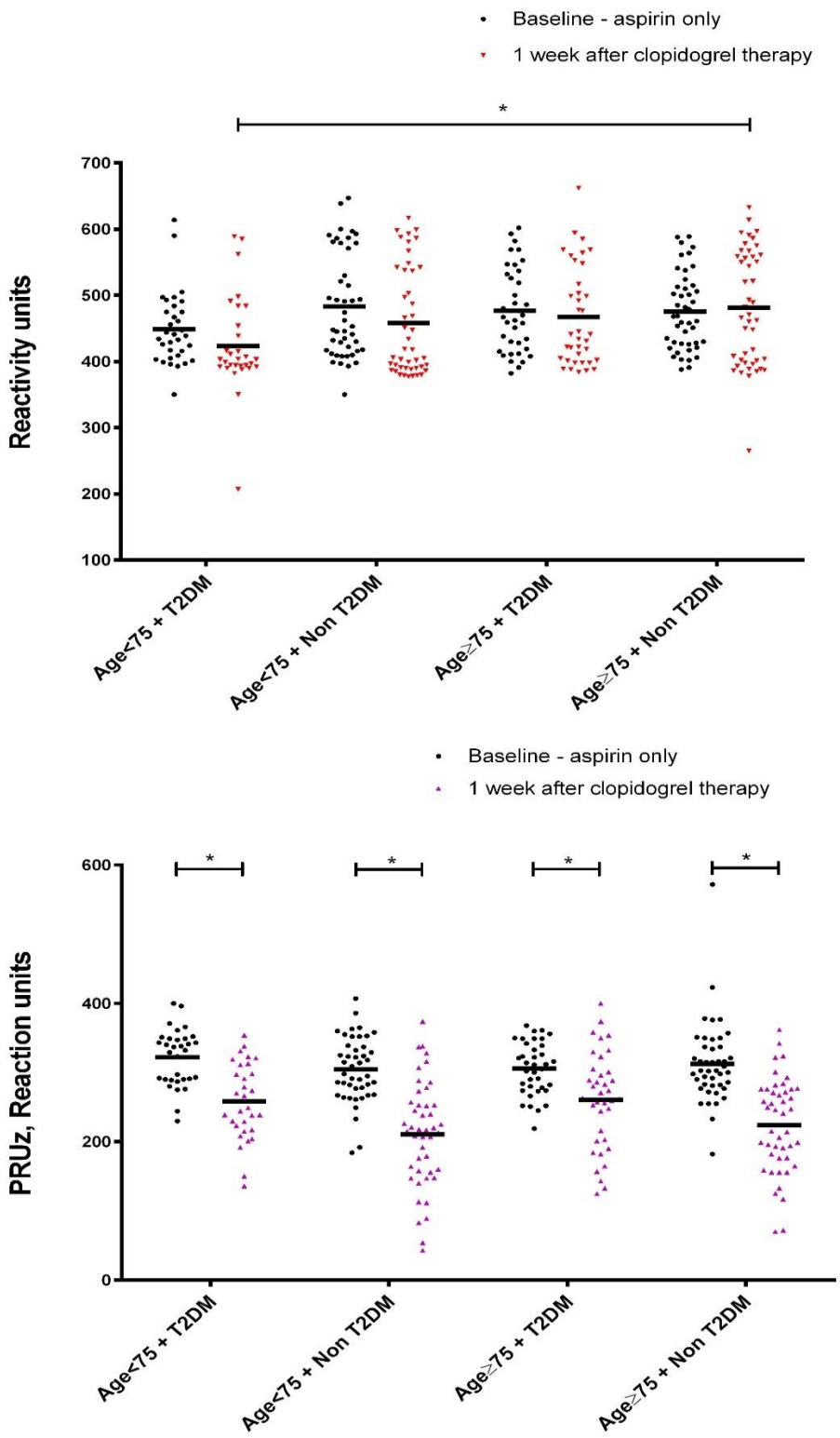


Figure 4.10 Changes in VerifyNow® indices

*p<0.05

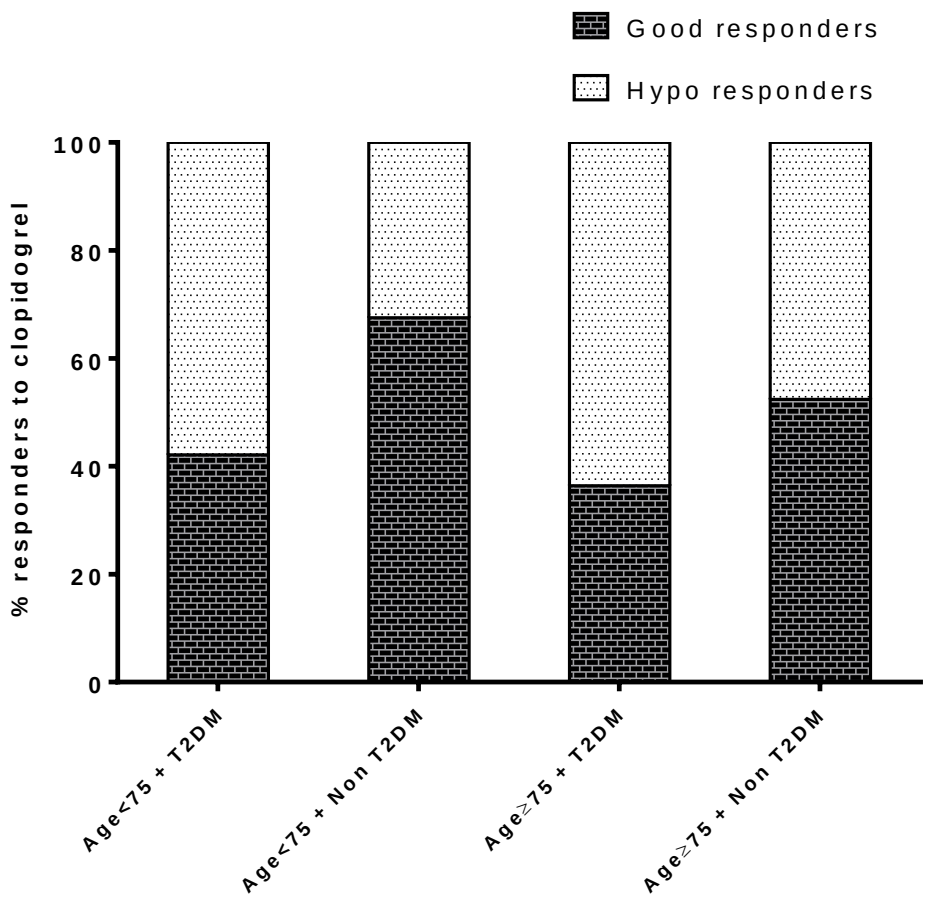


Figure 4.11 Percentage of good and hyporesponders to clopidogrel

Good responders to clopidogrel	Age < 75 + T2DM (n = 16)			Age < 75 + Non T2DM (n = 27)			Age ≥ 75 + T2DM (n = 12)			Age ≥ 75 + Non T2DM (n = 22)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	15579± 4650	11338± 4228	0.024 *	15629± 4968	12422± 4521	0.000 *	18217± 6227	14332± 5073	0.027 *	17069± 7575	13537± 4718	0.011 *
Low shear thrombus area, μ^2/mm	9858± 2259	7177± 1577	0.000 *	9924± 2821	7910± 2304	0.002 *	9331± 2087	7405± 1005	0.027 *	9210± 2546	8831± 1677	0.317
ARU	445± 52.8	422± 89.5	0.348	488± 79.5	462± 86.3	0.259	457± 70.6	460± 88.0	0.564	475± 65.6	486± 84.8	0.615
PRUz	300± 31.6	202± 37.9	0.000 *	302± 49.8	164± 54.4	0.000 *	271± 29.9	177± 30.1	0.000 *	312± 75.8	166± 41.2	0.000 *
Platelet inhibition, %	3.0± 1.9	30.9± 16.3	0.000 *	2.6± 5.0	41.7± 22.7	0.000 *	3.1± 4.9	38.1± 10.7	0.000 *	4.7± 6.6	45.2± 15.1	0.000 *

Table 4.22 Changes in thrombus area and VerifyNow® indices (good responders to clopidogrel)

*p<0.05

Good responders to clopidogrel	Age < 75 + T2DM (n = 16)	Age < 75 + Non T2DM (n = 27)	Age ≥ 75 + T2DM (n = 12)	Age ≥ 75 + Non T2DM (n = 22)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD					
High shear thrombus area, μ^2/mm	15579± 4650	15629± 4968	18217± 6227	17069± 7575	0.558
Low shear thrombus area, μ^2/mm	9858± 2259	9924± 2821	9331± 2087	9210± 2546	0.758
ARU	445± 52.8	488± 79.5	457± 70.6	475± 65.6	0.226
PRUz	300± 31.6	302± 49.8	271± 29.9	312± 75.8	0.230
Platelet inhibition, %	3.0± 1.9	2.6± 5.0	3.1± 4.9	4.7± 6.6	0.665
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD					
High shear thrombus area, μ^2/mm	11338± 4228	12422± 4521	14332± 5073	13537± 4718	0.337
Low shear thrombus area, μ^2/mm	7177± 1577	7910± 2304	7405± 1005	8831± 1677	0.064
ARU	422± 89.5	462± 86.3	460± 88.0	486± 84.8	0.183
PRUz	202± 37.9	164± 54.4	177± 30.1	166± 41.2	0.051
Platelet inhibition, %	30.9± 16.3	41.7± 22.7	38.1± 10.7	45.2± 15.1	0.107
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI					
Delta ARU	23, -27.4-73.2	27, -20.7-73.7	16, -42.4-73.5	-10, -53.0-32.1	0.630
Delta PRUz	97, 70.2-124.4	138, 114.2-160.8	96, 74.2-118.5	146, 113.8-178.9	0.084
Delta Platelet inhibition, %	29.1, 18.7-39.4	39.0, 31.1-46.9	34.7, 28.2-41.3	40.5, 33.9-47.1	0.206

Table 4.23 Comparison of thrombus area and VerifyNow® indices (good responders to clopidogrel)

Post hoc analysis (One way ANOVA); *p<0.05

Hypo responders to clopidogrel	**Age < 75 + T2DM (n = 22)**			**Age < 75 + Non T2DM (n = 13)**			**Age ≥ 75 + T2DM (n = 21)**			**Age ≥ 75 + Non T2DM (n = 20)**		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14321± 5899	12269± 4164	0.063	16113± 1820	14809± 1442	0.370	15113± 6561	12440± 4637	0.007 *	17596± 6101	14484± 5024	0.012 *
Low shear thrombus area, μ^2/mm	7983± 2350	6967± 1794	0.047 *	10150± 2471	8460± 2556	0.047 *	9661± 2481	8432± 1897	0.016 *	10786± 2545	8926± 2387	0.007 *
ARU	472± 58.0	438± 68.2	0.022 *	486± 76.5	463± 84.4	0.274	492± 54.6	488± 75.9	0.841	479± 52.7	488± 81.8	0.624
PRUz	344± 30.5	299± 31.8	0.000 *	318± 32.3	281± 36.4	0.000	317± 31.8	284± 30.1	0.000 *	317± 36.9	276± 27.8	0.000 *
Platelet inhibition, %	1.3± 3.7	12.2± 10.1	0.000 *	0.9± 1.6	11.5± 8.9	0.001	1.9± 3.6	11.8± 1.8	0.000 *	3.0± 4.3	14.1± 9.3	0.000 *

Table 4.24 Changes in thrombus area and VerifyNow® indices (hyporesponders to clopidogrel)

*p<0.05

Hypo responders to clopidogrel	Age < 75 + T2DM (n = 22)	Age < 75 + Non T2DM (n = 13)	Age ≥ 75 + T2DM (n = 21)	Age ≥ 75 + Non T2DM (n = 20)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD					
High shear thrombus area, μ^2/mm	14321± 5899	16113± 1820	15113± 6561	17596± 6101	0.390
Low shear thrombus area, μ^2/mm	7983± 2350*	10150± 2471	9661± 2481	10786± 2545*	0.009 *
ARU	472± 58.0	486± 76.5	492± 54.6	479± 52.7	0.729
PRUz	344± 30.5	318± 32.3	317± 31.8	317± 36.9	0.061
Platelet inhibition, %	1.3± 3.7	0.9± 1.6	1.9± 3.6	3.0± 4.3	0.346
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD					
High shear thrombus area, μ^2/mm	12269± 4164	14809± 1442	12440± 4637	14484± 5024	0.241
Low shear thrombus area, μ^2/mm	6967± 1794*	8460± 2556	8432± 1897	8926± 2387*	0.044 *
ARU	438± 68.2	463± 84.4	488± 75.9	488± 81.8	0.126
PRUz	299± 31.8	281± 36.4	284± 30.1	276± 27.8	0.135
Platelet inhibition, %	12.2± 10.1	11.5± 8.9	11.8± 1.8	14.1± 9.3	0.830
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI					
Delta ARU	34, 5.5-61.6	23, -20.8-66.9	4, -34.9-42.5	-9, -44.9-27.6	0.293
Delta PRUz	48, 31.1-64.2	37, 23.7-50.0	33, 22.7-43.7	40, 26.3-54.5	0.435
Delta Platelet inhibition, %	10.9, 5.7-16.1	10.7, 5.6-15.8	9.9, 6.3-13.5	11.2, 7.1-15.2	0.977

Table 4.25 Comparison of thrombus area and VerifyNow® indices (hyporesponders to clopidogrel)

Post hoc analysis (One way ANOVA); *p<0.05

	Correlation with ARU Rho (2-tailed p value)	Correlation with PRUz Rho (2-tailed p value)
BMI, Kg/m^2	-0.018 (0.911)	0.292 (0.105)
Haemoglobin, g/dl	-0.172 (0.289)	-0.684 (0.000) *
Platelet count x 1000 cells/mm^3	-0.085 (0.604)	-0.094 (0.609)
Fibrinogen, g/ml	-0.292 (0.072)	-0.343 (0.059)
High shear thrombus area, μ^2/mm	-0.059 (0.449)	-0.036 (0.663)
Low shear thrombus area, μ^2/mm	-0.094 (0.247)	0.127 (0.140)
R, min	0.081 (0.307)	-0.021 (0.804)
K, min	0.146 (0.066)	-0.094 (0.264)
MA, mm	-0.138 (0.081)	0.054 (0.523)
G Kdynes/sec	-0.112 (0.157)	0.088 (0.298)
CI	-0.133 (0.093)	0.053 (0.527)
MA-AA, mm	0.246 (0.002) *	-0.001 (0.988)
MA-ADP, mm	-0.006 (0.940)	0.105 (0.218)
% aggregation to AA	0.248 (0.002) *	-0.055 (0.519)
% aggregation to ADP	0.032 (0.689)	0.091 (0.287)
Thrombin generation	-0.016 (0.842)	-0.046 (0.588)
Maximum rate of thrombin generation, min	-0.073 (0.362)	-0.005 (0.951)
Thrombus retraction, L parameter mm/min	0.136 (0.183)	0.031 (0.773)
Maximum rate of thrombus retraction, min	-0.067 (0.515)	-0.020 (0.853)

Table 4.26 Correlation: VerifyNow® and thrombus area, TEG®, Platelet Mapping™, V-curve data at baseline (visit 1)

*p<0.05

	Correlation with ARU Rho (2-tailed p value)	Correlation with PRUz Rho (2-tailed p value)
BMI, Kg/m^2	-0.083 (0.608)	0.025 (0.891)
Haemoglobin, g/dl	-0.242 (0.127)	-0.649 (0.000) *
Platelet count x 1000 cells/mm^3	0.144 (0.367)	-0.020 (0.914)
Fibrinogen, g/ml	-0.028 (0.866)	-0.076 (0.685)
High shear thrombus area, μ^2/mm	-0.81 (0.317)	-0.008 (0.916)
Low shear thrombus area, μ^2/mm	-0.160 (0.053)	0.195 (0.018) *
R, min	0.035 (0.662)	-0.242 (0.002) *
K, min	0.104 (0.194)	-0.094 (0.239)
MA, mm	-0.089 (0.264)	0.030 (0.707)
G Kdynes/sec	-0.089 (0.264)	0.030 (0.710)
CI	-0.076 (0.340)	0.175 (0.027) *
MA-AA, mm	0.186 (0.020) *	0.084 (0.295)
MA-ADP, mm	0.090 (0.263)	0.181 (0.024) *
% aggregation to AA	0.132 (0.101)	0.019 (0.811)
% aggregation to ADP	0.149 (0.064)	0.225 (0.005) *
Thrombin generation	0.004 (0.964)	-0.054 (0.521)
Maximum rate of thrombin generation, min	-0.157 (0.048)	0.035 (0.680)
Thrombus retraction, L parameter mm/min	0.046 (0.629)	-0.006 (0.950)
Maximum rate of thrombus retraction, min	-0.012 (0.900)	-0.001 (0.996)

Table 4.27 Correlation: VerifyNow® and thrombus area, TEG®, Platelet Mapping™, V-curve data at visit 2

*p<0.05

Age<75 years vs. Age≥75 years

I divided the study population as young (age<75 years) and elderly (age≥75 years) to assess the effect of age on clopidogrel response and platelet dependent thrombus formation.

Demographic data and baseline characteristics of the patients who had their platelet functions measured using VerifyNow® remain the same as discussed in the earlier section (Table 4.4).

Young patients (age<75 years) comprised of 43 good responders to clopidogrel (55.1%) compared to only 34 good responders (45.3%) in the elderly group (age≥75 years). Percentage of hyporesponders to aspirin at baseline and after one week of clopidogrel is 26.7% vs. 24.1% in young patients and 36.5% vs. 38.4% in the elderly.

The absolute mean PRUz value showed a significant reduction in both the groups after one week of clopidogrel therapy (age<75: 313±44.8 to 230±72.5, p<0.001; age≥75: 308±50.6 to 240±70.0, p<0.001). Despite significant reduction in PRUz, the mean PRUz in the elderly group remained 240, establishing high platelet reactivity in the elderly population. Mean difference in PRUz (Mean, 95%CI) from visit 1 and visit 2 were similar between the groups; Age≥75: 78.2, 62.6-93.7 and in Age<75: 89, 74.0-103.0; p=0.952 (Table 4.28, Table 4.29).

Despite being on long term aspirin therapy, there was a significant reduction in ARU from visit 1 to visit 2 in the younger population (mean±SD, 471±70.7 to 444±79.0; p=0.015) demonstrating improved platelet inhibition to aspirin after the addition of clopidogrel as a second antiplatelet agent. There was no change in ARU value in the elderly population after the addition of clopidogrel.

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean PRUz < 240). There was a consistent reduction both in high shear (age<75: 15612± 4803 vs. 12052± 4401, p<0.01; age≥75: 17487± 7036 vs. 13826± 4786, p=0.001) and low shear thrombus area following one week of clopidogrel therapy. Mean PRUz was similar between the groups both at baseline (age<75 vs. age≥75: 301±43.5 vs. 298±66.7, p=0.845) and one week after clopidogrel therapy (179±51.8 vs. 169±37.5, p=0.402). Difference in PRUz from baseline to one week after clopidogrel (delta PRUz) were also similar

between the groups (mean, 95%CI: 89, 74.0-103.0 vs. 78.2, 62.6-93.7, p=0.952). Mean PRUz were much lower than the cut off of 240 in both the groups. There was a significant reduction in ARU from baseline to one week after clopidogrel therapy in the younger group (mean±SD: 471±70.7 to 444±79.0, p=0.015), but there was no change in the elderly group (Table 4.30, Table 4.31).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean PRUz ≥ 240) were compared, both younger (age<75) and elderly (age≥75) patients consistently demonstrated significant reduction in both high shear (age<75: 14987± 6120 to 13212± 4669, p=0.040; age≥75: 16293± 6390 to 13411± 4873, p<0.001) and low shear thrombus area. Mean PRUz at baseline was significantly higher in patients with age<75 when compared to the elder group (334±33.3 vs. 317±34.0, p=0.032) but one week after clopidogrel therapy this difference was not significant (291±33.5 vs. 280±28.9, p=0.116). Difference in PRUz from baseline to one week after clopidogrel (delta PRUz) was numerically lower in the elderly group but this difference was not statistically significant (mean, 95%CI: 43.5, -1-107 vs. 36.7, 0-109, p=0.323). Despite demonstrating significant reduction in PRUz, the mean PRUz remained ≥ 240 in both the groups indicating high platelet reactivity. There was a significant reduction in ARU from baseline to one week after clopidogrel therapy in the younger group (mean±SD: 477±65.7 to 448±74.5, p=0.012), but there was no change in the elderly group (Table 4.32, Table 4.33).

	Age < 75 (n = 87)			Age ≥ 75 (n = 86)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
ARU	471±70.7	444±79.0	0.015 *	476±59.9	476±82.6	0.879
PRUz	313±44.8	230±72.5	0.000 *	308±50.6	240±70.0	0.000 *
Platelet inhibition, %	2.2±5.2	25.8±21.2	0.000 *	3.2±4.9	24.6±18.6	0.000 *

Table 4.28 Changes in VerifyNow® indices (Age<75 vs. Age≥75)

*p<0.05

	Age < 75 (n = 87)	Age ≥ 75 (n = 86)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
ARU	471±70.7	476±59.9	0.584
PRUz	313±44.8	308±50.6	0.531
Platelet inhibition, %	2.2±5.2	3.2±4.9	0.232
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
ARU	444±79.0	476±82.6	0.016 *
PRUz	230±72.5	240±70.0	0.592
Platelet inhibition, %	25.8±21.2	24.6±18.6	0.896
Good responders, % (n)	55.1 (43)	45.3 (34)	0.259
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	26, 4.9-47.1	-2, -21.9-17.7	0.836
Delta PRUz	89, 74.0-103.0	78.2, 62.6-93.7	0.952
Delta Platelet inhibition, %	24.7, 20.0-29.4	23.0, 18.9-27.1	0.532

Table 4.29 Comparison of thrombus area and VerifyNow® indices (Age<75 vs. Age≥75)

*p<0.05

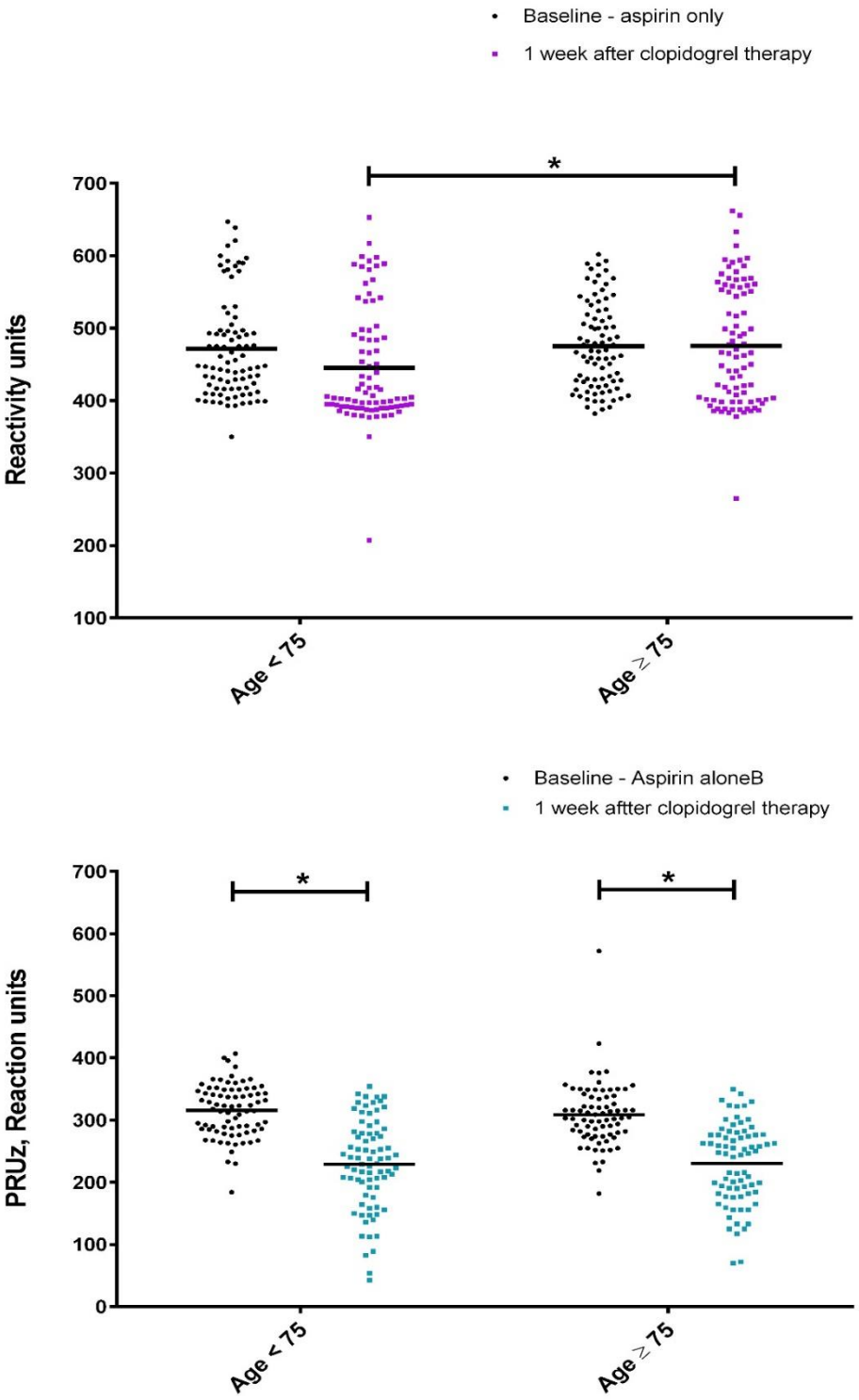


Figure 4.12 Changes in VerifyNow® indices (Age<75 vs. Age≥75)

Top graph: ARU, aspirin platelet reactivity units

Bottom graph: PRUz, clopidogrel platelet reactivity units

* P<0.05

Good responders to clopidogrel	**Age < 75 (n = 43)**			**Age ≥ 75 (n = 34)**		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	15612± 4803	12052± 4401	0.000	17487± 7036	13826± 4786	0.001
Low shear thrombus area, μ^2/mm	9900± 2602	7646± 2081	0.000	9248± 2379	8386± 1628	0.022
ARU	472±73.2	447±88.6	0.140	469±66.7	471±80.7	0.912
PRUz	301±43.5	179±51.8	0.000	298±66.7	169±37.5	0.000
Platelet inhibition, %	2.8±6.0	38.2±20.9	0.000	4.2±6.0	42.8±14.2	0.000

Table 4.30 Changes in thrombus area and VerifyNow® - good responders (Age<75 vs. Age≥75)

*p<0.05

Good responders to clopidogrel	Age < 75 (n = 43)	Age ≥ 75 (n = 34)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	15612±4803	17487±7036	0.197
Low shear thrombus area, μ^2/mm	9900±2602	9248±2379	0.274
ARU	472±73.2	469±66.7	0.852
PRUz	301±43.5	298±66.7	0.845
Platelet inhibition, %	2.8±6.0	4.2±6.0	0.314
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	12052±4401	13826±4786	0.105
Low shear thrombus area, μ^2/mm	7646±2081	8386±1628	0.098
ARU	447±88.6	471±80.7	0.145
PRUz	179±51.8	169±37.5	0.402
Platelet inhibition, %	38.2±20.9	42.8±14.2	0.213
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	25.2, -206-249	-1.8, -193-201	0.249
Delta PRUz	122.6, 38-267	129.7, 54-373	0.628
Delta Platelet inhibition, %	35.5, -12-74	38.6, 10-74	0.426

Table 4.31 Comparison of thrombus area and VerifyNow® - good responders (Age<75 vs. Age≥75)

*p<0.05

Hypo responders to clopidogrel	Age < 75 (n = 35)			Age ≥ 75 (n = 41)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14987± 6120	13212± 4669	0.040	16293± 6390	13411± 4873	0.000
Low shear thrombus area, μ^2/mm	8805± 2584	7551± 2125	0.004	10224± 2544	8679± 2141	0.000
ARU	477±65.7	448±74.5	0.012	486±53.4	488±77.8	0.857
PRUz	334±33.3	291±33.5	0.000	317±34.0	280±28.9	0.000
Platelet inhibition, %	1.1±3.1	12.0±9.5	0.000	2.4±4.0	12.9±8.8	0.000

Table 4.32 Thrombus area and VerifyNow® indices - hyporesponders (Age<75 vs. Age≥75)

*p<0.05

Hypo responders to clopidogrel	Age < 75 (n = 35)	Age ≥ 75 (n = 41)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	14987±6120	16293±6390	0.369
Low shear thrombus area, μ^2/mm	8805±2584	10224±2544	0.029
ARU	477±65.7	486±53.4	0.543
PRUz	334±33.3	317±34.0	0.032
Platelet inhibition, %	1.1±3.1	2.4±4.0	0.119
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	13212±4669	13411±4873	0.858
Low shear thrombus area, μ^2/mm	7551±2125	8679±2141	0.033
ARU	448±74.5	488±77.8	0.026
PRUz	291±33.5	280±28.9	0.116
Platelet inhibition, %	12.0±9.5	12.9±8.8	0.653
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	29.5, -106-187	-2.3, -162-181	0.063
Delta PRUz	43.5, -1-107	36.7, 0-109	0.323
Delta Platelet inhibition, %	-10.8, -30-16	-10.5, -27-0	0.886

Table 4.33 Thrombus area and VerifyNow® indices - hyporesponders (Age<75 vs. Age≥75)

*p<0.05

T2DM vs. Non DM

I divided the study population as T2DM and non DM to assess the effect of T2DM on clopidogrel response and platelet dependent thrombus formation.

Demographic data and baseline characteristics of the patients who had their platelet functions measured using VerifyNow® remain the same as discussed in the earlier section (Table 4.7).

T2DM group had only 28 good responders to clopidogrel (36.4%) compared to 49 good responders (63.6%) in the non DM group. Percentage of hyporesponders to aspirin at baseline and after one week of clopidogrel is 25.9% vs. 24.1% in T2DM patients and is 36.7% vs. 37.8% in non-DM patients.

The absolute mean PRUz value showed a significant reduction in both the groups after one week of clopidogrel therapy (T2DM: 314±39.3 to 261±61.8, p<0.001; non DM: 309±53.2 to 217±72.9, p<0.001). Despite significant reduction in PRUz, the mean PRUz in T2DM group remained 240, establishing high platelet reactivity in DM population. Mean difference in PRUz (Mean, 95%CI) from visit 1 and visit 2 were similar between the groups; T2DM: 63, 51.4-74.0 and in non DM: 100, 84.1-116.3; p<0.001 (Table 4.34, Table 4.35, Figure 4.13).

Despite being on long term aspirin therapy, there was a numerical reduction in ARU from visit 1 to visit 2 in T2DM population (mean±SD: 466±60.0 to 448±76.0, p=0.055). Though this reduction was not statistically significant, it does demonstrate improvement in platelet inhibition by aspirin after the addition of clopidogrel. There was no significant change in ARU value in non DM group after the addition of clopidogrel.

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean PRUz < 240). There was a consistent reduction both in high shear (T2DM: 16797± 5487 vs. 12720± 4789, p=0.001; non DM: 16259± 6212 vs. 12910± 4592, p<0.001) and low shear thrombus area following one week of clopidogrel therapy. Mean PRUz was similar between the groups at baseline (T2DM vs. non DM: 288±33.6 vs. 307±62.3, p=0.094) but after one week of clopidogrel therapy the PRUz in T2DM group remained significantly higher than non DM group (192±36.4 vs. 165±48.4, p=0.008), despite both the values being lower than the cut off of 240. Difference in PRUz from baseline to one week after

clopidogrel (delta PRUz) was significantly lower in T2DM compared to non DM (mean, 95%CI: 99, 81-116 in T2DM vs. 141, 123-160 in non DM, p=0.001), suggesting better platelet inhibition in non-DM patients compared to T2DM patients despite both the groups being good responders to clopidogrel. Though there was a small numerical reduction in ARU from baseline to one week after clopidogrel therapy in both the groups, this was not significant (Table 4.36, Table 4.37).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean PRUz ≥ 240) were compared, both T2DM and non DM patients consistently demonstrated significant reduction in both high shear (T2DM: 14708± 6169 to 12352± 4349, p=0.001; non DM: 16994± 6231 to 14616± 5015; p=0.011) and low shear thrombus area. Mean PRUz was similar at baseline and one week after clopidogrel therapy in both the groups. Difference in PRUz from baseline to one week after clopidogrel (delta PRUz) was similar between the groups (mean, 95%CI: 40.4, -1-107 vs. 39.0, 0-109.0, p=0.831). Despite demonstrating significant reduction in PRUz, the mean PRUz after one week of clopidogrel therapy remained ≥ 240 in both the groups indicating high platelet reactivity (T2DM: 331±33.6 to 290±30.8, p<0.001; non DM: 317±34.6 to 278±31.0, p<0.001). There was a small non-significant numerical reduction in ARU from baseline to one week after clopidogrel therapy in T2DM group (482±57.3 to 463±75.5, p=0.114) but in non DM group ARU remained unchanged (Table 4.38, Table 4.39).

	T2DM (n = 83)			Non DM (n = 90)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
ARU	466±60.0	448±76.0	0.055	479±68.9	469±84.4	0.351
PRUz	314±39.3	261±61.8	0.000	309±53.2	217±72.9	0.000
Platelet inhibition, %	2.3±5.1	18.9±15.1	0.000	3.0±5.1	29.7±21.9	0.000

Table 4.34 Changes in VerifyNow® indices (T2DM vs. Non-DM)

*p<0.05

	T2DM (n = 83)	Non DM (n = 90)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
ARU	466±60.0	479±68.9	0.174
PRUz	314±39.3	309±53.2	0.594
Platelet inhibition, %	2.3±5.1	3.0±5.1	0.493
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
ARU	448±76.0	469±84.4	0.144
PRUz	261±61.8	217±72.9	0.000
Platelet inhibition, %	18.9±15.1	29.7±21.9	0.001
Good responders, % (n)	36.4 (28)	63.6 (49)	0.009
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	18, -1.6-40.0	7, -13.9-28.8	0.027
Delta PRUz	63, 51.4-74.0	100, 84.1-116.3	0.000
Delta Platelet inhibition, %	18.7, 14.7-22.6	28.1, 23.7-32.6	0.013

Table 4.35 Comparison of thrombus area and VerifyNow® (T2DM vs. Non-DM)

*p<0.05

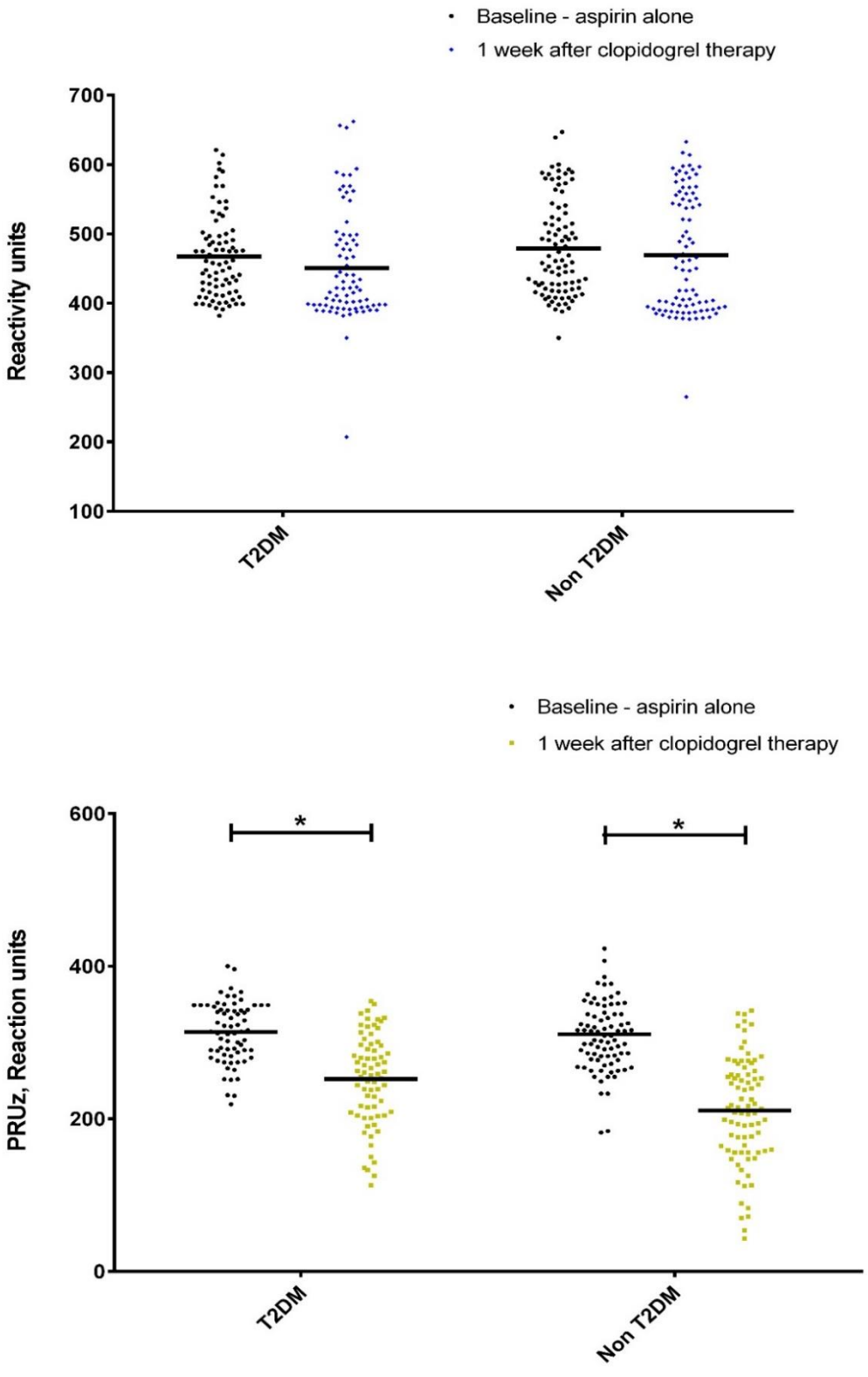


Figure 4.13 Changes in VerifyNow® indices (T2DM vs. Non-DM)

Good responders to clopidogrel	T2DM (n = 28)			Non DM (n = 49)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	16797± 5487	12720± 4789	0.001	16259± 6212	12910± 4592	0.000
Low shear thrombus area, μ^2/mm	9639± 2159	7272± 1347	0.000	9590± 2691	8341± 2067	0.001
ARU	450±59.7	438±89.3	0.259	483±73.1	473±85.6	0.531
PRUz	288±33.6	192±36.4	0.000	307±62.3	165±48.4	0.000
Platelet inhibition, %	3.0±6.5	34.0±14.4	0.000	3.6±5.8	43.3±19.6	0.000

Table 4.36 Thrombus area and VerifyNow® indices - good responders (T2DM vs. Non-DM)

*p<0.05

Good responders to clopidogrel	T2DM (n = 28)	Non DM (n = 49)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	16797±5487	16259±6212	0.703
Low shear thrombus area, μ^2/mm	9639±2159	9590±2691	0.934
ARU	450±59.7	483±73.1	0.041
PRUz	288±33.6	307±62.3	0.094
Platelet inhibition, %	3.0±6.5	3.6±5.8	0.728
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	12720±4789	12910±4592	0.869
Low shear thrombus area, μ^2/mm	7272±1347	8341±2067	0.011
ARU	438±89.3	473±85.6	0.105
PRUz	192±36.4	165±48.4	0.008
Platelet inhibition, %	34.0±14.4	43.3±19.6	0.020
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	14.2, -21-49	9.9, -22-42	0.670
Delta PRUz	99, 81-116	141, 123-160	0.001
Delta Platelet inhibition, %	-32, -37.7- -31.5	-40, -44.8- 34.6	0.042

Table 4.37 Thrombus area and VerifyNow® indices - good responders (T2DM vs. Non-DM)

*p<0.05

Hypo responders to clopidogrel	T2DM (n = 43)			Non DM (n = 33)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14708± 6169	12352± 4349	0.001	16994± 6231	14616± 5015	0.011
Low shear thrombus area, μ^2/mm	8845± 2532	7724± 1915	0.001	10553± 2495	8755± 2417	0.001
ARU	482±57.3	463±75.5	0.114	482±62.1	478±82.4	0.773
PRUz	331±33.6	290±30.8	0.000	317±34.6	278±31.0	0.000
Platelet inhibition, %	1.6±3.6	12.0±9.2	0.000	2.1±3.6	13.1±9.1	0.000

Table 4.38 VerifyNow® indices - hyporesponders (T2DM vs. Non-DM)

*p<0.05

Hypo responders to clopidogrel	T2DM (n = 43)	Non DM (n = 33)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	14708±6169	16994±6231	0.119
Low shear thrombus area, μ^2/mm	8845±2532	10553±2495	0.007
ARU	482±57.3	482±62.1	0.961
PRUz	331±33.6	317±34.6	0.097
Platelet inhibition, %	1.6±3.6	2.1±3.6	0.540
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	16994±6231	14616±5015	0.045
Low shear thrombus area, μ^2/mm	10553±2495	8755±2417	0.060
ARU	463±75.5	478±82.4	0.410
PRUz	290±30.8	278±31.0	0.073
Platelet inhibition, %	12.0±9.2	13.1±9.1	0.615
Difference in variables between Visit 1 and Visit 2 Mean change, 95% CI			
Delta ARU	18.6, -162-181	3.8, -137-187	0.403
Delta PRUz	40.4, -1-107	39.0, 0-109.0	0.831
Delta Platelet inhibition, %	-10.4, -30-16	-11.0, -27.0-0	0.795

Table 4.39 Thrombus area and VerifyNow® - hyporesponders (T2DM vs. Non-DM)

*p<0.05

4.3.2 Discussion – platelet reactivity indices

In my study, baseline (on aspirin alone) PRUz was similar in elderly (age≥75) and young patients (age<75), with or without T2DM. My study demonstrated a significant reduction in PRUz in elderly and young, again in patients with and without T2DM, after one week of clopidogrel (75mg daily) alongside aspirin (75 mg daily). Good response to clopidogrel was defined based on PRUz cut off of <240 units. Despite a significant reduction, mean PRUz remained significantly higher (≥240 U) both in elderly and young patients with T2DM when compared to non-diabetic individuals. This is the first study to compare VerifyNow indices with platelet dependent thrombus (PDT) from Badimon chamber (mimicking rheological flow conditions in moderately stenosed coronary arteries). This comparison of point of care platelet reactivity indices to ex-vivo whole blood thrombus generation is novel and will certainly be helpful in further exploring the use of these indices in the clinical setting to tailor antiplatelet therapy.

When comparing elderly and young patients, only 45.3% of elderly patients were good responders to clopidogrel versus 55.1% of young patients. PRUz was numerically higher in elderly cohort compared to the young. It is not surprising to see that there was a significant reduction in thrombus (measured as total thrombus area) among good responders to clopidogrel both in elderly and young cohort. Interestingly, my study has demonstrated a significant reduction in thrombus even among hypo responders to clopidogrel (PRUz ≥240 U), in elderly and young patients, with or without T2DM. This demonstrates that clopidogrel reduces thrombus formation despite high platelet reactivity as defined by VerifyNow cut off (PRUz ≥ 240 U).

When patients with T2DM were compared with non-diabetic individuals, only 36.4% were good responders in T2DM cohort versus 63.6% among non-diabetic. Mean PRUz among T2DM remained well above the cut off 240 U and was less than 240 U among non-DM. There was a significant reduction in thrombus area among good responders as well as hyporesponders to clopidogrel. Among good responders to clopidogrel, PRUz in T2DM remained significantly higher compared to non-diabetic individuals.

Haemoglobin levels negatively correlated with mean PRUz. This is the first study where thrombus area determined by Badimon chamber and TEG® parameters were compared to VerifyNow® data. There was a good correlation in the assessment of clopidogrel response by both the studies (MA-ADP and PRUz) after a week of

clopidogrel therapy. PRUz had a positive correlation with low shear thrombus but did not demonstrate any correlation with high shear thrombus.

Overall, data from my study confirm that on treatment platelet reactivity to clopidogrel as measured by PRUz has high variability in elderly patients and in the presence of T2DM. Mean PRUz value was numerically higher in elderly when compared to the young at 240 U, demonstrating high on-treatment platelet reactivity in the elderly cohort. Mean PRUz values were higher in patients with T2DM when compared to non-diabetic individuals, among both good responders and hypo responders. Even in hypo responders, there was a significant reduction in thrombus formation, both in elderly and young patients, with or without TDM. Antiplatelet response measured using TEG® (MA-ADP) correlated with the corresponding measures using VerifyNow (PRUz). Haemoglobin had a negative correlation with mean PRUz. VerifyNow® has been shown to be more reliable than TEG® in measuring responsiveness to antiplatelet therapy (Madsen *et al.*, 2010). Blais et al measured platelet reactivity using five different platelet function testing methods, before and one week after aspirin therapy. In this study TEG-PM showed higher prevalence of aspirin resistance compared to VerifyNow (Blais *et al.*, 2009).

The routine use of VerifyNow in clinical practice to guide the choice and monitoring of antiplatelet therapy in patients with CAD has been evaluated in a number of studies. The work of Silvain et al, SENIOR-PLATELET study conducted in patients who underwent coronary stent insertion, supported “age” as a factor independently influencing platelet reactivity during clopidogrel therapy alongside aspirin for ≥14 days. They also reported that the elderly patients had significantly higher platelet reactivity compared to young. In addition, the prevalence of high on-treatment platelet reactivity (HPR) was higher in the elderly whilst on clopidogrel and aspirin. In patients treated with 150 mg clopidogrel or 10 mg prasugrel, there was a trend towards higher platelet reactivity in the elderly compared to the young but this was not statistically significant (Silvain *et al.*, 2012). The discrepancy between the frequency of HPR reported in this study and others during prasugrel therapy may be related to the difference in treatment cohort - all ST-elevation myocardial infarction (STEMI) patients (see below). SENIOR-PLATELET study supports previous observations of the limited effectiveness of high-dose clopidogrel in overcoming high platelet reactivity (HPR) and the continued presence of high activity in acute coronary syndrome (ACS) patients treated with prasugrel (Michelson *et al.*, 2009; Price *et al.*, 2011a). The authors suggested that age

integrates several factors affecting clopidogrel metabolism, resulting in a higher prevalence of HPR. In their multivariate analysis, age was an independent predictor of HPR.

The report of Silvain et al is not the first evidence that platelet physiology is influenced by age during clopidogrel therapy. In a genome-wide association study in healthy subjects, increasing age was a significant factor for clopidogrel response (3.8% of variance, p=0.001) (Shuldiner *et al.*, 2009). In recent large translational studies, old age (defined as ≥65 or 75 years) was associated with high on-treatment platelet reactivity to ADP and also adverse ischaemic outcomes (Breet *et al.*, 2010; Price *et al.*, 2011a). However, the latter studies were not specifically designed to explore the influence of age on platelet reactivity.

Higher baseline pre-treatment platelet reactivity has been associated with higher post-treatment reactivity especially in the elderly (Gurbel *et al.*, 2003). However, no actual baseline platelet function measurements were made and the authors acknowledged this limitation. P2Y12 modulates the response to all agonists including TRAP that degranulate the platelet. Hence including baseline TRAP measurement as pre-treatment assessment of platelet function is not entirely correct. Therefore, the response to clopidogrel was not truly measured in this study. Therefore, the conclusion that elderly patients demonstrate an impaired 'response' to clopidogrel cannot be substantiated, although is implied by the data.

In large-scale ACS trials, the elderly patients have high ischaemic events despite treatment with more potent P2Y12 inhibitors. In the TRITON trial, patients aged ≥75 years (13% of the total population) had a 17.3% vs. 18.3% primary end-point compared with 8.1% vs. 10.6% in those < 65 years old during prasugrel vs. clopidogrel therapy, respectively (Wiviott *et al.*, 2007a). In Silvain's study, prasugrel (10 mg) was only administered to STEMI patients or diabetic patients presenting with non-STEMI. The 2011 ACCF/AHA Focused Update of the Guidelines for the Management of Patients with Unstable Angina/Non-STEMI states that prasugrel therapy may be considered in patients' ≥75 years of age who have 'high risk' situations: diabetes or prior myocardial infarction (Wright *et al.*, 2011).

STEMI, diabetes, and prior infarction are considered 'high risk' situations possibly due to HPR. Now we have further evidence that age is another 'high risk' component partly

due to a high prevalence of HPR. In this situation, it would be expected that the most pharmacodynamically effective agent would be associated with the greatest reduction in primary efficacy endpoint occurrence. However, this hypothesis is not supported by the data: in ACS setting ≥75 years of age (15% of the total population) had a 16.8% vs. 18.3% primary endpoint compared with 8.6% vs.10.4% in those < 75 years of age during ticagrelor vs. clopidogrel therapy, respectively (Wallentin *et al.*, 2009).

The majority of studies have shown adequate platelet inhibition post clopidogrel therapy in 20-25% of cohort, both in ACS and stable CAD setting. My study has demonstrated variable platelet inhibition with adequate inhibition in 36-64% among the stable CAD cohort. Difficulty in comparing data from various studies arises due to the differences in study design, varying dosage regimens, different baseline characteristics and clinical settings. Emerging data also suggest the association between low PRU and the risk of bleeding. International cardiac societies have therefore not included VerifyNow or other platelet reactivity indices as a routine standard of care in patients with CAD, except in high risk setting like post PCI stent thrombosis (ST) or recurrent ischaemic events following PCI despite DAPT (Bonello *et al.*, 2010; Tantry *et al.*, 2013). In addition to the occurrence of a greater number of ischaemic events, the elderly also have more frequent serious bleeding. Personalized antiplatelet therapy may have a significant clinical impact in the elderly given their overall high prevalence of treatment failure (ischaemia + bleeding). An interesting observation in Silvain's study was the low prevalence of low PRUz (<30) in elderly patients treated with thienopyridines.

Numerous studies have demonstrated an association between platelet reactivity on clopidogrel and cardiovascular events after coronary intervention and stent insertion. VerifyNow has been used to measure on-treatment platelet reactivity and several prospective observational studies have proposed optimal cut offs to identify high-risk patients. Migliorini et al identified PRUz value ≥240 as the only independent predictor of cardiac death (HR, 3.29; 95% CI, 1.16 to 9.37; p=0.025) and ST (HR, 3.44; 95% CI, 1.01 to 11.80; P=0.049) (Migliorini *et al.*, 2009). In the Gauging Responsiveness with A VerifyNow P2Y12 Assay: Impact on Thrombosis and Safety (GRAVITAS) trial, patients with PRU ≥230 U after PCI were treated with high-dose clopidogrel (150mg) instead of 75mg once daily. At 6 months, high-dose clopidogrel demonstrated a variable and modest pharmacodynamic effect but did not reduce the risk of CV death, MI and ST compared with standard-dose clopidogrel. After adjustment for other risk factors and significant predictors associated with adverse outcomes, PRUz <208 units

was significantly and independently associated with a markedly lower risk of the primary end point at 60-day follow-up (adjusted HR, 0.23; 95% CI, 0.05 to 0.98; P=0.047) and at 6 months (HR, 0.53; 95% CI, 0.28 to 1.04; P=0.06). It was also observed that, PRU <230 units was not significantly associated with the risk of the primary end point and this was consistent with the primary results of the trial. The analysis incorporated the dynamic characteristics of platelet reactivity by evaluating on-treatment reactivity as a time-varying covariate and supported the independent prognostic value of serial platelet function test post coronary intervention (Price *et al.*, 2009; Price *et al.*, 2011a).

These observations have important implications regarding the prognostic utility of platelet function testing and the identification of clinically effective strategies for individualized P2Y12 antagonist therapy. The attenuation in the association between reactivity and clinical outcome over longer-term follow-up could be due to ongoing variability in reactivity that weakened the relationship between the reactivity measured at 30-day follow-up and the actual reactivity at later time points. In addition, it has been suggested that platelet inhibition has greatest influence on cardiovascular events in the early post-PCI period. The influence of other clinical characteristics associated with longer-term outcome on the risk related to on-treatment reactivity also cannot be excluded.

A randomized pharmacodynamic study, SWitching Anti Platelet (SWAP) study demonstrated that switching patients with HPR after an ACS from clopidogrel to prasugrel resulted in earlier and better platelet inhibition (Angiolillo *et al.*, 2010). Similar effects were demonstrate with ticagrelor in patients who had HPR whilst on clopidogrel. Ticagrelor reduced PRU levels to <235 units among both good and hyporesponders to clopidogrel (Gurbel *et al.*, 2010a).

Assessment with a double Randomization of (1) a fixed dose versus a monitoring-guided dose of aspirin and Clopidogrel after DES implantation, and (2) Treatment Interruption versus Continuation, 1 year after stenting (ARCTIC) trial, studied the role of routine platelet function testing and dose adjustment of clopidogrel in those with HPR. In this trial 2440 patients scheduled for elective coronary stenting were randomly assigned to a strategy of VerifyNow P2Y12 and aspirin point-of-care platelet-function monitoring, with drug adjustment in patients who had a poor response to antiplatelet therapy, or to a conventional strategy without monitoring and drug adjustment. In the

monitoring group, HPR in patients taking clopidogrel (34.5% of patients, using a cut off of PRUz 208) or aspirin (7.6% of patients, using a cut off of ARU 550) led to the administration of an additional bolus of clopidogrel, prasugrel, or aspirin along with gp IIb/IIIa inhibitors during the procedure. The primary end point occurred in 34.6% of the patients in the monitoring group, as compared with 31.1% of those in the conventional-treatment group (hazard ratio, 1.13; 95% CI, 0.98 to 1.29; P=0.10). The main secondary end point, stent thrombosis or any urgent revascularization, occurred in 4.9% of the patients in the monitoring group and 4.6% of those in the conventional-treatment group (hazard ratio, 1.06; 95% CI, 0.74 to 1.52; P=0.77). The rate of major bleeding events did not differ significantly between groups. This study did not demonstrate any significant improvement in clinical outcomes with platelet-function monitoring and treatment adjustment for coronary stenting, as compared with standard antiplatelet therapy without monitoring (Collet *et al.*, 2011).

Assessment of Dual AntiPlatelet Therapy with Drug Eluting Stents) ADAPT-DES study, demonstrated that among 8582 patients who entered the study, 42.7% patients had HPR (PRU >208). Rate of ST was higher in patients with HPR after clopidogrel therapy (1.3% vs. 0.5%; HR, 2.54; 95% CI, 1.55-4.16; p=0.0002) but clinically relevant bleeding events were lower (5.6% vs. 6.7%; HR, 0.73; 95% CI, 0.61-0.89; p=0.002). Incidence of acute MI was higher in these patients (3.9% vs. 2.7%; p=0.01). One year mortality was again higher in patients with HPR after clopidogrel therapy (2.4% vs. 1.5%; p=0.002). By multivariable analysis, ST, MI, and clinically relevant bleeding were all strongly associated with all-cause mortality through 1-year follow-up but HPR was not independently related to mortality (Stone *et al.*, 2013).

Brar et al performed a meta-analysis of 6 studies and demonstrated that on a continuous scale, every 10 U increase in PRU was associated with a significantly higher rate of the primary endpoint death, MI or ST (HR: 1.04; 95% CI: 1.03-1.06; $p < 0.0001$). A PRU value ≥230 was associated with a higher rate of the composite primary endpoint (HR: 2.10; 95% CI: 1.62-2.73; $p < 0.0001$), as well as the individual endpoints of death (HR: 1.66; 95% CI: 1.04-2.68; $p = 0.04$), MI (HR: 2.04; 95% CI: 1.51-2.76; $p < 0.001$), and stent thrombosis (HR: 3.11; 95% CI: 1.50-6.46; $p = 0.002$). These findings suggest that VerifyNow might help in predicting short or medium CAD risk up to 1 year in patients undergoing coronary intervention, but not after 2 years (Brar *et al.*, 2011).

Park et al demonstrated a substantial interaction between platelet reactivity on clopidogrel and clinical presentations on cardiovascular events after PCI. The major findings of this study are that (1) in patients undergoing PCI for ACS, HPR on clopidogrel was independently associated with increased risks of primary end point (HR, 2.03; 95% CI, 1.30-3.18; p=0.002) and mortality (HR, 3.46; 95% CI, 1.18-10.18; p=0.02), whereas in patients with stable CAD, HPR did not significantly affect clinical outcomes, and (2) a significant interaction was present between HPR status and clinical presentations on the adjusted risks of the primary end point (p=0.02) mortality (p=0.04) (Park *et al.*, 2012).

In contrast to clopidogrel, aspirin induced platelet inhibition as measured by ARU units demonstrated less variability in young patients and in patients with T2DM. In my study, baseline (on aspirin alone) ARU were similar in elderly (age≥75) and young patients (age<75), with or without T2DM. ARU was numerically higher in the elderly cohort compared to the young. In young patients, there was a significant reduction in ARU after the addition of clopidogrel both among good and hyporesponders, a finding similar to the results from TEG®. This enhanced response to aspirin was not seen in the elderly cohort. In patients with T2DM there was a numerical reduction in ARU after adding clopidogrel to aspirin but this reduction was not statistically significant. This enhanced aspirin response was not seen in non-DM cohort. This non-significant reduction in ARU was also present among the cohort of patients who were hyporesponders with T2DM. Antiplatelet response to aspirin measured using TEG® (MA-AA) demonstrated a good correlation with the corresponding measures using VerifyNow (ARU). Nielson et al performed a small study with healthy volunteers and stable CAD in which all the patients were found to be good responders to aspirin using an ARU cut off of ≥550 (Nielsen *et al.*, 2008). In a small study with 201 stable CAD patients, platelet function tests varied significantly in measuring aspirin's antiplatelet effect and they correlated poorly amongst themselves (Lordkipanidzé *et al.*, 2007). In a large cohort of patients with stable CAD, age was independently correlated with reduced on-treatment aspirin responsiveness, determined using the VerifyNow Aspirin assay. Furthermore, patients aged ≥75 years exhibited higher mean ARU (450 ± 54 vs. 434 ± 53; p = 0.0007) and higher rates of aspirin resistance (19% vs 11%; p = 0.009) using an ARU cut off of ≥500 than patients aged <75 years. Older patients also consistently demonstrated higher platelet aggregation values than younger patients (Vaturi *et al.*, 2013).

Early prospective studies have suggested age as a potential risk factor in increasing incidence of aspirin resistance. There are several potential mechanisms by which age may modulate responsiveness to aspirin. First, it has been proposed that the high co-morbid disease burden in older patients may play a role. It has been demonstrated already that metabolic risk factors may be related to aspirin resistance in healthy subjects (Vaduganathan *et al.*, 2008). Second, platelet-level changes may occur in older patients that may predispose them to aspirin resistance. In addition, older patients may have enhanced basal platelet reactivity before aspirin exposure, which has been shown to be an important determinant of on-treatment platelet reactivity (Frelinger *et al.*, 2006). Significant microvascular dysfunction may also occur in these patients, with reductions in nitric oxide synthesis, impaired platelet responsiveness to vasoactive agents, and increased formation of platelet-monocyte aggregates (Goubareva *et al.*, 2007). Finally, increased platelet turnover in this population, possibly related to advanced atherosclerotic disease, may contribute to impaired responses to aspirin (Cesari *et al.*, 2008).

Stable CAD patients with T2DM, treated with low dose aspirin (81mg daily) exhibited higher aspirin resistance (ARU levels), higher ADP- and collagen-induced platelet aggregation and serum thromboxane levels than nondiabetic patients. Increased aspirin dosing resulted in similar reductions in resistance and platelet function levels between groups. These findings indicate that patients with T2DM exhibit a global high platelet reactivity phenotype that may be partially overcome by higher aspirin doses (DiChiara *et al.*, 2007).

Grove et al performed a comprehensive comparison of light transmission aggregometry (LTA), multiple electrode aggregometry (MEA), VerifyNow® Aspirin and urinary and serum thromboxane metabolites. Each of these tests were performed 8–10 times in healthy individuals and patients with CAD to evaluate the repeatability and concordance between these tests. This study demonstrated VerifyNow® Aspirin assay as the most reproducible test, and which had the strongest correlation with LTA. Furthermore, VerifyNow also demonstrated significant positive correlation with serum thromboxane levels, which is the most specific way to evaluate the pharmacological effect of aspirin on platelets (Grove *et al.*, 2010). However, my study did not show any correlation between ARU and thrombus area implying that ARU levels are a poor marker of thrombogenicity in those on aspirin. Except for a positive correlation between PRUz and low shear thrombus no significant association between PRUz or ARU and

thrombus has been demonstrated. This highlights the point that platelet reactivity indices using VerifyNow® is not useful in predicting thrombogenicity or future atherothrombotic risk in patients with stable CAD.

We desperately need a better understanding of how on-treatment platelet reactivity is related to thrombotic and bleeding events especially in the elderly population. Platelet function studies of adequate number have never been conducted in large-scale ACS trials to provide this answer. At this time, dedicated pharmacodynamic and clinical endpoint trials in the elderly specifically to address the issue of antiplatelet therapy are warranted. An individualized antiplatelet strategy based on serial platelet function testing merits investigation.

4.4 Multiplate® (Impedance aggregometry) - Elderly stable CAD study

4.4.1 Results – platelet reactivity indices

Baseline characteristics of the patients in all the four groups who had their platelet reactivity indices measured remain the same as discussed in the earlier section (Table 4.40). Published ADP AU cut-off value of 460 AU was used to classify patients as on treatment high platelet reactivity (hyporesponders). Patients with ADP AU<460 were classified as good responders to clopidogrel. Elderly T2DM group had the least number of good responders to clopidogrel, 17 patients (45.9%) and elderly non-diabetic group had the highest number of good responders, 29 patients (64.4%).

The absolute mean ADP AU was significantly lower in all the four groups after one week of clopidogrel therapy [median (IQR); young T2DM: 672 (552-941) to 452 (291-676), p=0.002; young non-diabetic: 822 (726-989) to 461 (268-626), p<0.001; elderly T2DM: 769 (653-974) to 488 (310-638), p<0.001; elderly non-diabetic: 762 (589-1012) to 386 (271-559), p<0.001]. Despite significant reduction in ADP AU, the mean AU in elderly T2DM group [488 (310-638)] still remained ≥460, establishing high platelet reactivity in the elderly diabetic population. In contrast, ADP AU was lowest in elderly non-diabetic group [386 (271-559)] after one week of clopidogrel treatment. Similar trends were seen with ASP AU, collagen AU and TRAP AU in young non-diabetics and older group (both T2DM and non-diabetic). These reductions were not demonstrated in the young T2DM group (Table 4.41, Table 4.42, Figure 4.14).

Despite being on long term aspirin therapy, it is interesting to note a significant reduction in ASP AU from visit 1 to visit 2 in the older population both in T2DM and non DM group and in younger non-diabetic group. This demonstrates improved platelet inhibition to aspirin after the addition of clopidogrel as a second antiplatelet agent. With VerifyNow this improved platelet inhibition was demonstrated in the younger group (both T2DM and non DM group), but Multiplate has demonstrated improved platelet inhibition in the elderly group (both T2DM and non DM group) and the young non DM group after the addition of clopidogrel. The percentage of good responders to aspirin increased after addition of clopidogrel in all four groups (young T2DM: 60% to 86.7%; young non-diabetic: 53.3% to 88.6%; old T2DM: 64.9% to 89.5%; old non-diabetic: 74.6% to 86.7%).

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean ADP AU < 460) from all the four groups. There was a consistent reduction both in high shear [median (IQR); age<75+T2DM: 13910 (8699-15594) to 10489 (7884-12106), p=0.022; age<75+non DM: 133323 (11210-18031) to 10942 (9373-13024), p=0.001; age≥75+T2DM: 15727 (9033-20021) to 11639 (8885-15453), p=0.041; age≥75+non DM: 17119 (11616-20464) to 13007 (8997-16029), p<0.001] and low shear thrombus area following one week of clopidogrel therapy. There was a significant reduction in ASP AU, Collagen AU and TRAP AU in young non DM group and in the elderly group (both T2DM and non DM) as shown in Table. Young T2DM group did not demonstrate these reductions (Table 4.43, Table 4.44, Table 4.45).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean PRUz ≥ 460) were compared, elderly non DM group demonstrated a numerical reduction in high shear thrombus area but this was not statistically significant [20387 (9947-27810) to 17565 (10728-21318), p=0.064]. All the other groups demonstrated significant reduction in thrombus area after one week of clopidogrel therapy [median (IQR); age<75+T2DM: 17451 (14577-18868) to 12295 (10272-16639), p=0.045; age<75+non DM: 16723 (11295-20299) to 12953 (11031-18355), p=0.021; age≥75+T2DM: 16411 (10454-19644) to 14069 (8705-15999), p=0.002]. All the four groups demonstrated significant reduction in PRUz but the mean ADP AU remained well above the cut off 460 (Table 4.46, Table 4.47, Table 4.48).

At baseline when patients were on aspirin alone, high shear thrombus area demonstrated a significant positive correlation with ADP AU (rho=0.191; p=0.025) and TRAP AU (rho=0.199; p=0.019). The parameters which measured aspirin induced platelet inhibition using TEG®, MA-AA and percentage aggregation to AA, correlated positively with ASP AU (aspirin induced platelet inhibition measurement using Multiplate®) (rho=0.452; p<0.001 and rho=0.425; p<0.001 respectively). MA-ADP correlated positively with ADP AU and Collagen AU. ARU and PRUz measured using VerifyNow correlated positively with ASU AU and ADP AU respectively (rho=0.309; p<0.001 and rho=0.183; p=0.040 respectively). Thrombin generation (TG) and maximum rate of thrombin generation (MRTG) demonstrated significant positive correlation with ADP AU, Collagen AU and TRAP AU (Table). Standard TEG

parameters R and K, demonstrated negative correlation with ADP AU, Collagen AU and TRAP AU whereas MA, G and CI demonstrated positive correlation with Multiplate indices (Figure 4.15).

After a week of clopidogrel therapy, high shear thrombus area continued to demonstrate a positive correlation with ADP AU (rho=0.170; p=0.046) and TRAP AU (rho=0.216; p=0.011). MA-AA correlated positively with ASP AU (rho=0.508; p<0.001), ADP AU (rho=0.180; p=0.033) and Collagen AU (rho=0.207; p=0.013) and percentage aggregation to AA correlated with ASP AU. MA-ADP correlated positively with ADP AU, Collagen AU and TRAP AU. ARU and PRUz measured using VerifyNow correlated positively with ASU AU and ADP AU respectively (rho=0.193; p=0.022 and rho=0.438; p<0.001 respectively). TG and MRTG demonstrated significant positive correlation with ADP AU and TRAP AU (Table). Correlation between standard TEG parameters with Multiplate indices (ADP AU, Collagen AU and TRAP AU) remained significant after the addition of clopidogrel (Figure 4.16, Figure 4.17).

	Age < 75 + T2DM (n = 15)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Demographic data: Mean ± SD or % (n)					
Age, years	64.1 ± 7.6	63 ± 7.3	78.6 ± 3.2	79.0 ± 3.7	
Male gender, % (n)	86.7 (13)	68.9 (31)	80.5 (33)	71.1 (32)	0.361
Body mass index, kg/m^2	33.0 ± 4.5	29.6 ± 3.7	29.1 ± 5.0	27.8 ± 3.6	0.001
Waist to hip ratio	1.0 ± 0.1*	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.1*	0.030*
Heart rate, beats per minute	72 ± 11*	62 ± 11*	68 ± 14	64 ± 10	0.010*
Systolic BP, mmHg	135 ± 19.2	137 ± 17	156 ± 22.5	152 ± 22.4	0.000
Diastolic BP, mmHg	76 ± 13.2	77 ± 7.8	76 ± 10	75± 9.0	0.756
Risk profile: % (n)					
Angina	80 (12)	77.8 (35)	80.5 (33)	60 (27)	0.101
Previous MI	40 (6)	48.9 (22)	46.3 (19)	66.7 (30)	0.187
Previous PCI	20 (3)	40 (18)	34.1 (14)	28.9 (13)	0.441

Previous CABG	26.7 (4)	11.1 (5)	39.0 (16)	26.7 (12)	0.016
H/o Hypertension	33.3 (5)	51.1 (21)	68.3 (28)	66.7 (30)	0.021
H/o CKD	0 (0)	0 (0)	14.6 (6)	2.2 (1)	0.014
H/o PVD	26.7 (4)	0 (0)	26.8 (11)	11.1 (5)	0.002
H/o CVA	15.4 (2)	4.4 (2)	14.6 (6)	8.9 (4)	0.519
Medications: % (n)					
Sulphonylurea	26.7 (4)		19.5 (8)		
Metformin	34.6 (9)		47.5 (19)		
Insulin	22.2 (6)		48.8 (20)		
Beta-blocker	73.3 (11)	82.2 (37)	68.3 (28)	66.7 (30)	0.462
ACE inhibitor / ARB	80 (12)	73.3 (33)	87.8 (36)	84.4 (38)	0.271
Statin	100 (15)	93.3 (42)	92.7 (38)	95.6 (43)	0.687
Laboratory data: Mean ± SD					
Haemoglobin, g/dl	13.7 ± 0.8	13.6 ± 1.2	12.6 ± 1.3	12.9 ± 1.2	0.000
Platelets x 1000 cells/mm^3	219 ± 40.0	238 ± 63.6	211 ± 48.8	232 ± 66.2	0.150

Fibrinogen, g/ml	3.2 ± 0.5	3.2 ± 0.6	3.4 ± 0.7	3.3 ± 0.6	0.554
HbA1c, mmol/mol	57.5 ± 15.3	39.3 ± 7.0	61.2 ± 15.1	41.3 ± 3.4	
Fasting plasma glucose, mmol/L	8.3 ± 3.4	5.2 ± 0.6	8.3 ± 4.3	5.2 ± 0.7	
Creatinine, micromol/L	87 ± 16.2	83 ± 25.2*	110 ± 53.5*	100 ± 27.7	0.004*
Total cholesterol, mmol/L	3.3 ± 0.6	4.2 ± 1.0	4.0 ± 0.9	4.2 ± 0.8	0.024
LDLc, mmol/L	1.7 ± 0.5	2.2 ± 0.9	2.1 ± 0.9	2.1 ± 0.7	0.310
HDLc, mmol/L	1.0 ± 0.2	1.3 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	0.000
Triglyceride, mmol/L	1.6 ± 0.5	1.4 ± 0.8	1.5 ± 0.7	1.3 ± 0.6	0.489
HsCRP, mg/L	2.6 ± 2.2	1.8 ± 1.8	4.3 ± 6.6	4.4 ± 7.7	0.137

Table 4.40 Baseline characteristics - Multiplate® sub study.

Body mass index, kg/m^2: Age<75+T2DM vs Age<75+non DM, p=0.042; Age<75+T2DM vs Age≥75+T2DM, p=0.015; Age<75+T2DM vs Age≥75+non DM, p<0.001

Systolic BP, mmHg: Age<75+T2DM vs Age≥75+T2DM, p=0.004; Age<75+T2DM vs Age≥75+non DM, p=0.042; Age<75+non DM vs Age≥75+T2DM, p<0.001; Age<75+non DM vs Age≥75+non DM, p=0.004

Total cholesterol, mmol/L: Age<75+T2DM vs Age<75+non DM, p=0.026; Age<75+T2DM vs Age≥75+non DM, p=0.041

HDLc, mmol/L: Age<75+T2DM vs Age<75+non DM, p=0.018; Age<75+T2DM vs Age≥75+non DM, p<0.001

*p<0.05

	Age < 75 + T2DM (n = 15)			Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14941 (11821-17778)	11450 (8554-13000)	0.011	15633 (11257-19736)	12918 (9516-15667)	0.000	15706 (9379-19977)	12593 (8793-15450)	0.000	17726 (11021-21806)	14128 (10354-18012)	0.000
Low shear thrombus area, μ^2/mm	9391 (7854-10095)	7139 (6517-7702)	0.938	9831 (7786-11809)	8010 (6127-9666)	0.004	9635 (7984-11373)	8260 (7224-9701)	0.000	10039 (8380-11947)	8967 (7509-10372)	0.000
ASP AU	356 (222-499)	264 (176-300)	0.143	385 (249-516)	198 (160-286)	0.000	312 (210-466)	220 (142-278)	0.004	298 (234-384)	196 (127-270)	0.000
ADP AU	672 (552-941)	452 (291-676)	0.002	822 (726-989)	461 (268-626)	0.000	769 (653-974)	488 (310-638)	0.000	762 (589-1012)	386 (271-559)	0.000
Collagen AU	547 (383-686)	497 (409-619)	0.653	702 (556-824)	571 (448-742)	0.000	711 (493-856)	601 (423-754)	0.034	623 (496-831)	531 (394-672)	0.001
TRAP AU	994 (656-1141)	1018 (883-1151)	0.468	1172 (1010-1252)	1065 (941-1276)	0.011	1140 (845-1405)	1015 (775-1250)	0.007	1128 (914-1319)	1052 (834-1282)	0.002

Table 4.41 Thrombus area and platelet reactivity indices - Multiplate® substudy.

*p<0.05

	Age < 75 + T2DM (n = 15)	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean (IQR)					
ASP AU	356 (222-499)	385 (249-516)	312 (210-466)	298 (234-384)	0.772
ADP AU	672 (552-941)	822 (726-989)	769 (653-974)	762 (589-1012)	0.757
Collagen AU	547 (383-686)	702 (556-824)	711 (493-856)	623 (496-831)	0.091
TRAP AU	994 (656-1141)	1172 (1010-1252)	1140 (845-1405)	1128 (914-1319)	0.142
Aspirin good responders, % (n)	60 (9)	53.3 (24)	64.9 (24)	75.6 (34)	0.175
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean (IQR)					
ASP AU	264 (176-300)	198 (160-286)	220 (142-278)	196 (127-270)	0.898
ADP AU	452 (291-676)	461 (268-626)	488 (310-638)	386 (271-559)	0.685
Collagen AU	497 (409-619)	571 (448-742)	601 (423-754)	531 (394-672)	0.180
TRAP AU	1018 (883-1151)	1065 (941-1276)	1015 (775-1250)	1052 (834-1282)	0.739
Aspirin good responders,% (n)	86.7 (13)	88.6 (39)	89.5 (34)	86.7 (13.3)	0.978
Clopidogrel Good responders, % (n)	60 (9)	50 (22)	45.9 (17)	64.4 (29)	0.330
Difference in variables between Visit 1 and Visit 2 Mean change, IQR					
Delta ASP AU	69 (-63-236)	125 (42-234)	85 (11-237)	101 (6-201)	0.786
Delta ADP AU	237 (143-443)	355 (157-530)	300 (190-428)	336 (210-336)	0.868
Delta Collagen AU	-39 (-145-104)	107 (-10-210)	38 (-68.5-207)	118 (3-273)	0.367
Delta TRAP AU	44 (-234-191)	80 (-2170	88 (-48-284)	100 (0-208)	0.056

Table 4.42 Comparison of Multiplate® indices; Post hoc analysis (One way ANOVA); *p<0.05

Good responders to clopidogrel	Age < 75 + T2DM (n = 9)			Age < 75 + Non T2DM (n = 22)			Age ≥ 75 + T2DM (n = 17)			Age ≥ 75 + Non T2DM (n = 29)		
Mean (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	13910 (8699-15594)	10489 (7884-12106)	0.022	133323 (11210-18031)	10942 (9373-13024)	0.001	15727 (9033-20021)	11639 (8885-15453)	0.041	17119 (11616-20464)	13007 (8997-16029)	0.000
Low shear thrombus area, μ^2/mm	8922 (7548-9658)	6600 (6341-7649)	0.011	9524 (7526-11809)	7438 (5782-8992)	0.004	8826 (7104-11387)	7523 (7127-9320)	0.051	9018 (7960-11086)	8341 (7243-9747)	0.005
ASP AU	356 (244-473)	241 (204-292)	0.293	311 (233-553)	175 (140-226)	0.000	236 (173-424)	151 (107-243)	0.002	285 (229-352)	191 (116-244)	0.001
ADP AU	598 (417-692)	307 (187-378)	0.026	776 (630-931)	269 (217-388)	0.000	670 (527-799)	302 (250-384)	0.000	647 (575-797)	314 (237-383)	0.000
Collagen AU	472 (246-647)	488 (362-581)	0.804	679 (554-728)	510 (416-583)	0.000	577 (408-713)	426 (315-581)	0.050	569 (487-724)	424 (355-535)	0.002
TRAP AU	856 (486-1080)	931 (819-1024)	0.370	1085 (958-1248)	975 (816-1151)	0.013	899 (735-1167)	779 (673-977)	0.004	1046 (843-1293)	915 (752-1197)	0.000

Table 4.43 Thrombus area and Multiplate® indices - good responders

*p<0.05

Good responders to clopidogrel	Age < 75 + T2DM (n = 9)	Age < 75 + Non T2DM (n = 22)	Age ≥ 75 + T2DM (n = 17)	Age ≥ 75 + Non T2DM (n = 29)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Median (IQR)					
High shear thrombus area, μ^2/mm	13910 (8699-15594)	133323 (11210-18031)	15727 (9033-20021)	17119 (11616-20464)	0.166
Low shear thrombus area, μ^2/mm	8922 (7548-9658)	9524 (7526-11809)	8826 (7104-11387)	9018 (7960-11086)	0.625
ASP AU	356 (244-473)	311 (233-553)	236 (173-424)	285 (229-352)	0.361
ADP AU	598 (417-692)	776 (630-931)	670 (527-799)	647 (575-797)	0.630
Collagen AU	472 (246-647)	679 (554-728)	577 (408-713)	569 (487-724)	0.145
TRAP AU	856 (486-1080)	1085 (958-1248)	899 (735-1167)	1046 (843-1293)	0.061
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Median (IQR)					
High shear thrombus area, μ^2/mm	10489 (7884-12106)	10942 (9373-13024)	11639 (8885-15453)	13007 (8997-16029)	0.281
Low shear thrombus area, μ^2/mm	6600 (6341-7649)	7438 (5782-8992)	7523 (7127-9320)	8341 (7243-9747)	0.120
ASP AU	241 (204-292)	175 (140-226)	151 (107-243)	191 (116-244)	0.189
ADP AU	307 (187-378)	269 (217-388)	302 (250-384)	314 (237-383)	0.784
Collagen AU	488 (362-581)	510 (416-583)	426 (315-581)	424 (355-535)	0.652
TRAP AU	931 (819-1024)	975 (816-1151)	779 (673-977)	915 (752-1197)	0.116

Table 4.44 Thrombus area and Multiplate® indices - good responders; Post hoc analysis (One way ANOVA); *p<0.05

Good responders to clopidogrel	Age < 75 + T2DM (n = 9)	Age < 75 + Non T2DM (n = 22)	Age ≥ 75 + T2DM (n = 17)	Age ≥ 75 + Non T2DM (n = 29)	P Value
Difference in variables between Visit 1 and Visit 2 Median (IQR)					
Delta ASP AU	120 (-45-218)	135 (52-238)	63 (15-222)	91 (14-157)	0.677
Delta ADP AU	227 (134-491)	512 (338-640)	331 (211-474)	365 (263-509)	0.486
Delta Collagen AU	-68 (-183-73)	127 (48-224)	70(-60-247)	118 (21-231)	0.175
Delta TRAP AU	16 (-333-140)	93 (3-228)	98 (-30-281)	127 (10-223)	0.019

Table 4.45 Comparison of change in Multiplate® indices - good responders

Post hoc analysis (One way ANOVA); *p<0.05

Hypo responders to clopidogrel	Age < 75 + T2DM (n = 6)			Age < 75 + Non T2DM (n = 22)			Age ≥ 75 + T2DM (n = 20)			Age ≥ 75 + Non T2DM (n = 16)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	17451 (14577-18868)	12295 (10272-16639)	0.045	16723 (11295-20299)	12953 (11031-18355)	0.021	16411 (10454-19644)	14069 (8705-15999)	0.002	20387 (9947-27810)	17565 (10728-21318)	0.064
Low shear thrombus area, μ^2/mm	10246 (7854-13165)	7654 (7370-8250)	0.158	9335 (8543-10521)	8797 (6729-9795)	0.009	10000 (8315-11816)	8493 (7184-10467)	0.004	11090 (8555-13287)	10180 (8011-12247)	0.269
ASP AU	354 (203-554)	191 (159-392)	0.327	412 (301-515)	233 (176-351)	0.017	380 (271-564)	259 (207-323)	0.145	356 (263-664)	256 (146-485)	0.160
ADP AU	905 (774-1171)	706 (512-867)	0.005	861 (774-997)	621 (550-812)	0.000	922 (729-1129)	628 (522-752)	0.000	1012 (747-1117)	670 (525-894)	0.015
Collagen AU	643 (517-687)	574 (339-640)	0.334	729 (608-900)	656 (549-808)	0.120	817 (615-952)	698 (599-891)	0.439	831 (595-1003)	694 (622-761)	0.114
TRAP AU	1093 (897-1597)	1169 (1028-1348)	0.999	1183 (1059-1261)	1234 (1004-1311)	0.431	1376 (1135-1444)	1218 (1037-1370)	0.307	1246 (1133-1319)	1231 (1025-1462)	0.770

Table 4.46 Thrombus area and Multiplate® indices - hyporesponders

*p<0.05

Hypo responders to clopidogrel	Age < 75 + T2DM (n = 9)	Age < 75 + Non T2DM (n = 22)	Age ≥ 75 + T2DM (n = 20)	Age ≥ 75 + Non T2DM (n = 16)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Median (IQR)					
High shear thrombus area, μ^2/mm	17451 (14577-18868)	16723 (11295-20299)	16411 (10454-19644)	20387 (9947-27810)	0.708
Low shear thrombus area, μ^2/mm	10246 (7854-13165)	9335 (8543-10521)	10000 (8315-11816)	11090 (8555-13287)	0.576
ASP AU	354 (203-554)	412 (301-515)	380 (271-564)	356 (263-664)	0.913
ADP AU	905 (774-1171)	861 (774-997)	922 (729-1129)	1012 (747-1117)	0.936
Collagen AU	643 (517-687)	729 (608-900)	817 (615-952)	831 (595-1003)	0.454
TRAP AU	1093 (897-1597)	1183 (1059-1261)	1376 (1135-1444)	1246 (1133-1319)	0.321
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Median (IQR)					
High shear thrombus area, μ^2/mm	12295 (10272-16639)	12953 (11031-18355)	14069 (8705-15999)	17565 (10728-21318)	0.345
Low shear thrombus area, μ^2/mm	7654 (7370-8250)	8797 (6729-9795)	8493 (7184-10467)	10180 (8011-12247)	0.042
ASP AU	191 (159-392)	233 (176-351)	259 (207-323)	256 (146-485)	0.903
ADP AU	706 (512-867)	621 (550-812)	628 (522-752)	670 (525-894)	0.873
Collagen AU	574 (339-640)	656 (549-808)	698 (599-891)	694 (622-761)	0.135
TRAP AU	1169 (1028-1348)	1234 (1004-1311)	1218 (1037-1370)	1231 (1025-1462)	0.548

Table 4.47 Thrombus area and Multiplate® indices – hyporesponders; Post hoc analysis (One way ANOVA); *p<0.05

Hypo responders to clopidogrel	Age < 75 + T2DM (n = 9)	Age < 75 + Non T2DM (n = 22)	Age ≥ 75 + T2DM (n = 20)	Age ≥ 75 + Non T2DM (n = 16)	P Value
Difference in variables between Visit 1 and Visit 2 Median (IQR)					
Delta ASP AU	-4 (-69-337)	122 (42-212)	92 (2-277)	169 (-44-262)	0.901
Delta ADP AU	311 (182-412)	193 (104-372)	230 (122-409)	213 (39-441)	0.860
Delta Collagen AU	26 (-41-335)	79 (-55-202)	24 (-85-98)	141 (-22-341)	0.779
Delta TRAP AU	103 (-281-248)	27 (-88-135)	34 (-134-276)	20 (-125-110)	0.876

Table 4.48 Comparison of change in thrombus area and Multiplate® indices - hyporesponders

Post hoc analysis (One way ANOVA)

*p<0.05

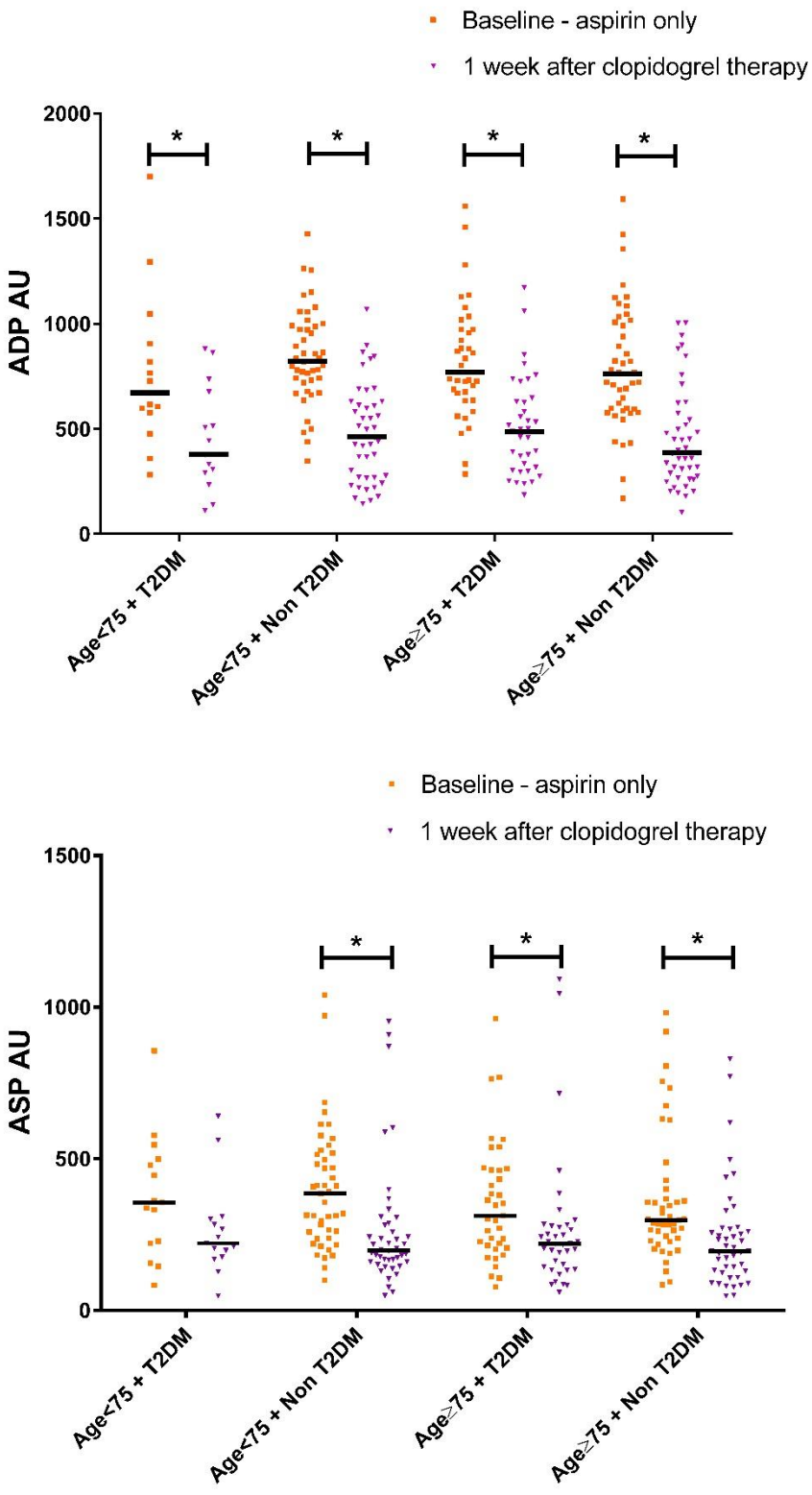


Figure 4.14 Changes in Multiplate® indices

*p<0.05

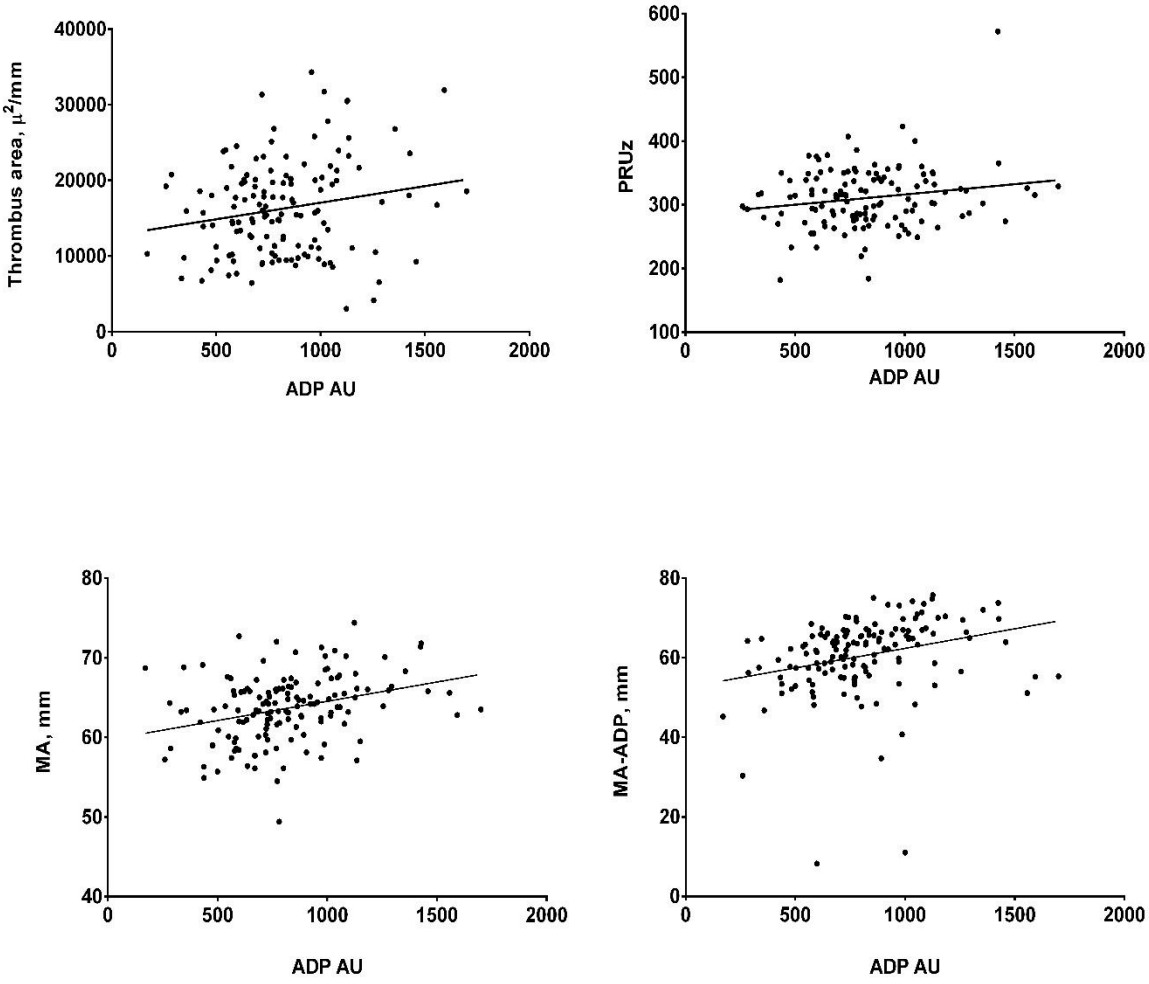


Figure 4.15 Correlation: ADP AU with thrombus area, PRUz, MA and MA-ADP (baseline)

ADP AU positively correlated with the following parameters

i. Thrombus area (rho= 0.191, p=0.025).
ii. PRUz (rho= 0.183, p=0.040)
iii. MA (rho= 0.316, p<0.001)
iv. MA-ADP (rho=0.274, p=0.001)

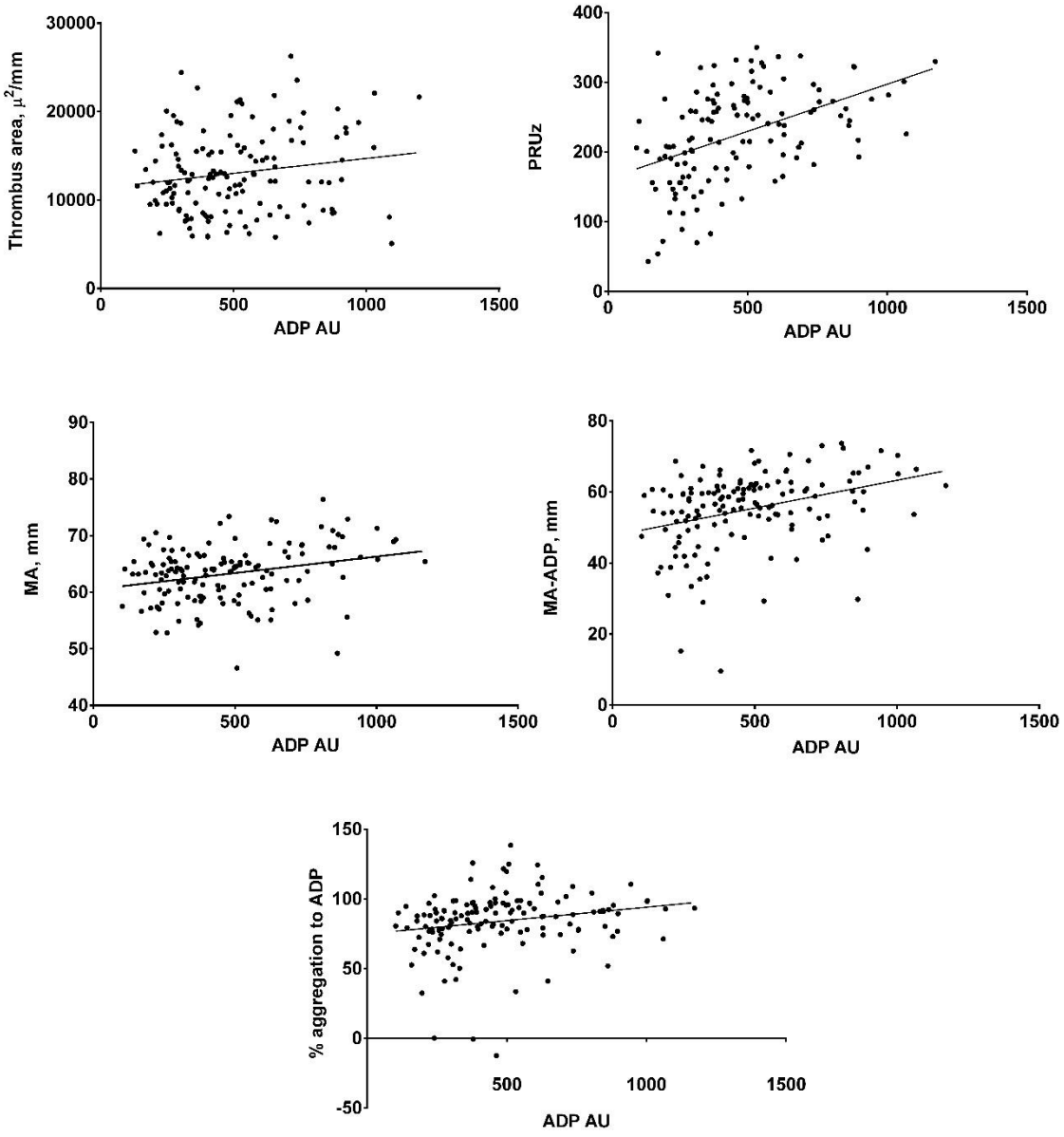


Figure 4.16 Correlation: ADP AU with thrombus, PRUz, MA & MA-ADP (after clopidogrel)

ADP AU positively correlated with the following parameters

i. Thrombus area (rho= 0.170, p=0.046)
ii. PRUz (rho= 0.438, p<0.001)
iii. MA (rho= 0.269, p=0.001)
iv. MA-ADP (rho=0.336, p<0.001)
v. Percentage aggregation to ADP (rho=0.204, p=0.016)

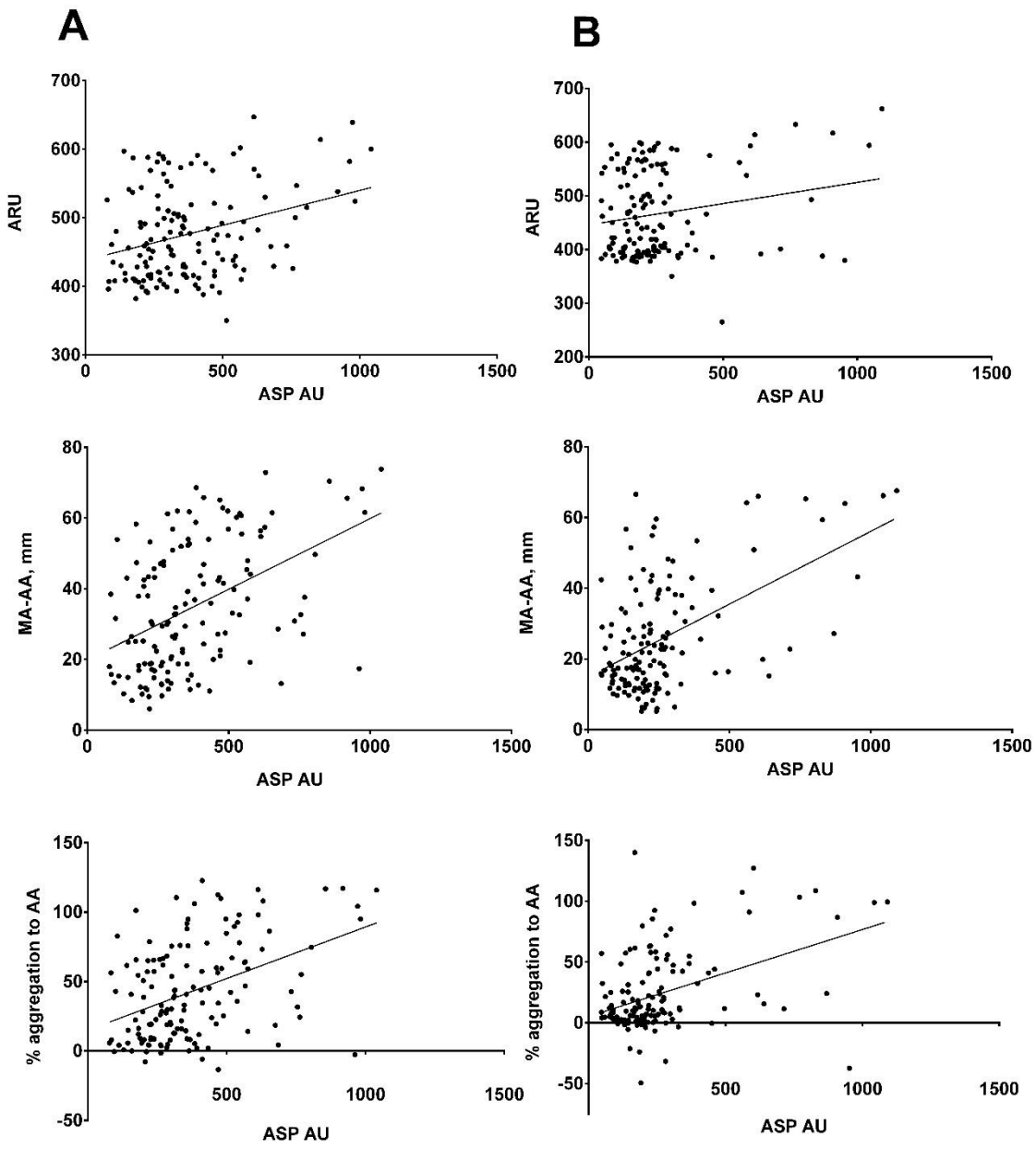


Figure 4.17 Correlation: ASP AU with ARU, MA and MA-AA

At baseline ASP AU positively correlated with the following parameters: ARU (rho= 0.183, p=0.040); MA-AA (rho= 0.452, p<0.001); % aggregation to AA (rho=0.425, p<0.001) (figures in column A). After a week of clopidogrel therapy positive correlation persisted with the following parameters: ARU (rho= 0.193, p=0.022); MA-AA (rho= 0.508, p<0.001); % aggregation to AA (rho=0.425, p<0.001) (figures in column B).

Age<75 years vs. Age≥75 years

I divided the study population as young (age<75 years) and elderly (age≥75 years) to assess the effect of age on clopidogrel response and platelet dependent thrombus formation.

Demographic data between these two groups are shown in Table 4.49. The elderly group had a significantly higher number of participants with a history of hypertension, PVD, DM, previous CABG and CKD. Mean systolic BP was higher in the elderly population. Laboratory data demonstrated significantly higher serum creatinine, hsCRP and HbA1c levels and lower haemoglobin level in the elderly group. Significantly higher number of patients in the elderly group were prescribed insulin (Table 4.49).

Young patients (age<75 years) had 31 good responders to clopidogrel (52.5%) compared to 46 good responders (56.1%) in the elderly group (age≥75 years). The absolute mean ADP AU showed a significant reduction in both the groups after one week of clopidogrel therapy (mean, IQR, age<75: 801, 669-987 to 443, 269-630, p<0.001; age≥75: 765, 598-995 to 428, 298-624, p<0.001). Mean difference in PRUz (Mean, IQR) from visit 1 and visit 2 were similar between the groups; Age≥75: 333, 152-506 and in Age<75: 325, 208-446; p=0.532 (Table 4.50, Table 4.51).

Despite being on long term aspirin therapy, there was a significant reduction in ASP AU from visit 1 to visit 2 in the younger population (mean, IQR: 359, 237-510 to 205, 166-288; p<0.001) demonstrating improved platelet inhibition to aspirin after the addition of clopidogrel as a second antiplatelet agent. There was a similar reduction in ASP AU in the elderly cohort as well from visit 1 and visit 2 (mean, IQR: 302, 225-440 to 212, 133-274; p<0.001). The percentage of good responders to aspirin increased after addition of clopidogrel in both the groups (young: 55% to 88.1% and elderly: 70.7% to 88%).

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean ADP AU < 460). There was a consistent reduction both in high shear (age<75: 13642, 10984-16405 vs. 10942, 8933-12455, p<0.001; age≥75: 16508, 10134-20021 vs. 12878, 9042-15679, p<0.001) and low shear thrombus area following one week of clopidogrel therapy. Mean ADP AU was numerically lower in the elderly group at baseline (age<75 vs. age≥75: 741,

535-835 vs. 659, 562-790; p=0.554) but was numerically higher one week after clopidogrel therapy (277, 220-378 vs. 311, 248-382, p=0.369), but this difference was not statistically significant. Difference in ADP AU from baseline to one week after clopidogrel (delta ADP AU) was low in the elderly cohort but was not significant (mean, IQR: 468, 222-571 vs. 339, 238-491, p=0.371). Mean ADP AU were much lower than the cut off of 460 in both the groups. There was a significant reduction in ASP AU from baseline to one week after clopidogrel therapy both in young (332, 237-499 vs. 196, 151-258, p<0.001) and elderly cohort (283, 203-364 vs. 176, 116-243, p<0.001) (Table 4.52, Table 4.53).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean ADP AU ≥ 460) were compared, both younger (age<75) and elderly (age≥75) patients consistently demonstrated significant reduction in both high shear (age<75: 17300, 12178-20093 vs. 12584, 11169-17002, p=0.002; age≥75: 16813, 9947-22867 vs. 14532, 9241-18753, p<0.001) and low shear thrombus area. The younger cohort of patients demonstrated a numerically lower mean ADP AU when compared to the elderly (863, 777-1016 vs. 957, 729-1124, p=0.682) but one week after clopidogrel therapy mean ADP AU were similar (631, 549-826 vs. 628, 522-837, p=0.696). The difference in ADP AU from baseline to one week after clopidogrel (delta ADP AU) was numerically lower in the elderly group but this difference was not statistically significant (mean, IQR: 221, 122-392 vs. 230, 100-409, p=0.935). Despite demonstrating significant reduction, the mean ADP AU remained ≥ 460 in both the groups indicating high platelet reactivity. There was a significant reduction in ASP AU from baseline to one week after clopidogrel therapy in both young (mean, IQR: 412, 261-518 to 219, 169-333, p=0.008 and elderly cohort (361, 265-567 to 256, 194-447, p=0.039) (Table 4.54, Table 4.55).

	Age < 75 (n = 60)	Age ≥ 75 (n = 83)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	63±7.3	79±3.4	
Male gender, % (n)	73.3 (44)	75.9 (63)	0.727
Body mass index, kg/m^2	30.5±4.1	28.4±4.4	0.004 *
Waist to hip ratio	1.0±0.1	0.9±0.1	0.121
Heart rate, beats per minute	64±11.9	66±12.2	0.546
Systolic BP, mmHg	137±18.0	155±22.6	0.000 *
Diastolic BP, mmHg	77±9.3	75±9.5	0.348
Risk profile: % (n)			
Angina	78.3 (47)	69.9 (58)	0.259
Previous MI	46.7 (28)	59.0 (49)	0.143
Previous PCI	35.0 (21)	32.5 (27)	0.758
Previous CABG	15 (9)	33.7 (28)	0.012 *
Diabetes Mellitus	25 (15)	38 (45.8)	0.011 *
Hypertension	46.7 (28)	69.9 (58)	0.005 *
Heart failure	1.7 (1)	10.8 (9)	0.034
CKD	0 (0)	7.2 (6)	0.033 *
PVD	6.7 (4)	19.3 (16)	0.032 *
CVA	6.7 (4)	10.8 (9)	0.391
Medications: % (n)			
Sulphonylurea	6.7 (4)	9.6 (8)	0.527
Metformin	15 (9)	20.5 (17)	0.402
Insulin	10 (6)	25.3 (21)	0.021 *

Beta-blocker	80 (48)	68.7 (57)	0.258
ACE inhibitor / ARB	75 (45)	86.7 (72)	0.072
Statin	95 (57)	94 (78)	0.793
Laboratory data: Mean ± SD			
Haemoglobin, g/dl	13.6±1.1	12.7±1.2	0.000 *
Platelets x 1000 cells/mm^3	234±59.9	222±59.8	0.218
Fibrinogen, g/ml	3.2±0.6	3.3±0.6	0.309
HbA1c, mmol/mol	43.4±12.0	50.7±14.8	0.002 *
Fasting plasma glucose, mmol/L	5.9±2.1	6.6±3.4	0.121
Creatinine, micromol/L	84±23.3	105±42.6	0.000 *
Total cholesterol, mmol/L	4.0±1.0	4.0±0.9	0.860
LDLc, mmol/L	2.1±0.9	2.1±0.8	0.644
HDLc, mmol/L	1.3±0.3	1.3±0.3	0.285
Triglyceride, mmol/L	1.5±0.8	1.4±0.7	0.662
HsCRP, mg/L	2.0±1.9	4.2±7.2	0.009 *

Table 4.49 Baseline characteristics – Multiplate® sub study (Age<75 vs. Age≥75)

*p<0.05

	Age < 75 (n = 60)			Age ≥ 75 (n = 83)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14796 (11298-19461)	120101 (9503-15110)	0.000	16545 (10134-20547)	13069 (9312-16759)	0.000
Low shear thrombus area, μ^2/mm	9302 (7830-11291)	7680 (6461-8912)	0.000	9585 (8290-11571)	8513 (7407-9881)	0.000
ASP AU	359 (237-510)	205 (166-288)	0.000	302 (225-440)	212 (133-274)	0.000
ADP AU	801 (669-987)	443 (269-630)	0.000	765 (598-995)	428 (298-624)	0.000
Collagen AU	685 (542-787)	559 (440-679)	0.001	677 (497-834)	539 (395-715)	0.000
TRAP AU	1126 (983-1246)	1060 (921-1237)	0.179	1135 (904-1365)	1027 (824-1257)	0.000

Table 4.50 Thrombus area and Multiplate® indices (Age<75 vs. Age≥75)

*p<0.05

	Age < 75 (n = 60)	Age ≥ 75 (n = 83)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Median (IQR)			
High shear thrombus area, μ^2/mm	14796 (11298-19461)	16545 (10134-20547)	0.178
Low shear thrombus area, μ^2/mm	9302 (7830-11291)	9585 (8290-11571)	0.764
ASP AU	359 (237-510)	302 (225-440)	0.332
ADP AU	801 (669-987)	765 (598-995)	0.653
Collagen AU	685 (542-787)	677 (497-834)	0.786
TRAP AU	1126 (983-1246)	1135 (904-1365)	0.418
Aspirin good responders, % (n)	55 (33)	70.7 (58)	0.040
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Median (IQR)			
High shear thrombus area, μ^2/mm	120101 (9503-15110)	13069 (9312-16759)	0.273
Low shear thrombus area, μ^2/mm	7680 (6461-8912)	8513 (7407-9881)	0.016
ASP AU	205 (166-288)	212 (133-274)	0.695
ADP AU	443 (269-630)	428 (298-624)	0.897
Collagen AU	559 (440-679)	539 (395-715)	0.933
TRAP AU	1060 (921-1237)	1027 (824-1257)	0.571
Aspirin good responders, % (n)	88.1 (52)	88 (73)	0.594
Clopidogrel Good responders, % (n)	52.5 (31)	56.1 (46)	0.406
Difference in variables between Visit 1 and Visit 2 Median (IQR)			
Delta ASP AU	120 (29-236)	90 (10-219)	0.568
Delta ADP AU	333 (152-506)	325 (208-446)	0.532
Delta Collagen AU	76 (-35-196)	90 (-55-222)	0.893
Delta TRAP AU	79 (-37-173)	93 (-28-252)	0.070

Table 4.51 Comparison of thrombus area and Multiplate® indices (Age<75 vs. Age≥75)

Good responders to clopidogrel	Age < 75 (n = 31)			Age ≥ 75 (n = 46)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	13642 (10984-16405)	10942 (8933-12455)	0.000	16508 (10134-20021)	12878 (9042-15679)	0.000
Low shear thrombus area, μ^2/mm	9152 (7526-11062)	7410 (5965-8504)	0.000	9018 (7585-11204)	7841 (7234-9621)	0.001
ASP AU	332 (237-499)	196 (151-258)	0.000	283 (203-364)	176 (116-243)	0.000
ADP AU	741 (535-835)	277 (220-378)	0.000	659 (562-790)	311 (248-382)	0.000
Collagen AU	575 (472-723)	490 (421-574)	0.009	570 (440-715)	425 (338-534)	0.000
TRAP AU	1037 (856-1203)	968 (819-968)	0.233	998 (789-1259)	861 (712-1116)	0.000

Table 4.52 Thrombus area and Multiplate® indices - good responders (Age<75 vs. Age≥75)

*p<0.05

Good responders to clopidogrel	Age < 75 (n = 31)	Age ≥ 75 (n = 46)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	13642 (10984-16405)	16508 (10134-20021)	0.118
Low shear thrombus area, μ^2/mm	9152 (7526-11062)	9018 (7585-11204)	0.907
ASP AU	332 (237-499)	283 (203-364)	0.118
ADP AU	741 (535-835)	659 (562-790)	0.554
Collagen AU	575 (472-723)	570 (440-715)	0.636
TRAP AU	1037 (856-1203)	998 (789-1259)	0.833
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	10942 (8933-12455)	12878 (9042-15679)	0.094
Low shear thrombus area, μ^2/mm	7410 (5965-8504)	7841 (7234-9621)	0.071
ASP AU	196 (151-258)	176 (116-243)	0.218
ADP AU	277 (220-378)	311 (248-382)	0.369
Collagen AU	490 (421-574)	425 (338-534)	0.201
TRAP AU	968 (819-968)	861 (712-1116)	0.196
Difference in variables between Visit 1 and Visit 2 Median, IQR			
Delta ASP AU	128 (49-236)	83 (14-186)	0.457
Delta ADP AU	468 (222-571)	339 (238-491)	0.371
Delta Collagen AU	73 (-42-172)	103 (9-231)	0.585
Delta TRAP AU	80 (-34-223)	124 (6-251)	0.118

Table 4.53 Comparison of thrombus area and Multiplate® - good responders (Age<75 vs. Age≥75)

Hypo responders to clopidogrel	Age < 75 (n = 28)			Age ≥ 75 (n = 36)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	17300 (12178-20093)	12584 (11169-17002)	0.002	16813 (9947-22867)	14532 (9241-18753)	0.000
Low shear thrombus area, μ^2/mm	9385 (8248-11283)	8026 (6976-9281)	0.003	10168 (8388-12313)	9281 (7484-10743)	0.003
ASP AU	412 (261-518)	219 (169-333)	0.008	361 (265-567)	256 (194-447)	0.039
ADP AU	863 (777-1016)	631 (549-826)	0.000	957 (729-1124)	628 (522-837)	0.000
Collagen AU	703 (598-850)	619 (540-768)	0.057	819 (615-952)	696 (607-790)	0.082
TRAP AU	1177 (1051-1278)	1186 (1014-1320)	0.579	1299 (1135-1411)	1231 (1025-1373)	0.293

Table 4.54 Thrombus area and Multiplate® indices - hyporesponders (Age<75 vs. Age≥75)

*p<0.05

Hypo responders to clopidogrel	Age < 75 (n = 28)	Age ≥ 75 (n = 36)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	17300 (12178-20093)	16813 (9947-22867)	0.496
Low shear thrombus area, μ^2/mm	9385 (8248-11283)	10168 (8388-12313)	0.336
ASP AU	412 (261-518)	361 (265-567)	0.861
ADP AU	863 (777-1016)	957 (729-1124)	0.682
Collagen AU	703 (598-850)	819 (615-952)	0.207
TRAP AU	1177 (1051-1278)	1299 (1135-1411)	0.065
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	12584 (11169-17002)	14532 (9241-18753)	0.661
Low shear thrombus area, μ^2/mm	8026 (6976-9281)	9281 (7484-10743)	0.058
ASP AU	219 (169-333)	256 (194-447)	0.508
ADP AU	631 (549-826)	628 (522-837)	0.696
Collagen AU	619 (540-768)	696 (607-790)	0.190
TRAP AU	1186 (1014-1320)	1231 (1025-1373)	0.145
Difference in variables between Visit 1 and Visit 2 Median, IQR			
Delta ASP AU	116 (23-216)	151 (-15-277)	0.517
Delta ADP AU	221 (122-392)	230 (100-409)	0.935
Delta Collagen AU	59 (-40-197)	38 (-71-194)	0.748
Delta TRAP AU	68 (-108-140)	20 (-122-240)	0.664

Table 4.55 Multiplate® indices - hyporesponders (Age<75 vs. Age≥75)

T2DM vs. Non DM

I divided the study population as patients with T2DM and non DM to assess the effect of T2DM on clopidogrel response and platelet dependent thrombus formation.

Demographic data between these two groups are shown in Table 4.56. Patients with T2DM had significantly higher number of participants with history of PVD, previous CABG and CKD. Heart rate and waist-hip ratio were higher in T2DM cohort. Laboratory data demonstrated significantly lower platelet count, total cholesterol and HDLc level in T2DM group (Table 4.56).

T2DM had 26 good responders to clopidogrel (50%) compared to 51 good responders (57.3%) in the non DM cohort. The absolute mean ADP AU showed a significant reduction in both the groups after one week of clopidogrel therapy (mean, IQR, T2DM: 737, 607-973 to 474, 303-643, p<0.001; non DM: 790, 658-994 to 425, 269-611, p<0.001). Mean difference in ADP AU (Mean, IQR) from visit 1 and visit 2 were similar between the groups; T2DM: 283, 174-433 and in non DM: 342, 193-511; p=0.496.

Despite being on long term aspirin therapy, there was a significant reduction in ASP AU from visit 1 to visit 2 in T2DM (mean, IQR: 342, 216-470 to 220, 157-283; p=0.002) and non DM (314, 238-484 to 196, 144-273, p<0.001) demonstrating improved platelet inhibition to aspirin after the addition of clopidogrel as a second antiplatelet agent. A similar reduction was also seen with collagen AU and TRAP AU in non DM patients but not in patients with T2DM. The percentage of good responders to aspirin increased after addition of clopidogrel in both the groups (T2DM: 63.5% to 88.7% and non DM: 63.7% to 87.6%) (Table 4.57, Table 4.58).

Good responders to clopidogrel:

I compared all the good responders to clopidogrel (mean ADP AU < 460). There was a consistent reduction both in high shear (T2DM: 16788, 13144-18871 vs. 13031, 8793-16262, p<0.001; non DM: 15301, 11210-19623 vs. 12076, 9373-14753, p<0.001) and low shear thrombus area following one week of clopidogrel therapy. Mean ADP AU was numerically lower in the non DM at baseline (T2DM vs. non DM: 914, 739-1109 vs. 732, 577-823; p=0.265) and there was a much greater reduction in mean ADP AU among non DM when compared to T2DM one week after clopidogrel therapy (638, 517-770 vs. 289, 221-380; p=0.683), but this difference was not statistically significant. Difference in ADP AU from baseline to one week after clopidogrel (delta ADP AU) in

T2DM cohort was not significant (mean, IQR: 266, 159-405 vs. 384, 301-551; p=0.212). Mean ADP AU were much lower than the cut off of 460 in both the groups. There was a significant reduction in ASP AU from baseline to one week after clopidogrel therapy both in T2DM (380, 233-555 vs. 239, 199-315, p=0.070) and non DM (287, 230-400 vs. 180, 129-236, p<0.001). There was a significant reduction in Collagen Au and TRAP AU in non DM cohort of good responders but this was not demonstrated in T2DM group (Table 4.59, Table 4.60).

Hyporesponders to clopidogrel:

When hyporesponders to clopidogrel (mean ADP AU ≥ 460) were compared, again both T2DM and non DM patients' consistently demonstrated significant reduction in both high shear (T2DM: 16788, 13144-18871 vs. 13030, 8793-16262, p=0.002; non DM: 17964, 10793-23227 vs. 14794, 11188-19172, p<0.001) and low shear thrombus area. At baseline, mean ADP AU was similar among T2DM and non DM patients (914, 739-1109 vs. 917, 774-1059, p=0.538) and one week after clopidogrel therapy mean ADP AU still remained similar between the groups (638, 517-770 vs. 629, 548-845, p=0.860). Despite demonstrating significant reduction, the mean ADP AU remained ≥ 460 in both the groups indicating high platelet reactivity. There was a reduction in ASP AU from baseline to one week after clopidogrel therapy in both T2DM (mean, IQR: 380, 233-555 to 239, 199-315; p=0.070 and non DM cohort (397, 266-521 to 242, 165-418; p=0.006). The hyporesponders in the non DM group demonstrated a reduction in Collagen AU but not in TRAP AU. T2DM group did not demonstrate any significant reduction in Collagen AU and TRAP AU (Table 4.61, Table 4.62).

	T2DM (n = 53)	Non DM (n = 90)	P Value
Demographic data: Mean ± SD or % (n)			
Age, years	75±8.2	71±9.9	0.019 *
Male gender, % (n)	83 (44)	70 (63)	0.083
Body mass index, kg/m^2	30.2±5.2	28.7±3.7	0.065
Waist to hip ratio	1.0±0.1	0.9±0.1	0.033 *
Heart rate, beats per minute	69±13.5	63±10.5	0.005 *
Systolic BP, mmHg	151±24.4	145±21.3	0.104
Diastolic BP, mmHg	76±10.9	76±8.4	0.945
Risk profile: % (n)			
Angina	81.1 (43)	68.9 (62)	0.109
Previous MI	47.2 (25)	57.8 (52)	0.219
PCI	32.1 (17)	34.4 (31)	0.772
CABG	37.7 (20)	18.9 (17)	0.013 *
Hypertension	62.3 (33)	58.9 (53)	0.691
Heart failure	11.3 (6)	4.4 (4)	0.119
CKD	9.4 (5)	1.1 (1)	0.017 *
PVD	28.3 (15)	14 (20)	0.000 *
CVA	13.2 (7)	6.7 (6)	0.189
Medications: % (n)			
Sulphonylurea	22.6 (12)	0	
Metformin	49.1 (26)	0	
Insulin	49.1 (26)	0	
Beta-blocker	71.7 (38)	74.4 (67)	0.419
ACE inhibitor / ARB	86.8 (46)	78.9 (71)	0.237

Statin	94.3 (50)	94.4 (85)	0.979
Laboratory data: Mean ± SD			
Haemoglobin, g/dl	12.8±1.1	13.2±1.2	0.069
Platelets x 1000 cells/mm^3	212±47.2	235±64.6	0.016 *
Fibrinogen, g/ml	3.4±0.6	3.2±0.6	0.295
HbA1c, mmol/mol	60.6±15.3	40.3±5.5	
Fasting plasma glucose, mmol/L	8.3±4.2	5.2±0.6	
Creatinine, micromol/L	104±48.5	91±27.7	0.083
Total cholesterol, mmol/L	3.8±0.9	4.2±0.9	0.017 *
LDLc, mmol/L	2.0±0.8	2.2±0.8	0.208
HDLc, mmol/L	1.1±0.3	1.4±0.3	0.000 *
Triglyceride, mmol/L	1.5±0.7	1.4±0.7	0.171
HsCRP, mg/L	3.7±5.7	3.1±5.7	0.551

Table 4.56 Baseline characteristics – Multiplate® sub study (T2DM vs. Non-DM)

*p<0.05

	T2DM (n = 53)			Non DM (n = 90)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
ASP AU	342 (216-470)	220 (157-283)	0.002	314 (238-484)	196 (144-273)	0.000
ADP AU	737 (607-973)	474 (303-643)	0.000	790 (658-994)	425 (269-611)	0.000
Collagen AU	662 (442-811)	559 (419-709)	0.047	681 (525-824)	555 (416-693)	0.000
TRAP AU	1107 (799-1390)	1000 (830-1189)	0.068	1147 (988-1308)	1065 (914-1276)	0.000

Table 4.57 Thrombus area and Multiplate® indices (T2DM vs. Non DM)

*p<0.05

	T2DM (n = 53)	Non DM (n = 90)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
ASP AU	342 (216-470)	314 (238-484)	0.551
ADP AU	737 (607-973)	790 (658-994)	0.792
Collagen AU	662 (442-811)	681 (525-824)	0.299
TRAP AU	1107 (799-1390)	1147 (988-1308)	0.335
Aspirin good responders, % (n)	63.5 (33)	63.7 (58)	0.524
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
ASP AU	220 (157-283)	196 (144-273)	0.681
ADP AU	474 (303-643)	425 (269-611)	0.483
Collagen AU	559 (419-709)	555 (416-693)	0.763
TRAP AU	1000 (830-1189)	1065 (914-1276)	0.320
Aspirin good responders, % (n)	88.7 (47)	87.6 (78)	0.540
Clopidogrel Good responders, % (n)	50 (26)	57.3 (51)	0.401
Difference in variables between Visit 1 and Visit 2 Median, IQR			
Delta ASP AU	81 (4-234)	114 (27.5-221)	0.348
Delta ADP AU	283 (174-433)	342 (193-511)	0.496
Delta Collagen AU	32 (-71-114)	118 (2-222)	0.097
Delta TRAP AU	68 (-56-254)	80 (-10-80)	0.898

Table 4.58 Comparison of Multiplate® indices (T2DM vs. Non-DM)

Good responders to clopidogrel	**T2DM (n = 26)**			**Non DM (n = 51)**		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	14549 (9033-18768)	11314 (8462-14236)	0.004	15301 (11210-19623)	12076 (9373-14753)	0.000
Low shear thrombus area, μ^2/mm	8922 (7263-10237)	7505 (6517-8494)	0.002	9140 (7670-11291)	7828 (6921-9546)	0.000
ASP AU	322 (173-450)	204 (129-253)	0.011	287 (230-400)	180 (129-236)	0.000
ADP AU	612 (497-745)	305 (246-377)	0.000	732 (577-823)	289 (221-380)	0.000
Collagen AU	509 (381-712)	445 (318-544)	0.180	575 (498-723)	472 (386-559)	0.000
TRAP AU	878 (725-1123)	854 (692-991)	0.210	1053 (880-1257)	958 (797-1165)	0.000

Table 4.59 Thrombus area and Multiplate® indices - good responders (T2DM vs. Non-DM)

*p<0.05

Good responders to clopidogrel	T2DM (n = 26)	Non DM (n = 51)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	14549 (9033-18768)	15301 (11210-19623)	0.138
Low shear thrombus area, μ^2/mm	8922 (7263-10237)	9140 (7670-11291)	0.166
ASP AU	322 (173-450)	287 (230-400)	0.507
ADP AU	612 (497-745)	732 (577-823)	0.265
Collagen AU	509 (381-712)	575 (498-723)	0.087
TRAP AU	878 (725-1123)	1053 (880-1257)	0.018
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	11314 (8462-14236)	12076 (9373-14753)	0.334
Low shear thrombus area, μ^2/mm	7505 (6517-8494)	7828 (6921-9546)	0.120
ASP AU	204 (129-253)	180 (129-236)	0.491
ADP AU	305 (246-377)	289 (221-380)	0.683
Collagen AU	445 (318-544)	472 (386-559)	0.923
TRAP AU	854 (692-991)	958 (797-1165)	0.033
Difference in variables between Visit 1 and Visit 2 Median, IQR			
Delta ASP AU	79 (17-219)	101 (30-233)	0.347
Delta ADP AU	283 (174-479)	384 (301-551)	0.240
Delta Collagen AU	38 (-87-135)	122 (39-209)	0.076
Delta TRAP AU	78 (-51-256)	111 (11-227)	0.285

Table 4.60 Comparison of Multiplate® indices - good responders (T2DM vs. Non-DM)

Hypo responders to clopidogrel	T2DM (n = 28)			Non DM (n = 38)		
Median (IQR)	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	16788 (13144-18871)	13030 (8793-16262)	0.000	17964 (10793-23227)	14794 (11188-19172)	0.003
Low shear thrombus area, μ^2/mm	10168 (8220-11750)	8183 (7333-9746)	0.001	9638 (8552-12428)	8951 (7539-10796)	0.007
ASP AU	380 (233-555)	239 (199-315)	0.070	397 (266-521)	242 (165-418)	0.006
ADP AU	914 (739-1109)	638 (517-770)	0.000	917 (774-1059)	629 (548-845)	0.000
Collagen AU	767 (602-914)	653 (570-785)	0.214	767 (614-932)	677 (574-771)	0.023
TRAP AU	1295 (1075-1456)	1189 (1050-1365)	0.374	1191 (1105-1315)	1233 (1018-1333)	0.435

Table 4.61 Thrombus area and Multiplate® indices - hyporesponders (T2DM vs. Non-DM)

*p<0.05

Hypo responders to clopidogrel	T2DM (n = 28)	Non DM (n = 36)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD			
High shear thrombus area, μ^2/mm	16788 (13144-18871)	17964 (10793-23227)	0.681
Low shear thrombus area, μ^2/mm	10168 (8220-11750)	9638 (8552-12428)	0.924
ASP AU	380 (233-555)	397 (266-521)	0.701
ADP AU	914 (739-1109)	917 (774-1059)	0.538
Collagen AU	767 (602-914)	767 (614-932)	0.948
TRAP AU	1295 (1075-1456)	1191 (1105-1315)	0.417
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD			
High shear thrombus area, μ^2/mm	13030 (8793-16262)	14794 (11188-19172)	0.148
Low shear thrombus area, μ^2/mm	8183 (7333-9746)	8951 (7539-10796)	0.204
ASP AU	239 (199-315)	242 (165-418)	0.929
ADP AU	638 (517-770)	629 (548-845)	0.860
Collagen AU	653 (570-785)	677 (574-771)	0.952
TRAP AU	1189 (1050-1365)	1233 (1018-1333)	0.405
Difference in variables between Visit 1 and Visit 2 Median, IQR			
Delta ASP AU	85 (-47-278)	131 (6-213)	0.547
Delta ADP AU	266 (159-405)	194 (90-394)	0.406
Delta Collagen AU	25 (-70-110)	80 (-32-222)	0.533
Delta TRAP AU	52 (-172-252)	27 (-108-130)	0.646

Table 4.62 Comparison of Multiplate® indices - hyporesponders (T2DM vs. Non-DM)

Post hoc analysis (One way ANOVA); *p<0.05

4.4.2 Discussion – platelet reactivity indices

I report the findings of Multiplate® parameters from my study. Baseline (on aspirin alone) ADP AU was similar in elderly (age≥75) and young patients (age<75), with or without T2DM. One week of clopidogrel therapy led to a significant reduction in ADP AU in elderly and young, again in patients with and without T2DM. An ADP AU cut off of 460 was used to define hyporesponders to clopidogrel. Despite a significant reduction, mean ADU AU remained greater than 460 in elderly diabetic patients. This is the first study to compare Multiplate® indices with platelet dependent thrombus. Similar to VerifyNow®, comparing Multiplate® derived platelet reactivity indices to ex-vivo whole blood thrombus generation is also novel and will certainly be helpful in further exploring the use of these indices in clinical setting to tailor antiplatelet therapy.

In elderly and young patients, 56.1% and 52.5% were good responders to clopidogrel respectively. Mean ADP AU were similar in both the elderly and the young cohorts. There was a significant reduction in thrombus (measured as total thrombus area) among good responders to clopidogrel both in the elderly and the young cohorts. Interestingly, similar to VerifyNow data there was a significant reduction in thrombus even among hyporesponders to clopidogrel (ADP AU ≥460), in elderly and young patients, with or without T2DM. This again demonstrates that clopidogrel reduces thrombus formation despite high platelet reactivity as defined by Multiplate cut off (ADP AU ≥ 460). These findings are novel and can potentially be explained by

i) additional pleiotropic anti-atherosclerotic roles of clopidogrel such as modulation of vascular tone, improvement of nitric oxide bioavailability as well as inhibition of inflammation and oxidative stress (Ostad *et al.*, 2011)
ii) reconstituted in-vitro thrombus in test cells involves different mechanism when compared to real time thrombus generated from ex-vivo Badimon chamber
iii) factors other than platelets involved in thrombogenesis e.g. fibrin cannot be assessed by Multiplate®

ASP AU cut off of 400 was used to define hyporesponders to aspirin. A significantly higher percentage of elderly patients were good responders to aspirin (70.7%) compared to young patients (55%) at baseline. After one week of clopidogrel this percentage increased to 88% and 88.1% in elderly and young respectively.

In patients with T2DM and non DM, 50% and 57.3% were good responders to clopidogrel. Mean ADP AU among T2DM remained well above the cut off 460 U but was <460 in non-DM cohort. There was a significant reduction in thrombus area among good responders as well as hyporesponders to clopidogrel. Among good responders to clopidogrel, ADP AU in patients with T2DM remained significantly higher compared to patients without diabetes. Percentage of good responders to aspirin increased with addition of clopidogrel to aspirin in T2DM (63.5% to 88.7%) and non DM patients (63.7 to 87.6%).

This is the first study where thrombus area was determined by Badimon chamber, and TEG® parameters and VerifyNow® indices were compared with Multiplate® data. High shear and low shear thrombus area correlated significantly with ADP AU both at baseline and one week after clopidogrel therapy. This possibly is due to the fact that platelets play a significant role in thrombus formation. There was a good correlation in the assessment of clopidogrel response by TEG® and Multiplate® (MA-ADP and ADP AU respectively) after a week of clopidogrel therapy. Assessment of aspirin response by TEG® and Multiplate® (MA-AA and ASP AU) also demonstrate significant correlation. VerifyNow® indices ARU and PRUz positively correlated with ASP AU and ADP AU respectively both at baseline and one week after clopidogrel therapy. These results suggest the usefulness of Multiplate® alongside other point of care tests in the assessment of thrombogenicity and platelet inhibition by various antiplatelet agents.

Overall, data from my study confirm high variability in on-treatment platelet reactivity as measured by ADP AU among elderly patients and in the presence of T2DM. Both elderly and young patients demonstrated significant reduction in ADP AU following clopidogrel therapy. Mean ADP AU value as a measure of platelet reactivity was similar in elderly and young, contrary to what was demonstrated by VerifyNow indices. Mean ADP AU value remained higher than 460 in patients with T2DM when compared to non-diabetics, among both good responders and hyporesponders. Even in hyporesponders, there was a significant reduction in thrombus formation, both in elderly and young patients, with or without TDM. Antiplatelet response for both aspirin and clopidogrel measured using TEG® (MA-AA and MA-ADP) and VerifyNow® (ARU and PRUz) correlated well with the corresponding measures using Multiplate® (ASP AU and ADP AU).

Multiplate has been shown to be useful in monitoring clopidogrel responsiveness in my study, both in younger group and in the elderly, in the presence and absence of T2DM. Multiplate was used to quantify platelet aggregation in a large prospective study of over 1000 patients who underwent successful coronary stenting after 600mg loading dose of clopidogrel. In this study, 11.7% demonstrated low response to clopidogrel. Independent predictors for low response were acute coronary syndrome (OR = 6.54), diabetes mellitus (OR = 2.07) and male gender (OR = 1.83). Age was not found to be a predictor (OR = 1.02, p=0.06) (Behr *et al.*, 2011). In a small study conducted on healthy volunteers, Multiplate® was shown to consistently and reliably detect the antiplatelet effect of aspirin and AA-induced platelet aggregation (Jámbor *et al.*, 2009).

Light transmission aggregometry (LTA) is considered to be the gold standard for assessing the platelet response to agonists such as adenosine diphosphate (ADP) and has been used to assess the drug response to clopidogrel in the initial dose-finding studies. In a study with 149 patients who had 600mg of clopidogrel loading dose prior to coronary stent insertion, Multiplate strongly correlated with LTA (rho = 0.7; p<0.0001) (Sibbing *et al.*, 2010b). Similar correlation was demonstrated in other large scale studies as well (Siller-Matula *et al.*, 2012).

Multiplate was also used to predict the risk of bleeding in patients taking clopidogrel. ADP AU cut off of 188 was found to be the optimal cut off to predict the occurrence of in-hospital major bleeding. The incidence of major bleeding was significantly higher in enhanced clopidogrel responders (very low ADP AU <188) as compared to the remaining patients (2.2% vs. 0.8%; OR 2.6; p=0.005) (Sibbing *et al.*, 2010b).

Karon et al performed a study on 40 healthy volunteers and 10 volunteers on aspirin and/or clopidogrel and assessed arachidonic acid–induced platelet function by LTA, Multiplate®, VerifyNow®, and TEG-PM®. ADP-induced platelet function was also measured using the same methods and flow cytometry. From this study, Multiplate® was the only method to demonstrate an acceptable reliability coefficient among healthy volunteers and those on both aspirin and clopidogrel therapy. TEG-PM® was found to be the least suited point of care test in monitoring the effects of antiplatelet agents (Karon *et al.*, 2014). In a meta-analysis, Multiplate® ADP AU significantly predicted acute MI (OR 4.03, 95% CI [1.16-14.00], P = 0.03) and stent thrombosis (OR 13.89, 95% CI [2.63-73.45], P = 0.002), but only a trend was observed regarding CV death (3.21 [0.86-12.00], P = .08).

In a study conducted by Mueller et al in patients with stable CAD, 57% of patients were hyporesponders to clopidogrel using a cut off of 460 AU. There was also a trend towards improved aspirin induced platelet inhibition after the addition of clopidogrel, which is in keeping with my findings (Mueller *et al.*, 2007). In my study, approximately 45 % of the whole cohort were hyporesponders to clopidogrel, with a higher proportion of good responders in the elderly and the non DM cohort. Awidi et al demonstrated that 26% of patients on aspirin 75 to 100mg daily for secondary prevention were hyporesponders (Awidi *et al.*, 2010). In my study 25-45% were hyporesponders to aspirin at baseline which reduced, falling to 10-15% after the addition of clopidogrel. This improvement could be due to two reasons:

i) enhanced aspirin induced platelet inhibition after the addition of clopidogrel
ii) improved compliance to aspirin along with clopidogrel during study period

Verdoia et al analysed HPR on 195 post-ACS patients who underwent coronary stenting and were on dual antiplatelet therapy with aspirin and ticagrelor. In this study the prevalence of HPR was significantly higher in elderly patients (age≥70 years) when compared to the young (21.5% vs. 5.2% respectively, p=0.002).Though patients with DM had numerically higher prevalence of HPR compared to the non DM cohort this was not statistically significant (14.6% vs. 11.5% respectively, p=0.61). Age ≥ 70 years was found to be an independent predictor of HPR with ticagrelor (OR [95%CI] = 4.6 [1.55–13.8], p=0.006) (Verdoia *et al.*, 2016).

In Evaluation of Clinical Risk Factors to Predict High On-Treatment Platelet Reactivity and Outcome in Patients with Stable Coronary Artery Disease (PREDICT-STABLE) study, 810 ACS patients and 739 stable CAD patients were recruited and platelet reactivity analysed. From this study, age was a significant predictor of HPR (OR 2.11, 95%CI [1.26–3.53], p=0.005) (Droppa *et al.*, 2015). In another small study, Multiplate ADP AU platelet reactivity was measured in 45 elderly (age ≥ 75 years) patients with NSTE-ACS undergoing coronary stenting. Almost half (49%) had low platelet reactivity (< 188 AU) consistent with increased risk of bleeding. Interestingly, platelet reactivity did not correlate with age or with diabetes status (Batty *et al.*, 2015).

In my study, very low platelet reactivity was identified in only a very small percentage of patients in the elderly cohort compared to the young (3.7% vs. 10.2% respectively) and this was not statistically significant (P=0.114). Among patients with T2DM and non

DM, percentage of participants with very low platelet reactivity were similar (5.8% vs. 6.7% respectively, p=0.562).

Over all, it is clear that the Multiplate® assay has demonstrated consistency, good reproducibility, low coefficient of variation and high probability of predicting adverse events, making it a suitable point of care assay to monitor pharmacodynamics of newer antiplatelet / antithrombotic agents in developmental trials.

4.5 Coagulation biomarkers - Elderly stable CAD study

4.5.1 Results

In patients with established CAD, three groups were analysed: age<75+non DM; age≥75+T2DM and age≥75+non DM.

Demographic data and baseline characteristics of the patients remains the same as discussed in the earlier section (Table 4.1). Plasma levels of fibrinogen, P selectin, CD40 ligand and PAI-1 were similar between the groups at baseline. Post chamber effluent blood levels of P selectin, CD40 ligand and PAI-1 were measured and compared with venous blood levels. A significant increase in levels of P selectin, CD40 ligand and PAI-1 was demonstrated in the post chamber effluent blood in all three groups (Table 4.64, Figure 4.18, Figure 4.19).

P selectin (ng/ml): age<75+non DM: 62.7±18.5 vs. 85.6±20.5, p<0.001; age≥75+T2DM: 62.8±19.3 vs. 80.4±23.1, p<0.001; age≥75+non DM: 65.5±19.3 vs. 86.1±24.2, p<0.001.

CD40 ligand (pg/ml): age<75+non DM: 2730±1683 vs. 5506±2044, p<0.001; age≥75+T2DM: 2447±2051 vs. 3818±2445, p<0.001; age≥75+non DM: 2471±2101 vs. 4457±1906, p<0.001.

PAI-1 (ng/ml): age<75+non DM: 24.8±25.3 vs. 42.1±30.0, p<0.001; age≥75+T2DM: 20.2±26.6 vs. 32.4±32.9, p<0.001; age≥75+non DM: 16.1±27.2 vs. 30.1±23.4, p<0.001.

Following one week of clopidogrel therapy, there was a significant reduction in levels of CD40 ligand in non DM group, both young (pg/ml, mean±SD: 2730±1683 vs. 2121±1368; p=0.05) and elderly (2471±2101 vs. 1733±1119; p=0.006). In the elderly T2DM cohort there was a numerical reduction in CD40 ligand but this reduction was not statistically significant (2447±2051 vs. 2075±1644; p=0.195). There was no significant change in the levels of fibrinogen, P selectin, and PAI-1 compared to baseline (Table 4.63, Table 4.65, Table 4.66).

There was a significant increase in post chamber effluent blood levels of P selectin, CD40 ligand and PAI-1 when compared with venous blood levels, similar to that

demonstrated at baseline. This increase was similar with and without clopidogrel treatment.

There were no significant correlations seen between thrombus area and levels of soluble P selectin and PAI-1both from venous blood samples and post chamber effluent blood samples. After one week of clopidogrel therapy, venous CD40 ligand and post chamber CD40 ligand correlated positively with low shear thrombus area (rho=0.190, p=0.018 and rho=0.178, p=0.028). Delta values of P selectin, CD40L and PAI-1 (pre and post chamber levels) showed no correlation to thrombus area.

	Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
Fibrinogen, g/l	3.2±0.6	3.2±0.7	0.814	3.5±0.7	3.5±0.7	0.685	3.3±0.6	3.3±0.8	0.555
Venous P selectin, ng/ml	62.7±18.5	63.7±15.1	0.613	62.8±19.3	59.9±26.6	0.462	65.5±19.3	60.3±21.9	0.038
Post chamber P selectin, ng/ml	85.6±20.5	73.7±24.1	0.000	80.4±23.1	78.6±26.4	0.618	86.1±24.2	85.2±31.0	0.819
Venous CD40 ligand, pg/ml	2730±1683	2121±1368	0.050	2447±2051	2075±1644	0.195	2471±2101	1733±1119	0.006
Post chamber CD40 ligand, Pg/ml	5506±2044	5827±2168	0.326	3818±2445	4071±2488	0.474	4457±1906	4096±1503	0.210
Venous PAI-1, ng/ml	24.8±25.3	25.5±32.8	0.776	20.2±26.6	18.4±23.0	0.422	16.1±27.2	12.1±14.2	0.221
Post chamber PAI-1, ng/ml	42.1±30.0	32.8±24.0	0.014	32.4±32.9	26.2±25.5	0.057	30.1±23.4	29.2±29.7	0.738

Table 4.63 Coagulation biomarkers – Elderly stable CAD study

	Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Venous sample**	**Post chamber sample**	**P Value**	**Venous sample**	**Post chamber sample**	**P Value**	**Venous sample**	**Post chamber sample**	**P Value**
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD									
P selectin, ng/ml	62.7±18.5	85.6±20.5	0.000 *	62.8±19.3	80.4±23.1	0.000 *	65.5±19.3	86.1±24.2	0.000 *
CD40 ligand, pg/ml	2730±1683	5506±2044	0.000 *	2447±2051	3818±2445	0.000 *	2471±2101	4457±1906	0.000 *
PAI-1, ng/ml	24.8±25.3	42.1±30.0	0.000 *	20.2±26.6	32.4±32.9	0.000 *	16.1±27.2	30.1±23.4	0.000 *
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD									
P selectin, ng/ml	63.7±15.1	73.7±24.1	0.002 *	59.9±26.6	78.6±26.4	0.001 *	60.3±21.9	85.2±31.0	0.000 *
CD40 ligand, Pg/ml	2121±1368	5827±2168	0.000 *	2075±1644	4071±2488	0.000 *	1733±1119	4096±1503	0.000 *
PAI-1, ng/ml	25.5±32.8	32.8±24.0	0.024 *	18.4±23.0	26.2±25.5	0.001 *	12.1±14.2	29.2±29.7	0.000 *

Table 4.64 Change in coagulation markers pre and post chamber; *p<0.05

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) **Mean ± SD** **Median (IQR)**				
Fibrinogen, g/l	3.2±0.6	3.5±0.7	3.3±0.6	0.283
Venous P selectin, ng/ml	62.7± 18.5 60.4 (50.9-73.2)	62.8± 19.3 60.1 (48.9-75.6)	65.5± 19.3 68.5 (49.3-77.9)	0.736
Post chamber P selectin, ng/ml	85.6± 20.5 86.1 (73.7-99.6)	80.4± 23.1 78.4 (62.5-98.7)	86.1± 24.2 85.2 (69.7-102.4)	0.439
Venous CD40 ligand, pg/ml	2730± 1683 2151 (1527-3734)	2447± 2051 1697 (1223-2997)	2471± 2101 1762 (1122-2929)	0.753
Post chamber CD40 ligand, pg/ml	5506± 2044 5296 (4135-6803) *ł	3818± 2445 3289 (2021-5216) *	4457± 1906 4288 (2839-5558) ł	0.001 *
Venous PAI-1, ng/ml	24.8± 25.3 16.6 (6.04-35.9)	20.2± 26.6 9.5 (3.6-25.9)	16.1± 27.2 8.5 (1.9-18.6)	0.298
Post chamber PAI-1, ng/ml	42.1± 30.0 40.0 (18.8-52.1)	32.9± 32.6 24.8 (12.9-38.7)	30.1± 23.4 23.7 (11.3-49.7)	0.123

Table 4.65 Comparison of coagulation biomarkers at baseline

*ł p<0.05

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) **Mean ± SD** **Median (IQR)**				
Fibrinogen, g/l	3.2±0.7	3.5±0.7	3.3±0.8	0.193
Venous P selectin, ng/ml	63.7± 15.1 65.8 (52.0-71.2)	59.9± 26.6 54.0 (39.6-74.7)	60.3± 21.9 57.2 (44.7-68.9)	0.666
Post chamber P selectin, ng/ml	73.7± 24.1 71.1 (59.3-82.0)	78.6± 26.4 80.6 (59.3-93.8)	85.2± 31.0 80.0 (65.2-100.6)	0.138
Venous CD40 ligand, pg/ml	2121± 1368 1595 (1337-2204)	2075± 1644 1410 (946-2737)	1733± 1119 1251 (1050-2341)	0.355
Post chamber CD40 ligand, pg/ml	5827± 2168 5791 (4072-7265) *ł	4071± 2488 3466 (2080-5611) *	4096± 1503 3816 (2860-5093) ł	0.001
Venous PAI-1, ng/ml	25.5± 32.8 15.3 (3.8-35.0) *	18.4± 23.0 10.6 (3.3-24.4)	12.1± 14.2 5.3 (2.2-17.6) *	0.037
Post chamber PAI-1, ng/ml	32.8± 24.0 27.2 (12.2-44.4)	26.2± 25.5 16.8 (6.3-35.6)	29.2± 29.7 20.3 (6.2-37.9)	0.519

Table 4.66 Comparison of coagulation biomarkers after clopidogrel

*ł $p<0.05$

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Difference in variables between Visit 1 and Visit 2 **Mean ± SD** **Median (IQR)**				
Delta Venous P selectin, ng/ml	-0.98±12.85 -4.04 (-8.09-6.67)	2.83±24.39 8.92 (-2.49-16.64)	5.16±16.18 7.4 (-2.23-16.68)	0.277
Delta Post chamber P selectin, ng/ml	11.85±16.46 13.48 (5.51-22.55) *	1.73±22.09 -0.74 (-9.43-15.68)	0.86±25.05 1.92 (-15.37-16.42) *	0.030 *
Delta Venous CD40 ligand, pg/ml	608.41±2025.52 326.43(-107.49-1766.44)	372.33±1809.95 187.97 (-266.62-938.27)	738.47±1702.45 336.58 (-388.51-1183.35)	0.653
Delta Post chamber CD40 ligand, pg/ml	-320.98±2166.57 -206.71 (-1469.91-757.19)	-253.41±2243.12 -202.66 (-1833.92-986.33)	360.88±1859.45 142.54 (-822.35-1440)	0.253
Delta Venous PAI-1, ng/ml	-0.75±17.62 -0.04 (-6.57-7.28)	1.80±14.23 0.02 (-3.03-6.35)	4.02±21.73 0.13 (-1.12-2.63)	0.465
Delta Post chamber PAI-1, ng/ml	9.27±24.29 7.39 (-6.22-28.79)	6.21±19.77 2.86 (-3.54-18.46)	1.52±30.18 3.78 (-5.98-12.54)	0.350

Table 4.67 Change in coagulation biomarkers (delta)

* $p<0.05$

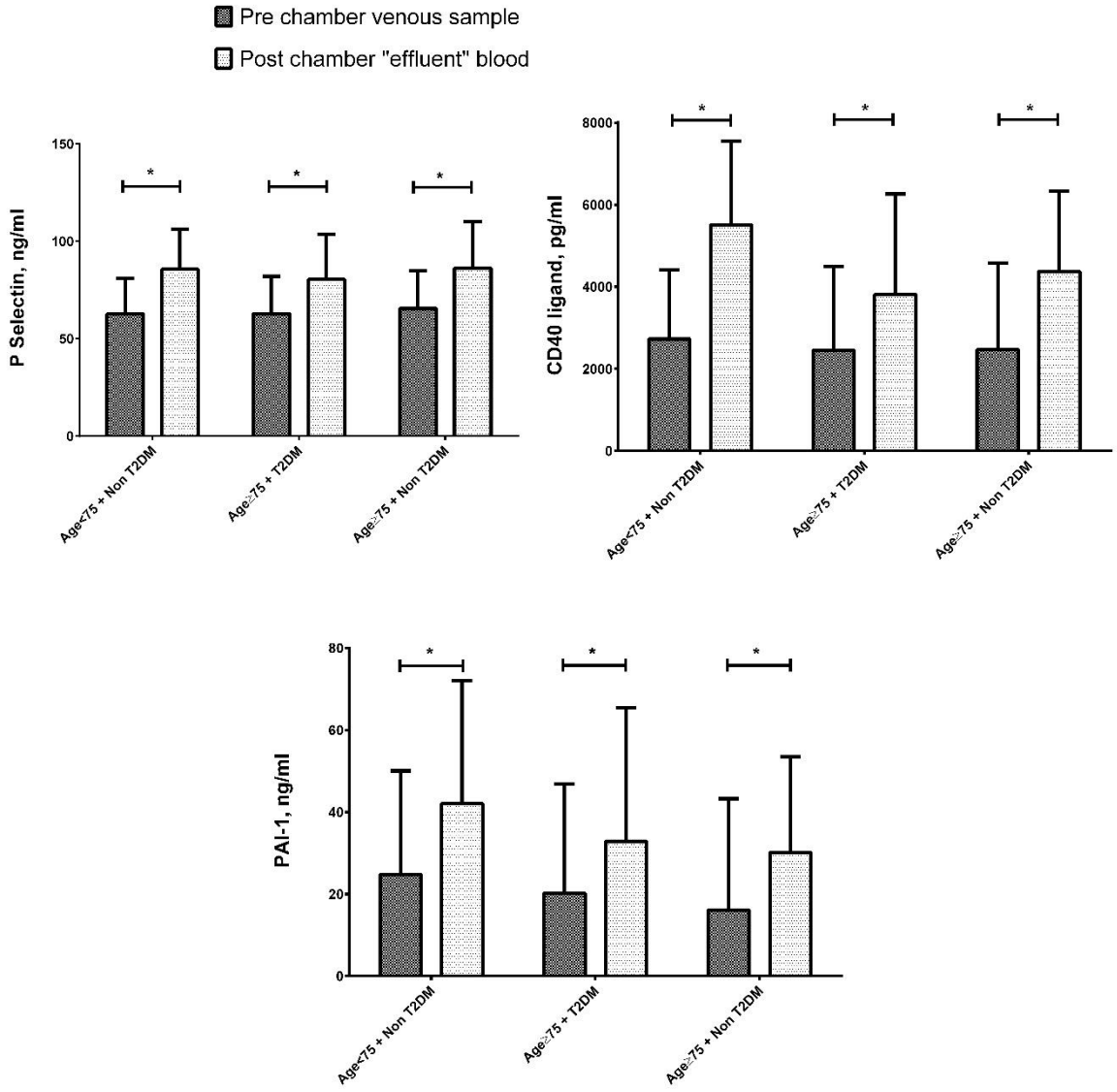


Figure 4.18 P Selectin, CD40 ligand & PAI-1 levels – pre- vs. post-chamber at baseline

*p<0.05

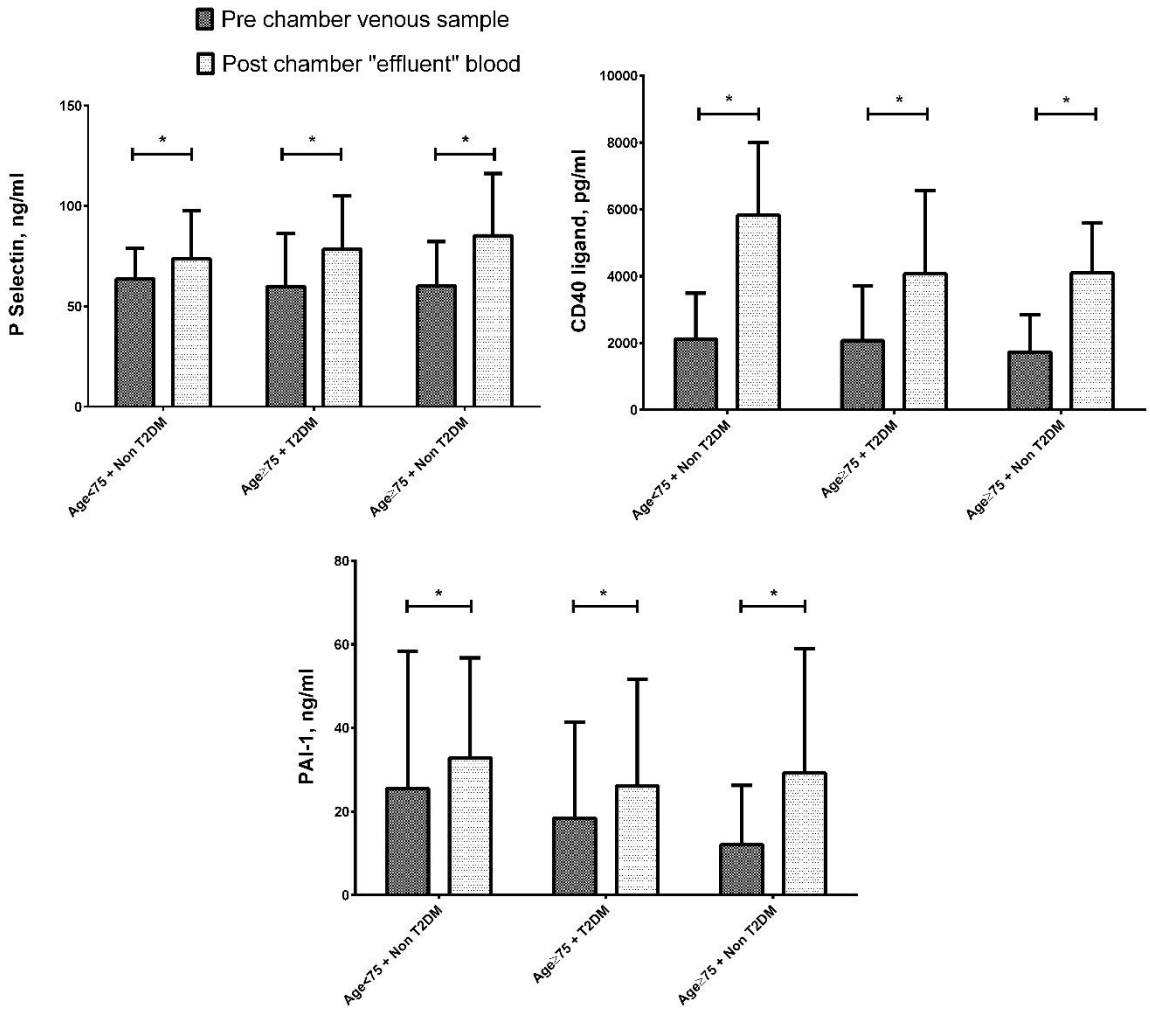


Figure 4.19 P Selectin, CD40 ligand & PAI-1 levels – pre- vs. post-chamber after clopidogrel

*p<0.05

4.5.2 Discussion

To explore the mechanisms involved in platelet dependent thrombus formation in elderly individuals and also to determine the effects of antiplatelet therapy on other markers of thrombus formation, I measured the levels of coagulation markers namely fibrinogen, P selectin and CD40. The levels of these biomarkers were similar between the young and the elderly cohort and also between patients with T2DM and without DM in the elderly cohort. One week of clopidogrel therapy did not affect the levels of fibrinogen, P selectin or PAI-1 but CD40 levels demonstrated a significant reduction in the non-diabetic cohort, both the elderly and the young.

P selectin, earlier known as α-granule membrane protein-140 (GMP-140) is now a well-studied biomarker of atherothrombosis, especially in patients with established coronary artery disease or T2DM. One of the first studies performed by Wu et al demonstrated significantly raised levels of GMP-140 in patients after acute MI or cerebral thrombosis (Wu *et al.*, 1993). Following this study, numerous studies were conducted in various subsets of patients and soluble P selectin levels were found to be raised in patients with unstable angina (Ikeda *et al.*, 1995; Aoki and Tomoda, 1998), acute coronary syndrome (Ikeda *et al.*, 1994), stable CAD and peripheral arterial disease (Blann *et al.*, 1995). Blann et al also demonstrated that increased soluble P-selectin is also a marker of disease progression in patients who have survived a myocardial infarction (Blann *et al.*, 1997). Levels of soluble P selectin were higher in patients with stable CAD compared to healthy volunteers (Furman *et al.*, 1998).

P-selectin is stored in the α-granules of platelets and the Weibel Palade bodies of endothelial cells and is rapidly translocated to the cell surface upon activation by a variety of mediators including thrombin and histamine (Bevilacqua and Nelson; Kansas, 1996). A number of studies have confirmed higher plasma levels of P selectin in patients with T2DM and hyperglycemia is an independent predictor of P selectin in patients with T2DM (Yngen *et al.*, 2001). A significant reduction in P selectin following improved glycemic control has suggested a direct impact of hyperglycemia on platelet activation (Santilli *et al.*, 2010). In a study of 667 patients with symptomatic CAD who underwent coronary intervention, Stellos et al reported high levels of platelet derived P selectin in ACS patients especially in those with STEMI. P selectin levels correlated with myocardial necrosis markers troponin I and creatinine kinase (CK-MB) (Stellos *et al.*, 2010). New recombinant monoclonal antibodies against P-selectin such as

Inclacumab are being tested in clinical trials and have shown promising results by reducing myocardial damage after PCI in patients with non–ST-segment elevation myocardial infarction (Tardif *et al.*, 2013).

There are very few published studies in the literature regarding the effect of increasing age on P selectin levels. The few studies that do exist provide conflicting data. Although the underlying mechanism of ageing is poorly understood, the oxidative stress hypothesis, an imbalance due to increased oxidative stress and a weakened anti-oxidative defense system, is the most accepted theory behind the ageing process. Endothelium tops the list of tissues vulnerable to stress and hence it was proposed that endothelial derived soluble adhesion molecules (SAMs) levels increase as a result of oxidative stress due to ageing. Zou and colleagues demonstrated a 2.45-fold higher level of P selectin in older rats (24 months of age; equivalent to human age of 70 – 75 years) compared to the younger ones (6 months of age) confirming age related alterations to sAMs as a result of endothelial activation / dysfunction (Zou *et al.*, 2004). On the contrary, Blann et al could only demonstrate a significant correlation between vascular cell adhesion molecule-1 (VCAM-1) and ageing. P selectin levels did not correlate with ageing (Blann *et al.*, 1996). In another study in patients with atrial fibrillation, plasma von Willebrand factor (vWF, a marker of endothelial damage / dysfunction) levels were associated with four recognized risk factors for thrombotic events which included advancing age, prior stroke, previous heart failure and DM, but soluble P selectin (a marker of platelet activation) was associated only with DM and not advancing age (Conway *et al.*, 2002).

The role of CD40 ligand (CD40L) in atherosclerosis and coronary artery disease has been well established (Tousoulis *et al.*, 2010). It has been demonstrated that CD40L is expressed on the surface of platelets within seconds of platelet activation (Henn *et al.*, 1998). It is stored in platelet granules and is responsible for 95% of circulating sCD40 levels. Following platelet activation, the granules are translocated rapidly to the platelets' surface where they are activated then cleaved and shed from the surface as sCD40L (Schonbeck and Libby, 2001). Increased levels of CD40L have been found in patients with asymptomatic hypercholesterolemia, unstable angina, and acute MI. Elevated levels of sCD40L in patients with unstable CAD, indicates an increased risk of major cardiovascular events, including AMI, sudden death, and recurrent angina (Heeschen *et al.*, 2003; Yan *et al.*, 2004b). In patients with ACS, elevated levels of sCD40L levels have also been shown to be associated with higher risk of major

adverse cardiovascular events (Vishnevetsky *et al.*, 2004; Antoniades *et al.*, 2009). In vivo studies have demonstrated the role of sCD40L in destabilization of atheromatous plaque (Erbel *et al.*, 2007) and hence sCD40L may be used as a valuable marker for predicting the severity of ACS (Aukrust *et al.*, 1999; Yan *et al.*, 2002; Wang *et al.*, 2007). Diabetes mellitus and smoking were independently associated with elevated sCD40L levels (Tousoulis *et al.*, 2007a).

At a clinical level, elevated levels of sCD40L identify patients at high risk of UA and ACS. Patients with ACS have higher levels of sCD40L when compared to patients with stable CAD and controls (Tousoulis *et al.*, 2007b). Levels of sCD40L were much higher following coronary stent insertion in ACS patients with high ADP induced platelet aggregation (Obradovic *et al.*, 2009). Levels of sCD40L have also been correlated with complex coronary stenosis ($r = 0.60$, $P < 0.01$) in ACS patients undergoing coronary intervention (Yan *et al.*, 2004a). In these patients, intracoronary CD40L measured from the blood sample drawn from the culprit artery were higher when compared with that from the peripheral circulation (Aggarwal *et al.*, 2004; Tousoulis *et al.*, 2007b). Elevated levels of sCD40L 1 month after PCI also predict angiographic restenosis (L'Allier *et al.*, 2005).

Despite these findings, there is a growing body of evidence questioning the clinical significance of circulating sCD40L in the ACS setting. Hence measuring sCD40L levels routinely still remains controversial. In a large population-based study, 1524 patients treated with a GPIIb/IIIa receptor inhibitor, higher sCD40L levels were not associated with higher risk of nonfatal MI or re-hospitalization for ACS (Morrow *et al.*, 2008). In another study on patients presenting with ST-elevation MI (STEMI), elevated levels of sCD40L at admission were not associated with the onset of STEMI and did not provide any prognostic information regarding future mortality (Tan *et al.*, 2008). In the Dallas Heart Study, a large and representative multiethnic population study, sCD40L was not associated with established atherosclerotic risk factors suggesting that sCD40L measurement cannot be used as a screening tool (de Lemos *et al.*, 2005). Adding to this, studies have also found that in non-ACS patients, higher sCD40L levels are associated with a reduction in the risk of developing CAD (Rondina *et al.*, 2008), but in ACS cohort, no significant association was demonstrated between sCD40L and cardiovascular outcomes (Olenchock *et al.*, 2008). All these results have raised questions regarding the role of sCD40L in the development of CAD (Zirlik *et al.*, 2007).

Literature on sCD40L in elderly individuals (age≥75 years) is minimal. There is no published literature on sCD40L in elderly population with stable CAD or in ACS setting either. Hence my study findings are important in that respect. Deterioration of immune function in old age is a common finding (Lio *et al.*, 1998). This progressive decline of the immune system, defined `immunosenescence' (Pawelec *et al.*, 1997), is one of the several physiological changes thought to occur with increasing age. It has been demonstrated that CD40–CD40L pathways become dysfunctional with ageing (Effros, 2000; Weiskopf *et al.*, 2009). Levels of CD40, and CD40L decline with increasing age (Fernandez-Gutierrez *et al.*, 1999).

Levels of sCD40L are higher in both T1DM and T2DM than in non-diabetic individuals (Santilli *et al.*, 2007). Significantly increased co-expression of CD40 and CD40L on diabetic platelets has also been documented (Santilli *et al.*, 2007). In patients with T2DM, CD40L levels correlated significantly with urinary excretion rate of 11-dehydro-thromboxane B2, which is a marker of in vivo platelet activation (Santilli *et al.*, 2006).

In my study there was no difference in the levels of sCD40L between the elderly and young cohort or between T2DM and non DM. In the non DM cohort, addition of clopidogrel for 7 days resulted in a significant reduction in sCD40L levels. This finding is in keeping with the previous published data where administration of clopidogrel for one week in an ACS setting reduced platelet monocyte interaction and sCD40L levels (Xiao and Theroux, 2004). Quinn et al demonstrated low serum CD40L levels with clopidogrel pre-treatment with 300-600mg in patients who underwent PCI and these changes were more marked in flow cytometry assay after stimulation with ADP (Quinn *et al.*, 2004). In the PROCLAIM study (Pilot Study to Examine the Effects of Clopidogrel on Inflammatory Markers in Patients with Metabolic Syndrome Receiving Low-Dose Aspirin), the effect of 9 week treatment with dual antiplatelet therapy (aspirin and clopidogrel) was compared with aspirin and placebo, in participants with metabolic syndrome and who were clinically stable on appropriate medications. A significant reduction in CD40L was demonstrated after 9 weeks without changes in P selectin and other inflammatory markers (Willerson *et al.*, 2009).

PAI-1 is an independent risk factor in the development of cardiovascular disease and insulin resistance (Thögersen *et al.*, 1998). Serum PAI-1 levels are associated with cardiovascular morbidity and mortality in both men and women in prospective studies (Speiser *et al.*, 1988). In vivo, PAI-1 is synthesized by endothelial cells and

megakaryocytes (Sawdey and Loskutoff, 1991; Konkle *et al.*, 1993). PAI-1 is then sequestered in platelets and stored in the α-granules (Erickson *et al.*, 1984). Platelets form the main reservoir of PAI-1 in blood and the concentration of PAI-1 is extremely low in plasma (Kruithof *et al.*, 1986). From these data, it can be deduced that activation of platelets and release of the contents of the a-granules would result in a high local PAI-1 concentration in thrombi (Stringer *et al.*, 1994).

The age-associated increased risk of atherothrombotic disease may be related to reduced fibrinolytic potential as evidenced by reduction in tPA activity and/or increase in PAI-1 (Hashimoto *et al.*, 1987; Abbate *et al.*, 1993; Gleerup and Winther, 1995; Yamamoto *et al.*, 2005). PAI-1 levels are elevated in patients with T2DM (Mcgill *et al.*, 1994; Sobel *et al.*, 1998). In the IRAS (Insulin Resistance Atherosclerosis Study) study, PAI-1 predicted the incidence of T2DM and insulin resistance (Festa *et al.*, 2006). In poorly controlled T2DM patients, higher levels of PAI-1 have been reported (Lemkes *et al.*, 2010). In patients with acute coronary syndrome, levels of PAI-1 were increased (Yazici *et al.*, 2005). PAI-1 has also been demonstrated to play a significant role in evolution and outcomes in patients with T2DM following ACS (Sobel *et al.*, 1998; Sobel, 1999; Schneider and Sobel, 2012).

Long term dual antiplatelet therapy with aspirin and clopidogrel reduces PAI-1 levels (Sakata and Kario., 2011). Other strategies which successfully demonstrated a significant reduction in PAI-1 levels include improved glycaemic control, weight reduction and use of insulin sensitising agents (Schneider and Sobel, 2012). Statins increase the expression of tissue-type plasminogen activator and inhibit the expression of PAI-1 (Essig *et al.*, 1998). This inhibitory effect of statins on PAI-1 expression is mediated through the Phosphatidylinositol 3-kinase/protein kinase Akt pathway (Mukai *et al.*, 2007).

In my study, PAI-1 levels at baseline in the elderly cohort was similar to the young cohort. Within the elderly cohort, T2DM patients had similar PAI-1 levels when compared to patients without DM. All participants in my study were on good secondary prevention therapy including statin and this could be the reason for low PAI-1 levels over all.

Post chamber "effluent blood" (activated blood) from the Badimon chamber

When the blood flows through the chamber, it is subjected to high shear force and is exposed to tunica media, where contact activation of platelets with collagen occurs. Hence this effluent blood represents "activated blood" and should theoretically have increased levels of biomarkers that are released during platelet activation and involved in platelet dependent thrombus formation. Since the flow conditions within the chamber mimic the rheological flow conditions in a moderately stenosed coronary artery with a plaque rupture, the post chamber effluent blood may be equivalent to the blood sample from coronary sinus in patients with established CAD and acute plaque rupture. For P selectin, sCL40L and PAI-1 the gradient between pre and post chamber blood sample might be useful to quantify the amount of these biomarkers released by the activated platelets inside the chamber.

My study has demonstrated a significant increase in levels of P selectin, sCD40L and PAI-1 in the effluent blood both in the elderly and the young and in patients with T2DM and non DM. This may represent the amount of these biomarkers released from activated platelets inside the chamber. This increase was demonstrated at baseline and at one week after clopidogrel therapy. Addition of clopidogrel did not result in any significant reduction in release of these biomarkers from activated platelets as measured by the delta values of these biomarkers (pre and post chamber difference). However, I could not demonstrate any correlation between the venous levels, post chamber levels or the delta values (pre and post chamber difference) of P selectin, CD40L, PAI-1 and the thrombus area. This might be due to the small sample size in my study. The ELISA method used in my study has previously demonstrated more heterogeneous spread of P selectin measurements when compared to results from flow cytometry (Ritchie *et al.*, 2000). A better correlation could potentially have been demonstrated between thrombus area and levels of P selectin and CD40 ligand if the sample size was much higher and if flow cytometric methods were used in this study.

4.6 Inflammatory biomarkers - Elderly stable CAD study

4.6.1 Results

In patients with established CAD, three groups were analysed: age<75+non DM; age≥75+T2DM and age≥75+non DM.

Demographic data and baseline characteristics of the patients remains the same as discussed in the earlier section (Table 4.1). At baseline, venous blood levels of IFNγ, IL-6 and IL-1 were similar between the groups. Venous TNFα levels were higher in elderly diabetic group compared to the young non diabetic group (mean±SD, ng/ml: 3.99±1.12 vs. 4.96±1.42, p=0.002). Post chamber effluent blood levels of IFNγ, IL-6, IL-1 and TNFα were measured and compared with venous blood levels. This demonstrated significant reduction in IL-6 levels in patients with age<75+non DM (3.84±3.71 vs. 3.0±2.68, p<0.01) and age≥75+DM (6.21±6.27 vs. 4.82±4.47, p=0.014). Patients with age≥75+non DM also demonstrated a numerical reduction in IL-6 level but this was not statistically significant (5.27±5.92 vs. 4.88±5.34, p=0.182) (Table 4.69, Table 4.70).

Following one week of clopidogrel therapy, the elderly diabetic population interestingly demonstrated a slight but significant increase in venous blood levels of IFNγ and TNFα. The elderly non diabetic population also demonstrated a slight but significant increase in levels of TNFα. Post chamber effluent blood again demonstrated significant reduction in IL-6 levels in patients with age<75+non DM (4.82±5.21 vs. 4.22±4.68, p<0.01). Patients with age≥75+DM and age≥75+non DM also demonstrated a numerical reduction in IL-6 level but this was not statistically significant (Table). Venous TNFα levels remained higher in elderly diabetic group compared to young non diabetic group (5.66±1.80 vs. 3.97±0.96, p<0.01) (Figure 4.20, Table 4.68, Table 4.71).

There were modest but significant correlations between hsCRP and thrombus area (rho=0.158, p=0.05) at baseline and IL-6 levels and thrombus area (rho=0.191, p=0.017), 1 week after clopidogrel therapy. The difference in IL-6 levels between baseline and after 1 week of clopidogrel therapy (delta IL-6) also demonstrated a modest but significant correlation with thrombus area (rho=0.281, p=0.019) (Table 4.73).

	Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
Venous IFNγ, ng/ml	1.55±1.96	4.35±11.75	0.113	1.16±0.92	1.86±1.06	0.000 *	1.49±1.74	3.43±10.4	0.219
Post chamber IFNγ, ng/ml	1.56±1.83	4.80±13.66	0.116	1.33±0.81	1.76±0.91	0.001 *	1.54±1.75	3.7±11.72	0.241
Venous IL-1, ng/ml	0.20±0.33	0.23±1.54	0.880	0.11±0.28	0.23±1.32	0.607	0.26±0.79	0.03±0.13	0.072
Post chamber IL-1, ng/ml	0.84±2.92	0.04±0.13	0.075	0.36±1.22	0.17±0.30	0.330	0.22±0.43	1.0±5.47	0.339
Venous IL-6, ng/ml	3.84±3.71	4.82±5.21	0.106	6.21±6.27	6.22±5.55	0.978	5.27±5.92	4.84±4.86	0.653
Post chamber IL-6, ng/ml	3.0±2.68	4.22±4.68	0.032 *	4.82±4.47	5.63±4.63	0.188	4.88±5.34	4.77±4.56	0.909
Venous TNFα, ng/ml	3.99±1.12	3.97±0.96	0.910	4.96±1.42	5.66±1.80	0.011 *	4.41±1.30	4.89±1.65	0.003 *
Post chamber TNFα, ng/ml	3.71±1.37	4.07±1.13	0.070	4.75±2.72	5.30±1.67	0.185	5.17±7.58	4.7±1.45	0.693

Table 4.68 Inflammation biomarkers – Elderly stable CAD study;

* $p<0.05$

	Age < 75 + Non T2DM (n = 45)			Age ≥ 75 + T2DM (n = 41)			Age ≥ 75 + Non T2DM (n = 45)		
Mean ± SD	**Venous sample**	**Post chamber sample**	**P Value**	**Venous sample**	**Post chamber sample**	**P Value**	**Venous sample**	**Post chamber sample**	**P Value**
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD									
IFNγ, ng/ml	1.55±1.96	1.56±1.83	0.908	1.16±0.92	1.33±0.81	0.062	1.49±1.74	1.54±1.75	0.513
IL-1, ng/ml	0.20±0.33	0.84±2.92	0.152	0.11±0.28	0.36±1.22	0.196	0.26±0.79	0.22±0.43	0.849
IL-6, ng/ml	3.84±3.71	3.0±2.68	0.000 *	6.21±6.27	4.82±4.47	0.014 *	5.27±5.92	4.88±5.34	0.182
TNFα, ng/ml	3.99±1.12	3.71±1.37	0.114	4.96±1.42	4.75±2.72	0.572	4.41±1.30	5.17±7.58	0.534
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD									
IFNγ, ng/ml	4.35±11.75	4.80±13.66	0.201	1.86±1.06	1.76±0.91	0.282	3.43±10.4	3.7±11.72	0.323
IL-1, ng/ml	0.23±1.54	0.04±0.13	0.403	0.23±1.32	0.17±0.30	0.789	0.03±0.13	1.0±5.47	0.252
IL-6, ng/ml	4.82±5.21	4.22±4.68	0.000 *	6.22±5.55	5.63±4.63	0.071	4.84±4.86	4.77±4.56	0.160
TNFα, ng/ml	3.97±0.96	4.07±1.13	0.002 *	5.66±1.80	5.30±1.67	0.192	4.89±1.65	4.7±1.45	0.383

Table 4.69 Change in inflammation markers pre and post chamber

* $p<0.05$

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) **Mean ± SD** **Median (IQR)**				
Hs CRP mg/l	1.8± 1.8 1.2 (0.6-2.6)	4.3±6.6 2.0 (1.0-4.8)	4.4±7.7 1.5 (0.7-3.9)	0.075
Venous IFNγ, ng/ml	1.55± 1.96 1.13 (0.47-2.1)	1.16± 0.92 0.93 (0.46-1.87)	1.49± 1.74 1.13 (0.58-1.60)	0.494
Post chamber IFNγ, ng/ml	1.56± 1.83 1.11 (0.89-1.50)	1.33± 0.81 1.18 (0.64-1.94)	1.54± 1.75 1.08 (0.66-1.6)	0.747
Venous IL-1, ng/ml	0.20± 0.33 0 (0-0.26)	0.11± 0.28 0	0.26± 0.79 0	0.465
Post chamber IL-1, ng/ml	0.84± 2.92 0 (0-0.34)	0.36± 1.22 0 (0-0.22)	0.23± 0.43 0 (0-0.26)	0.269
Venous IL-6, ng/ml	3.84± 3.71 3.21 (1.86-4.21)	6.21± 6.27 4.39 (2.4-6.23)	5.27± 5.92 3.06 (2.49-5.17)	0.126
Post chamber IL-6, ng/ml	3.0± 2.68 2.12 (1.54-3.39)	4.82± 4.47 3.34 (2.04-4.75)	4.88± 5.34 2.94 (2.09-4.41)	0.081
Venous TNFα, ng/ml	3.99± 1.12 3.73 (3.26-4.66) *	4.96± 1.42 4.85 (4.18-5.6) *	4.41± 1.30 4.26 (3.46-4.86)	0.003 *
Post chamber TNFα, ng/ml	3.71± 1.37 3.32 (2.96-4.38)	4.75± 2.72 4.22 (3.42-4.88)	5.17± 7.58 3.9 (3.12-4.5)	0.349

Table 4.70 Comparison of inflammation biomarkers at baseline

* $p<0.05$

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) **Mean ± SD** **Median (IQR)**				
Venous IFNγ, ng/ml	4.35± 11.75 1.15 (0.74-2.13)	1.86± 1.06 1.7 (1.09-2.47)	3.43± 10.4 1.41 (0.94-2.41)	0.454
Post chamber IFNγ, ng/ml	4.80± 13.66 1.21 (0.94-2.0)	1.76± 0.91 1.62 (1.09-2.2)	3.7± 11.72 1.62 (1.09-2.4)	0.406
Venous IL-1, ng/ml	0.23± 1.54 0	0.23± 1.32 0	0.03± 0.13 0	0.660
Post chamber IL-1, ng/ml	0.04± 0.13 0	0.17± 0.30 0 (0-0.24)	1.0± 5.47 0 (0-0.21)	0.312
Venous IL-6, ng/ml	4.82± 5.21 3.46 (2.11-4.87)	6.22± 5.55 4.79 (2.73-8.07)	4.84± 4.86 3.45 (2.32-5.59)	0.368
Post chamber IL-6, ng/ml	4.22± 4.68 2.96 (1.77-4.72)	5.63± 4.63 4.43 (2.57-7.54)	4.77± 4.56 3.48 (2.22-5.38)	0.367
Venous TNFα, ng/ml	3.97± 0.96 3.89 (3.26-4.53) *ɬ	5.66± 1.80 5.22 (4.41-6.8) *	4.89± 1.65 4.62 (3.8-5.63) ɬ	0.000*
Post chamber TNFα, ng/ml	4.07± 1.13 3.83 (3.36-4.46) *	5.30± 1.67 4.98 (3.99-6.06) *	4.7± 1.45 4.27 (3.75-5.11)	0.001

Table 4.71 Comparison of inflammation biomarkers one week after clopidogrel therapy

*ɬ $p<0.05$

	Age < 75 + Non T2DM (n = 45)	Age ≥ 75 + T2DM (n = 41)	Age ≥ 75 + Non T2DM (n = 45)	P Value
Difference in variables between Visit 1 and Visit 2 **Mean ± SD** **Median (IQR)**				
Delta Venous IFNγ, ng/ml	-2.8±11.61 -0.03 (-0.82-0.22)	-0.70±1.01 -0.54 (-1.06- -0.04)	-1.94±10.46 -0.42 (-0.89-0.15)	0.570
Delta Post chamber IFNγ, ng/ml	-3.24±13.56 -0.14 (-0.84-0.22)	-0.43±0.79 -0.36 (-1.41-0.19)	-2.14±11.81 -0.45 (-0.97- -0.10)	0.464
Delta Venous IL-1, ng/ml	-0.04±1.61 0 (0-0.26)	-0.11±1.37 0	0.22±0.81 0	0.456
Delta Post chamber IL-1, ng/ml	0.80±2.94 0 (0-0.34)	0.19±1.25 0 (-0.15-0.17)	-0.78±5.32 0 (-0.08-0.05)	0.120
Delta Venous IL-6, ng/ml	-0.98±3.96 -0.40 (-1.07-0.56)	-0.016±3.73 -0.25 (-1.64-1.18)	0.43±6.38 -0.004 (-1.08-0.91)	0.380
Delta Post chamber IL-6, ng/ml	-1.22±3.69 -0.61 (-1.28-0.09)	-0.81±3.89 -0.48 (-1.73-0.25)	0.11±6.03 -0.31 (-0.84-0.30)	0.400
Delta Venous TNFα, ng/ml	0.01±0.85 0.06 (-0.63-0.73)	-0.70±1.68 -0.37 (-1.41-0.19)	-0.48±1.01 -0.34 (-1.10-0.21)	0.021*
Delta Post chamber TNFα, ng/ml	-0.36±1.29 -0.41 (-0.96-0.15)	-0.54±2.58 -0.81 (-1.55- -0.11)	0.46±7.66 -0.58 (-1.23- -0.08)	0.578

Table 4.72 Change in inflammatory biomarkers (delta)

* $p<0.05$

	Correlation with high shear thrombus area Rho (2-tailed p value)	Correlation with low shear thrombus area Rho (2-tailed p value)
Stable CAD Patients on Aspirin alone		
Venous IFNγ, ng/ml	-0.157 (0.043) *	-0.050 (0.539)
Hs CRP, mg/l	0.033 (0.674)	0.158 (0.050) *
Stable CAD Patients on Aspirin + 7 days of Clopidogrel		
Post chamber IFNγ, ng/ml	-0.178 (0.022) *	-0.092 (0.257)
Venous IL-1, ng/ml	0.014 (0.858)	-0.270 (0.001) *
Venous IL-6, ng/ml	-0.002 (0.983)	0.191 (0.017) *
Delta IL-6, ng/ml	0.108 (0.368)	0.281 (0.019) *
Post chamber TNFα, ng/ml	-0.104 (0.183)	-0.213 (0.008) *

Table 4.73 Correlation between inflammatory biomarkers and thrombus area

* $p<0.05$

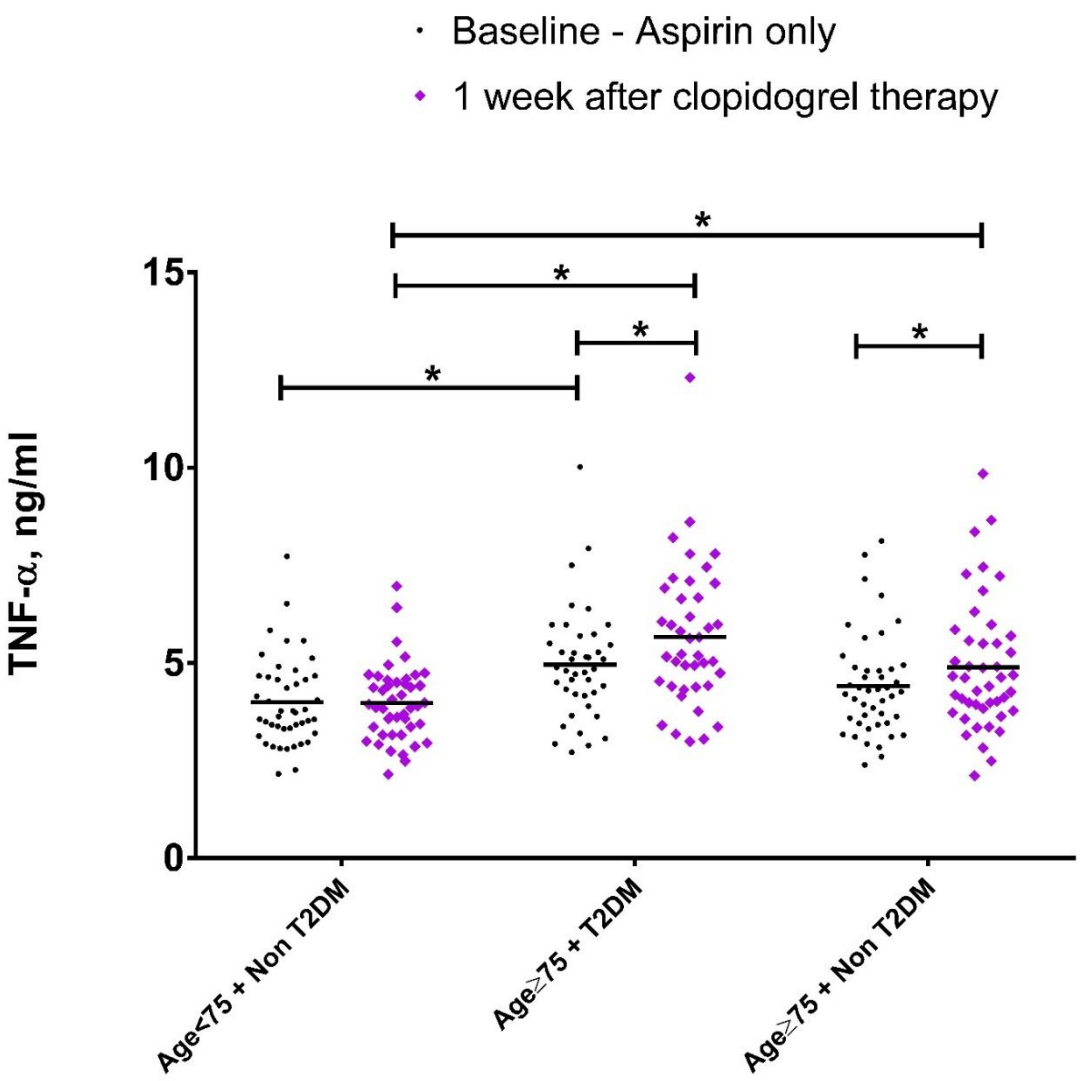


Figure 4.20 Change in venous TNFα levels with clopidogrel therapy

*p<0.05

At baseline: significantly higher TNFα levels in patients with age≥75+T2DM compared to age<75+non DM (4.96± 1.42 vs. 3.99± 1.12, p=0.003)

In patients with age≥75+T2DM there was a significant increase in TNFα one week after clopidogrel therapy compared to baseline (5.66±1.80 vs. 4.96±1.42, p=0.011)

In patients with age≥75+non DM there was a significant increase in TNFα one week after clopidogrel therapy compared to baseline (4.89±1.65 vs. 4.41±1.30, p=0.003)

After 1 week of clopidogrel therapy: significantly higher levels of TNFα were measured in patients with age≥75+T2DM (5.66±1.80 vs. 3.97±0.96, p<0.01) and age≥75+non-DM (4.89±1.65 vs. 3.97±0.96, p<0.01), when compared with age<75+non DM.

4.6.2 Discussion

Ageing has been associated with chronic low grade inflammation which in turn has been hypothesized to play a significant role in the pathogenesis of atherosclerosis and T2DM in the elderly. T2DM is also associated with inflammation and thrombotic events. I wanted to explore the role of inflammation in thrombus formation in elderly and CAD patients, with and without T2DM. Tumour necrosis factor-α (TNFα) levels were significantly higher in the elderly cohort compared to the young at baseline (whilst on aspirin alone) and continued to be high in the elderly cohort after addition of clopidogrel for one week. High sensitivity CRP (hs CRP) and interleukin-6 (IL-6) levels were numerically higher in the elderly cohort but were not statistically significant at baseline. IL-6 levels continued to remain high in the elderly compared to the young after the addition of clopidogrel but this again was not statistically significant. Levels of TNFα and IL-6 were highest among elderly diabetic patients.

Increasing age has been associated with increased circulating levels of inflammatory markers, including proinflammatory and anti-inflammatory cytokines, cytokine antagonists and acute phase proteins (Bruunsgaard *et al.*, 2001). Elevated levels of proinflammatory cytokines such as TNF-α (Paolisso *et al.*, 1998; Bruunsgaard *et al.*, 1999) and IL-6 (Wei *et al.*, 1992; Cohen *et al.*, 1997) are particularly important as they are associated with increased morbidity and mortality in the elderly (Brüünsgaard and Pedersen, 2003). Plasma TNF-α concentration also predicts impaired insulin action with advancing age (Paolisso *et al.*, 1998). Various reports have indicated that inflammatory markers might predict future cardiovascular events, especially in the elderly population (De Martinis *et al.*, 2006). Harris et al demonstrated that high circulating levels of IL-6 and CRP were associated with increased cardiovascular mortality in the elderly population (Harris *et al.*, 1999). High levels of IL-6 and CRP were also noted in patients with unstable angina and depending on the fall or raise of their levels in the blood the outcome of the patients was good or bad respectively (Biasucci *et al.*, 1996).

An increase in tissue factor expression caused by inflammation tends to shift the hemostatic balance in favour of thrombogenicity. Prothrombotic effect of TNFα and IL-6 are due to their ability to induce tissue factor expression, primarily on monocytes and macrophages (Walsh, 1987; Edgington *et al.*, 1991). TNFα also increases the production of ultra-large von Willebrand factor (vWF) multimers and fibrinogen, thereby

shifting haemostasis towards thrombus formation (Bernardo *et al.*, 2004). Normally these multimers of vWF are processed into smaller forms which are less thrombogenic. IL-6 prevents this process, thereby maintaining a thrombogenic milieu (Bernardo *et al.*, 2004). Increased levels of IL-6 have been associated with increased platelet production. The newly formed platelets appear to be more thrombogenic as they can be activated at lower concentrations of thrombin. Thus both platelet count and platelet reactivity are increased, thereby increasing their thrombogenic potential (Burstein, 1997). TNFα and IL-1 down regulate natural anticoagulant pathways involving protein C and thrombomodulin (Conway and Rosenberg, 1988; Fukudome and Esmon, 1994). Substantial experimental evidence suggests the association of TNFα and IL-6 with hyperglycaemia, insulin resistance and T2DM (Sandler *et al.*, 1990; Grau *et al.*, 1996; Pickup *et al.*, 1997; Tsigos *et al.*, 1997; Festa *et al.*, 2000). In another study conducted by Pradhan et al, CRP and IL-6, were determinants of risk for T2DM. CRP was a powerful independent predictor of T2DM after adjustment for obesity, clinical risk factors, and fasting insulin levels (Dovio and Angeli, 2001; Pradhan *et al.*, 2001).

Inflammatory cytokines and hsCRP levels appear to fall after treatment with aspirin and clopidogrel in a number of studies (Mehta *et al.*, 2001; Yusuf *et al.*, 2001a; Steinhubl *et al.*, 2002; Chen *et al.*, 2005b; Sabatine *et al.*, 2005b; Sabatine *et al.*, 2005c). Clopidogrel does not have any direct anti-inflammatory actions, hence its clinical benefits may be due to indirect anti-inflammatory effect in addition to its well characterised antiplatelet effect. Interestingly in my study, contrary to what was expected, after one week of clopidogrel there was a modest but significant increase in TNFα levels in elderly cohort (both in T2DM and non DM). Failure of clopidogrel to demonstrate any significant reduction in inflammatory biomarkers is probably due to the fact that 75 mg daily dose of clopidogrel for 7 days is not adequate enough to suppress inflammation.

Failure of DAPT to reduce inflammatory biomarkers was seen in Evaluation of Long-term clopidogrel AntiPlatelet and Systemic anti-inflammatory Effects (ELAPSE) study, a prospective study involving 26 patients who received DAPT for 12 months following coronary stent insertion. Levels of hsCRP, IL-10 and IL-18 initially increased for a few days but remained unchanged at 12 months but the contradictory finding was an increase in sP selectin level at 12 month follow up (Saw *et al.*, 2008).

I found that the levels of hsCRP and IL-6 demonstrated significant positive correlation with thrombus area (hsCRP: rho=0.158, p=0.05; IL-6: rho=0.191, p=0.017). Delta IL-6 also demonstrated a positive correlation with thrombus area (rho=0.281, p0.019). These findings further emphasise the potential role of these inflammatory markers in thrombus generation.

Post chamber "effluent blood" (activated blood) from the Badimon chamber

I compared the levels of inflammatory biomarkers from venous blood sample and from post chamber "effluent" blood. As explained before, post chamber blood may be equivalent to the blood sample from coronary sinus in patients with established CAD and acute plaque rupture. Cytokines are neither synthesised nor released from the blood that is flowing through the chamber. Hence any change in the levels of biomarkers pre and post chamber would represent the usage / consumption of these cytokines in the formation of thrombus within the chamber.

From my study, I observed a reduction in IL-6 in post chamber blood compared to venous blood sample (pre-chamber blood) both at baseline (when on aspirin alone) and after one week of clopidogrel alongside aspirin. This reduction was seen in the elderly and the young cohort, both in patients with T2DM and non DM. Levels of TNFα, IL-1 and IFNy remain unchanged in elderly and young patients both in the presence and absence of T2DM. This negative observation could be due to average low levels of inflammatory markers in my cohort of patients with stable CAD, as they all were on appropriate secondary prevention therapy including statins.

4.7 Scanning electron microscopy (SEM) – Elderly stable CAD study

4.7.1 Results - Ultrastructural analysis of thrombus

To explore the differences in ultrastructural characteristics of the formed thrombus, I performed a sub-study using scanning electron microscopy (SEM), in all three groups of patients: age<75+non DM, age≥75+T2DM and age≥75+non DM.

I randomly chose 20 patients from each group (n=20 in each group). Demographic profiles, metabolic profiles and cardiovascular risk factors are shown in Table 4.74. Overall, baseline characteristics are similar between the groups except for significantly higher systolic blood pressure and lower haemoglobin in the elderly cohort compared to the young. The elderly non DM cohort demonstrated a modest but significantly higher HDLc level compared to the elderly T2DM group.

SEM image analysis of high shear thrombus was performed to quantify the platelet content of thrombus and this was measured as proportion of platelet rich areas to fibrin rich areas at 60X magnification. At baseline, percentage of platelet rich areas were similar between the three groups (Table 4.76). After a week of clopidogrel therapy, a significant reduction in platelet rich area was demonstrated in all the groups (age<75+non T2DM: 75.9% to 66.7%, mean difference [MD]: 9.2%; 95%CI: 6.9-11.5; p<0.001; age≥75+T2DM: 77.6% to 69.3%; MD: 8.3%; 95%CI: 7.11-9.46; p<0.001; age≥75+Non T2DM: 77.7 % to 69.6%; MD: 8.1%; 95%CI: 5.8-10.3; p<0.001). The mean difference in reduction in platelet rich area of thrombus was similar in all the three groups.

Quantitative assessment of fibrin was performed by measuring the following parameters: fibrin diameter (µm), fibrin fibre density (n/μ^2), fibrin spokes (n/μ^2), fibrin hubs (n/μ^2). Platelet diameter was also measured (Table 4.75, Table 4.76, Table 4.77, Figure 4.24).

Fibrin diameter increased in all the three groups following one week of clopidogrel therapy (µm, mean±SD; age<75+T2DM: 218.1±20.2 to 243.4±16.6, p<0.001; age≥75+T2DM: 198.9± 70.2 to 245.0± 32.9, p=0.038; age≥75+non DM: 204.8± 22.9 to 233.2±27.9, p<0.001). There was no significant difference in fibrin diameter between the groups at baseline or at one week after clopidogrel therapy. Platelet

diameter was similar between the groups at baseline and remained unchanged after one week of clopidogrel therapy.

Fibrin fibre density (n/μ^2) increased in all three groups following one week of clopidogrel therapy (n/μ^2, mean±SD; age<75+T2DM: 38.5±4.3 to 43.2±7.1, p=0.026; age≥75+T2DM: 40.5±2.9 to 44.7±8.7, p=0.039; age≥75+non DM: 37.4±3.3 to 42.8±7.6, p=0.035)

Spokes of fibrin (n/μ^2 of thrombus), increased in all three groups following one week of clopidogrel therapy (mean±SD; age<75+T2DM: 26.3± 3.1 to 31.2±6.7, p=0.031; age≥75+T2DM: 25.3±3.8 to 29.1±6.4, p=0.023; age≥+non DM: 24.8±3.1 to 30.7±7.3, p=0.041) (Figure 4.25)

Hubs of fibrin (n/μ^2 of thrombus), increased in all three groups following one week of clopidogrel therapy (mean±SD; age<75+T2DM: 16.4±1.6 to 18.7±2.8, p=0.039; age≥75+T2DM: 17.4±2.3 to 19.4±2.6, p=0.014; age≥+non DM: 16.3±1.5 to 18.2±3.1, p=0.009)

In this cohort of patients, there was a significant reduction in platelet dependent thrombus (PDT) in both young non-diabetic and elderly diabetic group. In the elderly non-diabetic group there was a numerical reduction in thrombus area, but because of small number of patients in this substudy the reduction in thrombus did not reach statistical significance. At baseline, the number of spokes per μ^2 of thrombus demonstrated a strong negative correlation with low shear thrombus area (rho= -0.374, p=0.005) and high shear thrombus area (rho= -0.327, p=0.013). After a week of clopidogrel therapy percentage platelet rich area from SEM correlated significantly with high shear thrombus area (rho=0.408, p=0.002) (Figure 4.26).

Thromboelastography (TEG®) data demonstrated minimal numerical reduction in maximum strength of the thrombus (MA) and clot index (CI) with a trend towards increased clot lysis. These changes again were not statistically significant due to small sample size. The maximum amplitude of viscoelastic force of thrombus upon stimulation with ADP (MA-ADP) was significantly lower after one week of clopidogrel therapy (Table 4.78). At baseline, fibrin fibre density demonstrated a significant negative correlation with MA (rho= -0.459, p=0.048), G (rho= -0.469, p=0.043) and rate of thrombin generation (TG) (rho= -0.458, p=0.049).

VerifyNow® PRUz and percentage platelet inhibition demonstrated significant reduction after a week of clopidogrel therapy. ARU demonstrated a trend towards reduction but this was not statistically significant (Table 4.79). VerifyNow® indices did not show any correlation with SEM data both at baseline and after clopidogrel therapy.

Multiplate® ADP AU demonstrated a significant reduction after clopidogrel therapy. ASP AU demonstrated a significant reduction in the young non-diabetic cohort. In the elderly cohort there was a trend towards reduction but this was not statistically significant. At baseline, percentage platelet rich area with SEM demonstrated a strong positive correlation with ADP AU (rho=0.499, p=0.030).

	Age < 75 + Non T2DM (n = 19)	Age ≥ 75 + T2DM (n = 20)	Age ≥ 75 + Non T2DM (n = 19)	P Value
Demographic data: Mean ± SD or % (n)				
Age, years	56.4 ± 5.4	80.2 ± 2.6	82.0 ± 2.9	
Male gender, % (n)	84.2 (16)	80 (16)	89.5 (17)	0.716
Body mass index, kg/m^2	30.1 ± 3.5	28.7 ± 4.7	27.7 ± 2.8	0.158
Waist to hip ratio	1.0 ± 0.1	1.0 ± 0.08	0.9 ± 0.06	0.628
Heart rate, beats per minute	64 ± 13	71 ± 18	62 ± 10	0.117
Systolic BP, mmHg	131 ± 17 *ł	158 ± 21 *	154 ± 25 ł	0.000 *
Diastolic BP, mmHg	78 ± 7	78 ± 11	73 ± 10	0.268
Risk profile: % (n)				
Angina	26.3 (5)	15 (3)	26.3 (5)	0.617
Previous MI	57.9 (11)	50 (10)	63.2 (12)	0.423
Previous PCI	36.8 (7)	30 (6)	31.6 (6)	0.894
Previous CABG	5.3 (1)	35 (7)	31.6 (6)	0.062

H/o Hypertension	47.4 (9)	75 (15)	68.4 (13)	0.175
H/o CKD	0 (0)	15 (3)	5.3 (1)	0.171
H/o PVD	0 (0)	30 (6)	21.1 (4)	0.040
H/o CVA	10.5 (2)	0 (0)	5.3 (1)	0.333
Medications: % (n)				
Sulphonylurea	0 (0)	10 (2)	0 (0)	0.140
Metformin	0 (0)	40 (8)	0 (0)	
Insulin	0 (0)	40 (8)	0 (0)	
Beta-blocker	84.2 (16)	70 (14)	68.4 (13)	0.512
ACE inhibitor / ARB	68.4 (13)	95 (19)	89.5 (17)	0.055
Statin	94.7 (18)	95 (19)	94.7 (18)	0.999
Laboratory data: Mean ± SD				
Haemoglobin, g/dl	14.1 ± 0.8 *ł	12.5 ± 1.0 *	12.8 ± 0.9 ł	0.000 *
Platelets x 1000 cells/mm^3	240 ± 66	195 ± 49	219 ± 52	0.055
Fibrinogen, g/ml	3.2 ± 0.8	3.3 ± 0.6	3.3 ± 0.5	0.732

HbA1c, mmol/mol	40 ± 3.6	58.5 ± 15.6	41.4 ± 3.5	
Fasting plasma glucose, mmol/L	5.5 ± 0.7	8.8 ± 5.4	5.1 ± 0.6	
Creatinine, micromol/L	89 ± 14.9	101 ± 27.1	107 ± 36.7	0.139
Total cholesterol, mmol/L	4.1 ± 1.0	3.9 ± 1.1	4.0 ± 0.6	0.865
LDLc, mmol/L	2.2 ± 0.8	2.0 ± 0.9	2.0 ± 0.6	0.553
HDLc, mmol/L	1.3 ± 0.4	1.2 ± 0.3 *	1.5 ± 0.3 *	0.035 *
Triglyceride, mmol/L	1.3 ± 0.9	1.6 ± 0.7	1.2 ± 0.5	0.090
HsCRP, mg/L	1.5 ± 1.0	3.0 ± 3.3	2.7 ± 3.3	0.187

Table 4.74 Baseline characteristics – SEM substudy

*p<0.05

LDLc – Low density lipoprotein cholesterol; HDLc – High density lipoprotein cholesterol; HsCRP – high-sensitive C-reactive protein; H/o – History of

Systolic BP: Age<75+Non DM vs. Age≥75+T2DM, p=0.001; Age<75+Non DM vs. Age≥75+Non DM, p=0.003

Haemoglobin: Age<75+Non DM vs. Age≥75+T2DM, p<0.001; Age<75+Non DM vs. Age≥75+Non DM, p<0.001

HDLc: Age≥75+Non DM vs. Age≥75+T2DM, p=0.040

	Age < 75 + Non T2DM (n = 19)			Age ≥ 75 + T2DM (n = 20)			Age ≥ 75 + Non T2DM (n = 19)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
High shear thrombus area, μ^2/mm	16697± 5134	12712± 4991	0.000 *	15625± 7805	12711± 4683	0.020 *	16108± 6407	14143± 5131	0.094
Low shear thrombus area, μ^2/mm	9414± 2667	8250± 2249	0.044 *	9036± 2410	8059± 1706	0.076	9883± 2380	9081± 1690	0.068
Fibrin diameter, nm	218.1± 20.2	243.4± 16.6	0.000 *	198.9± 70.2	245.0± 32.9	0.038 *	204.8± 22.9	233.2± 27.9	0.000 *
Platelet diameter, µm	1.87± 0.15	1.82± 0.18	0.198	1.85± 0.11	1.84± 0.08	0.743	1.86± 0.08	1.86± 0.1	0.954
Fibrin fibre density, n/μ^2 of thrombus	38.5± 4.3	43.2±7.1	0.026 *	40.5±2.9	44.7±8.7	0.039 *	37.4±3.3	42.8±7.6	0.035 *
Spokes of fibrin, n/μ^2 of thrombus	26.3± 3.1	31.2±6.7	0.031 *	25.3±3.8	29.1±6.4	0.023 *	24.8±3.1	30.7±7.3	0.041 *
Hubs of fibrin, n/μ^2 of thrombus	16.4± 1.6	18.7±2.8	0.039 *	17.4±2.3	19.4±2.6	0.014 *	16.3±1.5	18.2±3.1	0.009 *

Table 4.75 Thrombus area and fibrin ultrastructure parameters

* $p<0.05$

	Age < 75 + Non T2DM (n = 19)	Age ≥ 75 + T2DM (n = 20)	Age ≥ 75 + Non T2DM (n = 19)	P Value
Stable CAD Patients on Aspirin alone (Visit 1) Mean ± SD				
Fibrin diameter, nm	218.1±20.2	198.9±70.2	204.8±22.9	0.401
Platelet diameter, µm	1.87±0.15	1.85±0.11	1.86±0.08	0.829
Fibrin fibre density, n/μ^2 of thrombus	38.5±4.3	40.5±2.9	37.4±3.3	0.458
Spokes of fibrin, n/μ^2 of thrombus	26.3±3.1	25.3±3.8	24.8±3.1	0.718
Hubs of fibrin, n/μ^2 of thrombus	16.4±1.6	17.4±2.3	16.3±1.5	0.351
Platelet rich area, %	75.9±7.1	77.6±8.6	77.7±7.8	0.302

Table 4.76 Comparison of fibrin and platelet parameters at baseline

* $p<0.05$

	Age < 75 + Non T2DM (n = 19)	Age ≥ 75 + T2DM (n = 20)	Age ≥ 75 + Non T2DM (n = 19)	P Value
Stable CAD Patients on Aspirin + 7 days of Clopidogrel (Visit 2) Mean ± SD				
Fibrin diameter, nm	243.4±16.6	245.0±32.9	233.2±27.9	0.341
Platelet diameter, µm	1.82±0.18	1.84±0.08	1.86±0.1	0.421
Fibrin fibre density, n/μ^2 of thrombus	43.2±7.1	44.7±8.7	42.8±7.6	0.562
Spokes of fibrin, n/μ^2 of thrombus	31.2±6.7	29.1±6.4	30.7±7.3	0.201
Hubs of fibrin, n/μ^2 of thrombus	18.7±2.8	19.4±2.6	18.2±3.1	0.694
Platelet rich area, %	66.7±8.2	69.3±9.2	69.6±9.8	0.587

Table 4.77 Comparison of fibrin and platelet parameters one week after clopidogrel therapy

* $p<0.05$

	Age < 75 + Non T2DM (n = 19)			Age ≥ 75 + T2DM (n = 20)			Age ≥ 75 + Non T2DM (n = 19)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
Thromboelastography data									
R, minutes	6.8±2.0	7.1±1.3	0.504	6.9±2.1	6.4±1.6	0.149	6.2±1.4	6.6±.1.8	0.233
MA, mm	62.7±5.6	62.7±5.2	0.994	63.4±3.3	63.0±5.3	0.745	64.6±3.6	63.7±3.9	0.190
MA-AA, mm	36.8±21.2	24.8±16.6	0.010 *	27.5±14.6	26.4±16.8	0.740	27.2±17.2	20.8±12.5	0.164
MA-ADP, mm	57.4±12.8	51.7±9.4	0.124	59.2±5.6	56.4±7.6	0.017 *	62.3±6.2	56.6±8.8	0.001 *
G, Kdynes/sec	8.7±1.9	8.6±1.8	0.884	8.7±1.2	8.8±2.3	0.834	9.3±1.5	8.9±1.2	0.139
CI	-0.57±2.6	-0.66±1.7	0.841	-0.30±1.8	0.12±1.6	0.220	0.58±1.1	0.04±1.4	0.126
L Parameter, mm/min	78.6±14.2	84.4±17.1	0.166	87.3±26.4	107.6±43.2	0.115	85.6±21.2	81.7±22.1	0.339

Table 4.78 TEG® and Platelet Mapping™ - SEM substudy

* $p<0.05$

	Age < 75 + Non T2DM (n = 19)			Age ≥ 75 + T2DM (n = 20)			Age ≥ 75 + Non T2DM (n = 19)		
Mean ± SD	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**	**Visit 1**	**Visit 2**	**P Value**
VerifyNow® data									
ARU	489±94.2	453±84.2	0.157	480±56.2	476±82.5	0.853	489±64	500±79	0.631
PRUz	299±39.9	178±72.0	0.000 *	306±29.8	258±51.7	0.000 *	309±49	220±78	0.000 *
Platelet inhibition, %	4.2±6.9	33±27.4	0.000 *	2.7±4.2	16.3±12.4	0.000 *	3.6±5.3	29±23.3	0.000 *
Multiplate® data									
Arachidonic acid, AU	465±249	307±252	0.013 *	349±243	289±240	0.090	359±195	245±163	0.070
ADP, AU	831±180	493±272	0.000 *	843±315	530±254	0.000 *	748±194	453±230	0.000 *

Table 4.79 VerifyNow® and Multiplate® indices - SEM substudy

* $p<0.05$

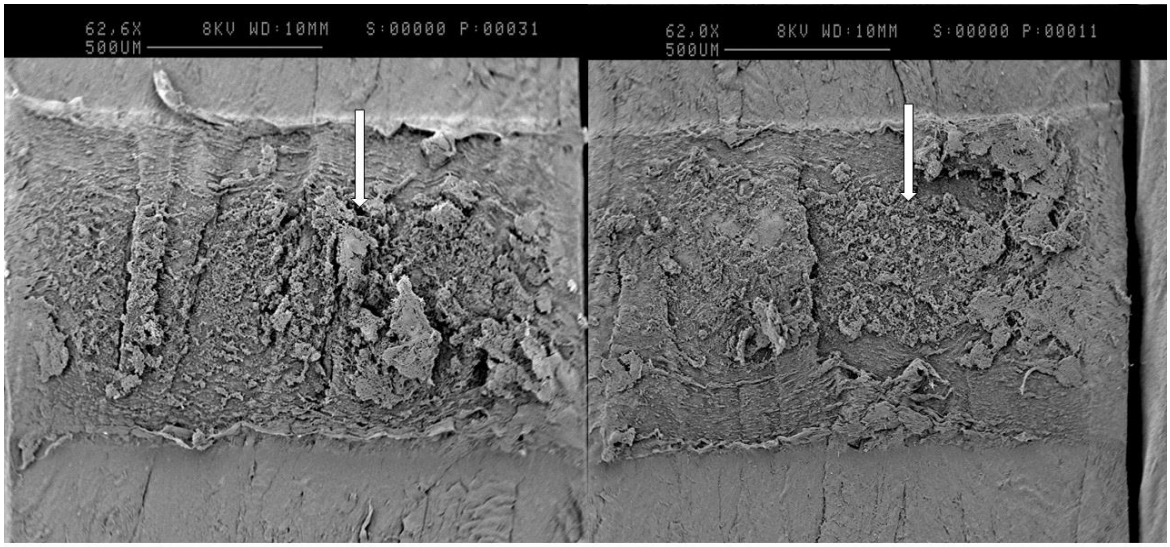


Figure 4.21 Platelet and fibrin content of thrombus - scanning electron microscopy

SEM image of platelet and fibrin content of thrombus at baseline (left) and one week (right) after clopidogrel therapy (60x magnifications). Solid white arrow in first picture is platelet rich area. A reduction in platelet rich area can be seen after one week of clopidogrel therapy.

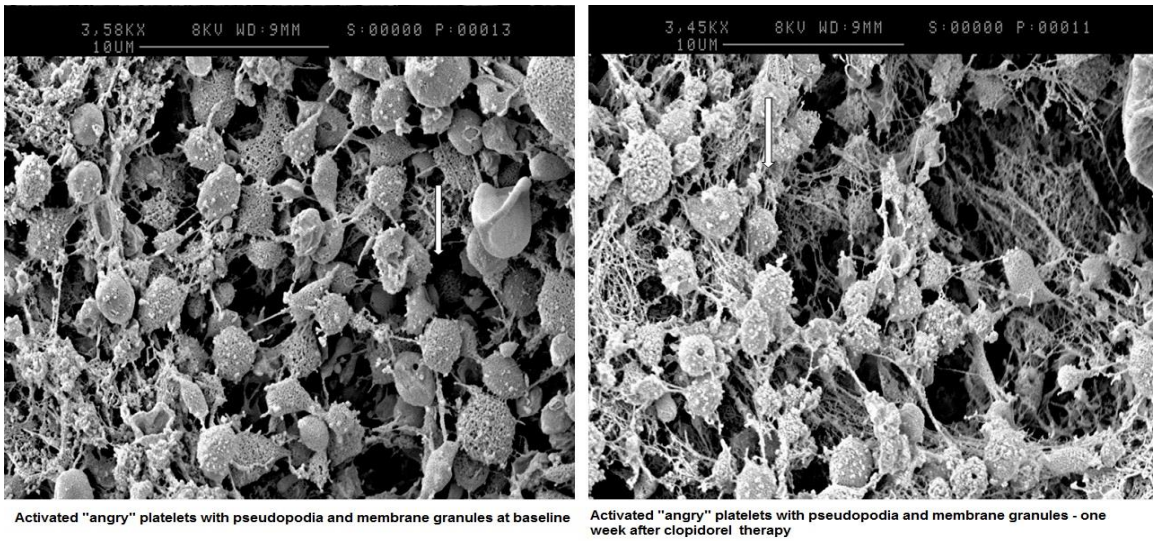


Figure 4.22 Ultrastructure of platelet at baseline and one week after clopidogrel therapy

Platelet ultrastructure at $3.4x10^3$ to $3.6x10^3$ magnification. Solid arrows represent activated “angry” platelets with pseudopodia and membrane granules. Platelet diameter remained unchanged after clopidogrel therapy.

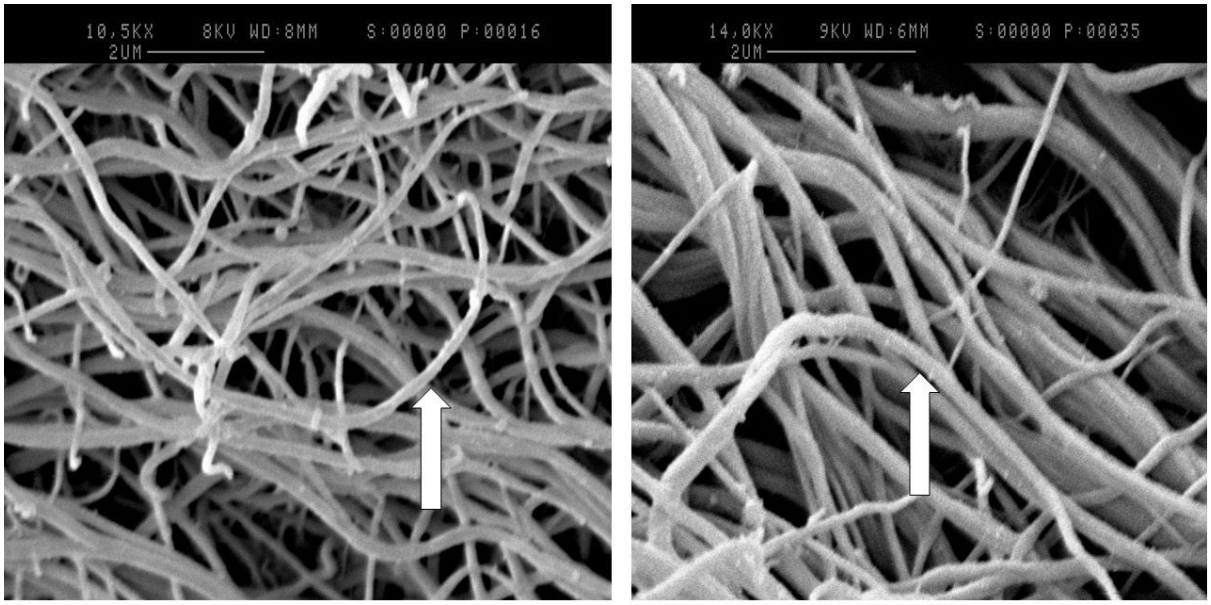


Figure 4.23 Fibrin ultrastructure at baseline and one week after clopidogrel therapy

Fibrin architecture at 10.5 $x10^3$ to 14.0$x10^3$ magnifications. Solid arrows represent fibrin fibres. After one week of clopidogrel therapy, there was an overall increase in fibrin fibre diameter, density, number of hubs and spokes and overall susceptibility to fibrinolysis.

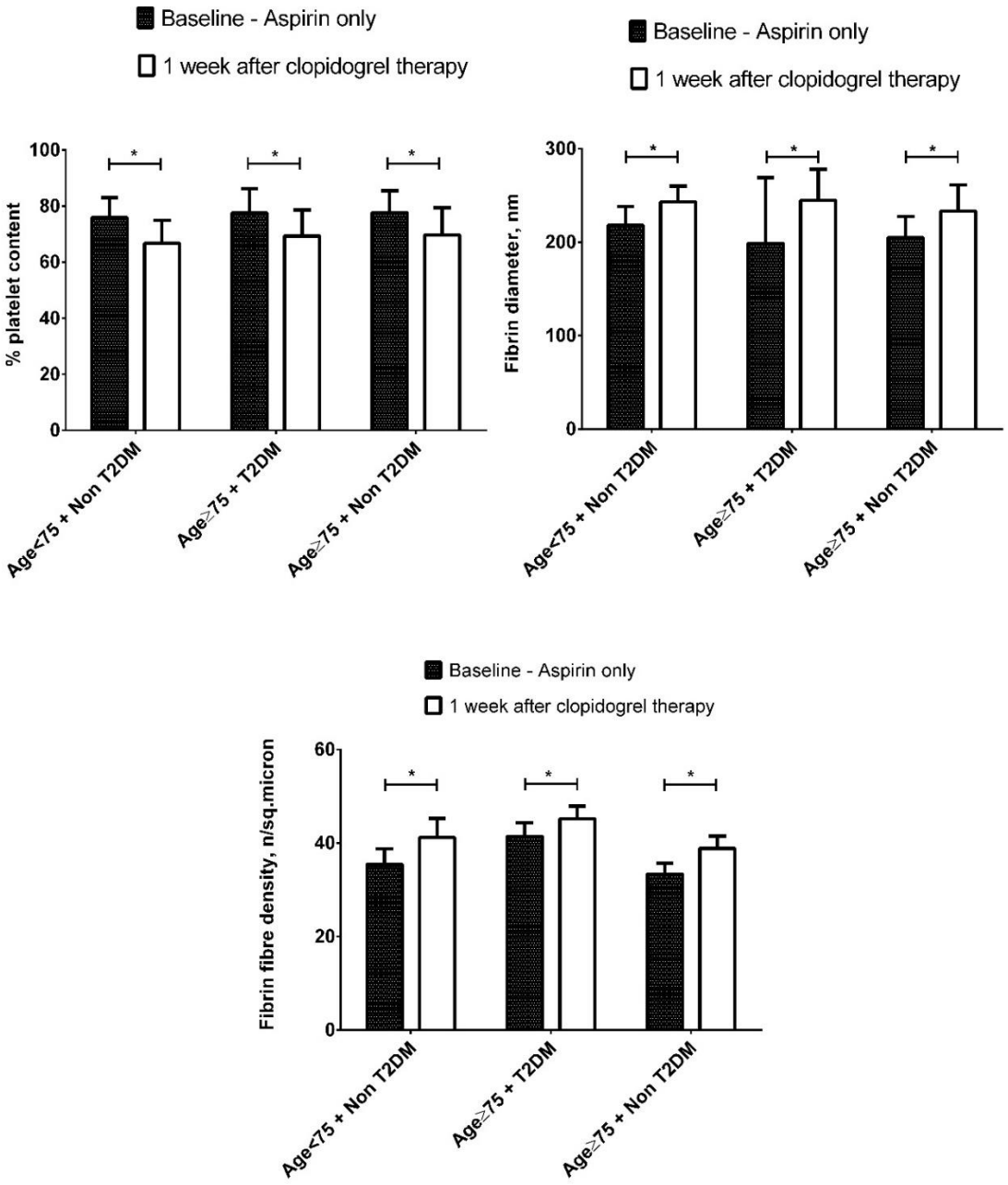


Figure 4.24 Change in platelet content of thrombus, fibrin diameter and fibrin fibre density

There was a significant reduction in percentage platelet content and increase in fibrin diameter and fibrin fibre density after a week of clopidogrel therapy. (Table 4.78)

*p<0.05

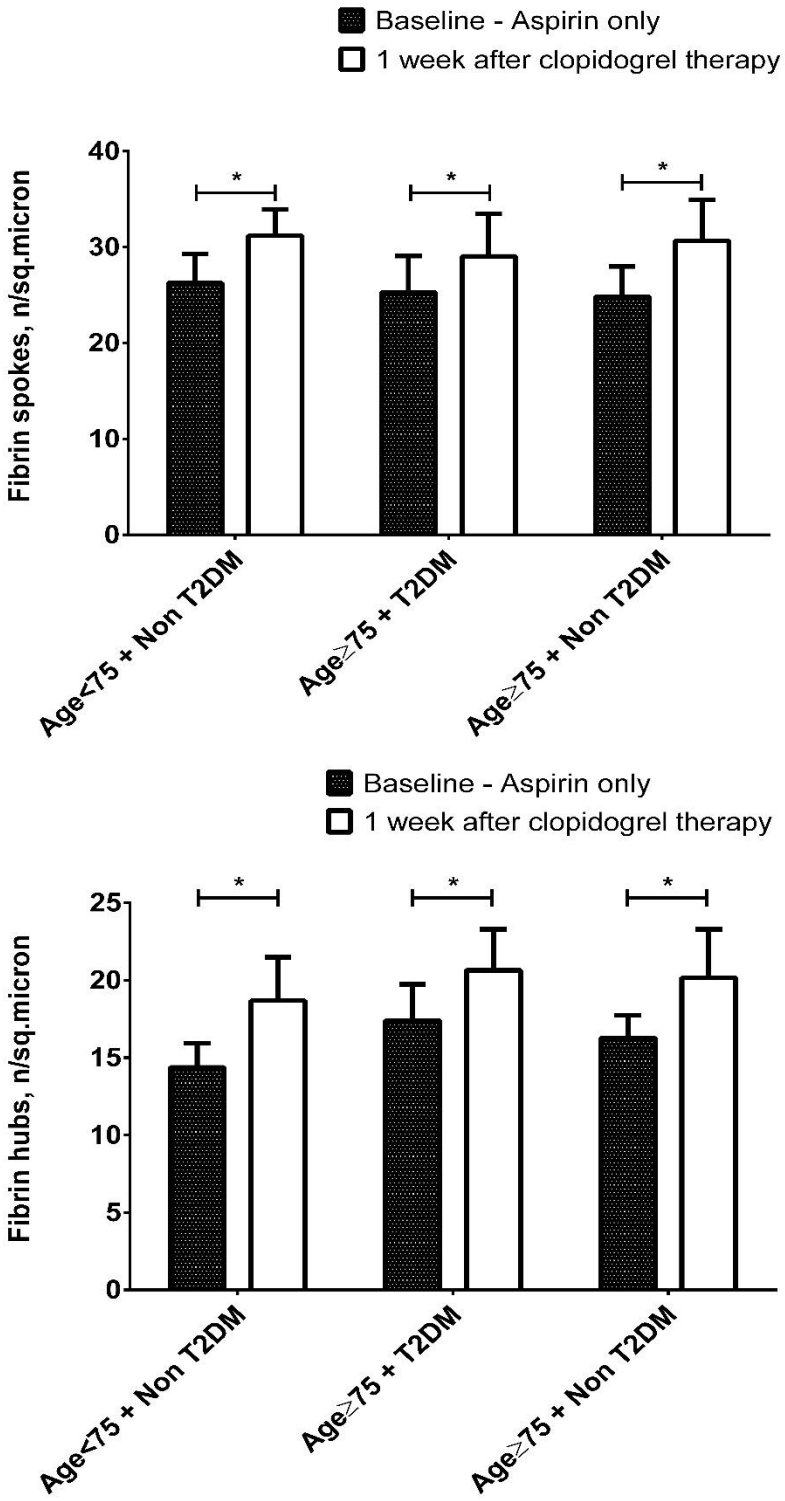


Figure 4.25 Changes to number of hubs and spokes per square micron of thrombus

There was a significant increase in fibrin hubs and spokes per square micron of thrombus after a week of clopidogrel therapy. (Table 4.78)

*p<0.05

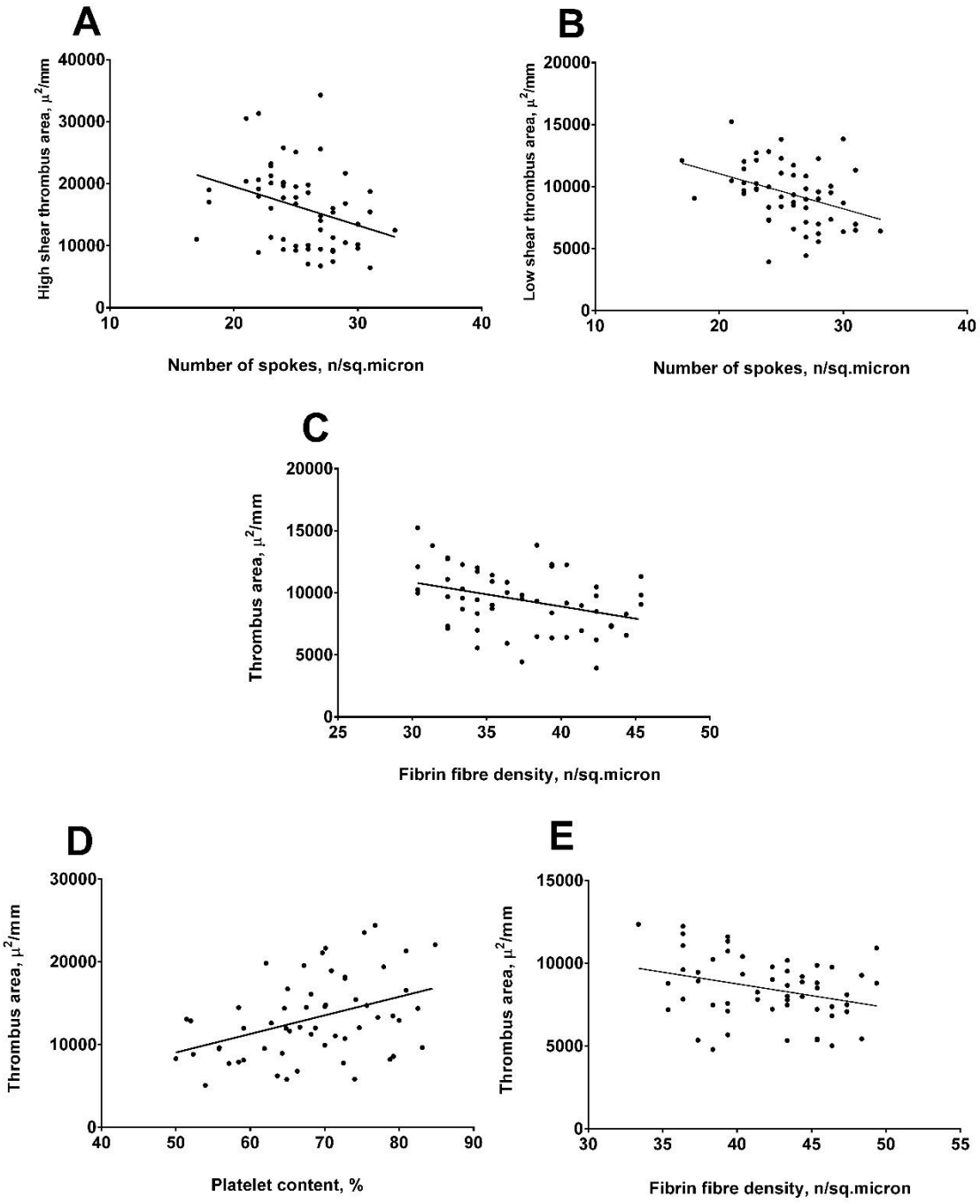


Figure 4.26 Correlation: SEM measured parameters and thrombus area

At baseline, number of spokes negatively correlated with high shear thrombus area (rho= -0.327, p=0.013) and low shear thrombus area (rho= -0.374, p=0.005); fibrin fibre density also correlated negatively with thrombus area (rho= -0.356, p=0.008).

After one week of clopidogrel therapy, negative correlation between fibrin fibre density and thrombus area persisted (rho= -0.309, p=0.022); percentage platelet content correlated positively with thrombus area (rho=0.408, p=0.008).

4.7.2 Discussion - Ultrastructural analysis of thrombus

I report the ultrastructural characteristics of platelet dependent thrombus formed in Badimon chamber from elderly patients (age≥75 years) with and without T2DM. My study has shown a significant reduction in platelet content of thrombus following addition of clopidogrel in addition to aspirin for one week. There was a significant increase in fibrin fibre diameter, fibrin fibre density and increase in number of spokes and hubs per square micron of thrombus following one week of clopidogrel therapy. These changes in fibrin architecture were seen consistently in both elderly and young population, with and without T2DM. These assessment methods were novel and hence full internal validation was performed at every step. Our previous work in young T2DM patients also demonstrated a similar result in patients treated with clopidogrel (Viswanathan *et al.*, 2013).

After one week of clopidogrel therapy, the percentage platelet content in the thrombus reduced significantly in all the groups, similar to the reduction in platelet dependent thrombus formation observed in my main study. As explained before, Badimon chamber simulates rheological flow conditions in a moderately stenosed coronary artery (high shear) and hence the thrombus formed inside the chamber is platelet rich, similar to the “white thrombus” or platelet rich thrombus as described by Davies et al in patients after an acute MI (Davies and Thomas, 1984). This result suggests that one of the ways of achieving a reduction in the quantity of thrombus is by altering the cellular contents of the thrombus. Numerous studies have revealed that, antiplatelet therapy resulted in a reduction of platelet activation, smaller mean platelet volume and low platelet counts, thereby altering the ultrastructure of the thrombus (Standeven *et al.*, 2005; Undas and Ariëns, 2011).

An important step in clot formation is thrombin production. This in turn stimulates the conversion of fibrinogen into a 3-dimensional fibrin fibre network. Further cross-linking and stabilisation of fibrin is mediated by thrombin-activated factor (F XIII). Clot structure has a role in atherothrombotic disease. Alterations in clot structures in coronary artery disease (CAD) was first demonstrated by Fatah et al in 1992 (Fatah *et al.*, 1992). In my study fibrin fibre architecture was altered by clopidogrel therapy in elderly and young, with and without T2DM. Individual fibrin fibre diameter increased following one week of clopidogrel therapy. It is already well established from previous studies that increase in fibrin fibre diameter favours fibrinolysis and reduces the stability of the

thrombus. Clots with thin fibers, small pores, and compact structure are associated with the development of premature and more severe coronary artery disease, which may be related to slower lysis of clots (Williams *et al.*, 1995; Collet *et al.*, 2000). Aspirin treatment has been demonstrated to promote an increase in fibrin fibre diameter (Ajjan *et al.*, 2009). Progressive fragmentation of the fibrin fibre as a result of lateral transection initially led to an increase in fibre diameter. This then resulted in progressive bending of the fibre, sometimes splaying of the edges, and finally disaggregation into small chunks (Collet *et al.*, 2000). Polymerisation of fibrin fibres results in a network of fibrins with stretching, twisting, branching and lateral aggregation. This has already been demonstrated by SEM and TEM (Weisel and Litvinov, 2013). The point where twisting of fibrin fibre occurs around an axis is termed as “hub”. The viscoelastic force in the fibrin strand is reduced at “hub” points as it limits the elastic recoiling of the fibres. With increasing hubs and branching points, the number of binding sites for fibrinolytic agonists such as tissue plasminogen activator (tPA) and plasmin increases, thereby reducing the threshold for fibrinolysis (Ajjan *et al.*, 2009). In my study, addition of clopidogrel to aspirin resulted in increased number of hubs and branches (“spokes”), thereby allowing the thrombus to respond well to fibrinolysis.

With increasing age there is heightened coagulation enzyme activity, accompanied by enhanced fibrin formation. The impaired fibrinolytic potential in old age has been described as a state of ‘‘thrombotic preparedness’’ (Gharacholou and Becker, 2009). Pretorius et al studied fibrin ultrastructure in healthy individuals with age > 75 years and compared it to healthy individuals < 25 years of age. They concluded that with age, major fibres become sparse and thinner and minor, thin fibres dominate forming a fine mesh-like structure. Thicker, fibrin fibres were also identified at irregular intervals. This is possibly due to enhanced fibrin formation and heightened coagulation enzyme activity (Pretorius *et al.*, 2010). In people with diabetes, non-enzymatic glycation of fibrinogen resulted in increased fibrin polymerisation, reduction in binding of tPA and plasminogen and slowing the conversion of plasminogen to plasmin, thereby increasing overall blood thrombogenicity (Dunn *et al.*, 2005).

My study has demonstrated a significant positive correlation between percentage platelet content and thrombus area from Badimon perfusion chamber. A significant negative correlation between fibrin fibre density and thrombus area was demonstrated at baseline and one week after clopidogrel therapy. At baseline, number of spokes per

square micron of thrombus demonstrated significant negative correlation with thrombus area. These findings suggest that reduction in platelet content reduces thrombus area. Increase in fibrin fibre density and number of spokes results in a reduction in thrombus possibly due to increased susceptibility to fibrinolysis as discussed below.

The viscoelastic properties of thrombus are some of the most sensitive measures that reflect small changes in clot structure (Weisel, 2007). I measured viscoelastic properties using thromboelastography (TEG®) and Platelet Mapping™. At baseline when patients were on aspirin alone, I demonstrated a significant negative correlation between fibrin fibre density and maximum strength of thrombus after stimulation with kaolin (MA), viscoelastic force of the thrombus (G) and rate of thrombin generation (TG). Increase in fibrin fibre density indirectly reflects increase in fibrin thickness and increase in total number of “hubs” and “spokes”, with overall increase in binding sites to fibrinolytic agonists. This reduced the overall viscoelasticity of the formed thrombus as published in previous studies (Collet *et al.*, 2005; Gersh *et al.*, 2009). VerifyNow® PRUz showed a significant reduction in PRU after the addition of clopidogrel for one week. However, no significant associations were noted between VerifyNow® indices and SEM parameters.

Multiplate® results demonstrated significant reduction in ADP AU and a trend towards reduction in ASP AU. I was able to demonstrate a significant positive correlation between percentage platelet rich area on the thrombus and ADP AU. This is not surprising, because the platelet inhibitory effect of clopidogrel reduces platelet aggregation and hence a reduction in platelet rich area. No significant associations were noted between Multiplate® indices and fibrin measurements based on SEM were noted. This could possibly be due to no impact or minimal impact of alterations in fibrin architecture on electrical impedance aggregometry.

Platelet diameter remained unchanged after a week of clopidogrel therapy both in elderly and young patients, in patients with T2DM and non DM cohorts. Mean platelet volume increases with increasing age and in the presence of T2DM (Lippi *et al.*, 2012). Higher MPV indicates larger platelet size, which in turn is deemed to suggest increased platelet activation. Little is known about effect of various antiplatelet agents on platelet size and mean platelet volume (MPV). Previous in vitro studies found no effect of aspirin on platelet size (Sharpe *et al.*, 1994; Jagroop *et al.*, 2003). However, it has been

demonstrated that clopidogrel significantly inhibits the ADP-induced increase in MPV in vitro (Jagroop and Mikhailidis, 2003), similar to the findings in my study. One previous study demonstrated that platelet diameter decreases with clopidogrel therapy in non-diabetic healthy volunteers (Papanas *et al.*, 2004). The possible explanations for unchanged platelet diameter in my study could be because in the elderly and T2DM populations, platelets are under constant stimulation by a number of naturally circulating agonists alongside low grade inflammation (Bruunsgaard *et al.*, 2001) and one week of maintenance dose clopidogrel may not be sufficient to cause any significant reduction in platelet size.

Platelet function tests such as TEG, VerifyNow and Multiplate are being used widely in clinical research and also in some specific clinical settings. My SEM substudy has demonstrated some correlation between ultrastructure characteristics of thrombus and some platelet reactivity indices.

My findings from this substudy are novel and the findings may be helpful to develop future studies. The main limitation in my study is that the sample size is very small and the patient cohort is carefully chosen with strict inclusion and exclusion criteria. For these reasons the findings from this study cannot be generalised. Experiments with newer antiplatelet agents (Ticagrelor and Prasugrel) need to be performed to assess their effect on fibrin architecture.

4.8 Rafigrelide study – Platelet dependant thrombosis and viscoelastic properties

4.8.1 Results

Rafigrelide, a novel, highly selective and potent platelet-lowering agent was used to reduce the platelet count. Baseline demographic data of participants in treatment period 1 and 2, panel A and panel B are shown in Table 4.80.

Platelet count:

Following administration of rafigrelide in Period 1, participants in Panel A demonstrated a reduction in platelet count (mean±SD) from 283±17 $x10^9$/L at baseline to 125±47 $x10^9$/L at Day 14. Rafigrelide was not administered after Day 14, and by Day 21 the platelet count had partially recovered to 206±48 $x10^9$/L, but remained significantly lower than at baseline. A similar pattern was seen in Panel A when the drug was co-administered with aspirin (Period 2); platelet count decreased from 280±36 $x10^9$/L at baseline to 134±27 $x10^9$/L at Day 14, and recovered to 239±41 $x10^9$/L after treatment cessation, at Day 21.

There was also a reduction in platelet count in treatment Period 1, in Panel B, from 250±25 $x10^9$/L at baseline to 160±51 $x10^9$/L at Day 14, when treatment was stopped. Platelet counts were taken until Day 18 for volunteers in Panel B, and no recovery was seen at this time point. Similar results were seen in Period 2, after co-treatment with rafigrelide and aspirin (Table 4.81, Figure 4.27and Figure 4.29).

Mean platelet volume:

Mean platelet volume (MPV) was assessed along with platelet count during all the visits (Table 4.81). In treatment period 1, following administration of rafigrelide, participants in panel A demonstrated an increase in MPV (mean±SD, fL) from 10.7±0.9 fL at baseline to 12.2±0.8 fL at day 14. Rafigrelide was no longer administered after day 14, and by day 21 mean MPV was 12±0.9 fL, higher than at baseline. A similar pattern was seen in panel A when the drug was co-administered with aspirin (period 2); mean MPV increased from 10.7±0.6 fL at baseline to 12.2±0.9 fL at day 14, and reduced to 11.8±0.8 fL after treatment cessation, at day 21.

There was also an increase in mean MPV in treatment period 1, in panel B, from 10.1±0.6 fL at baseline to 11.5±1.1 fL at day 14. MPVs were taken until day 18 for volunteers in panel B, and no recovery was seen after treatment cessation at day 14. Similar results were seen in period 2, after co-treatment of rafigrelide and aspirin.

Platelet-dependent thrombus formation:

Under both high and low shear stress conditions, treatment with rafigrelide generally led to reductions in thrombus area (Table 4.82, Table 4.83). These reductions tended to occur in conjunction with reductions in platelet count under both high and low shear conditions, with moderate correlations between the parameters (Figure 4.31).

Under high shear conditions, there was a reduction from baseline in mean thrombus area at Day 14 in Panel A after administration of rafigrelide alone (thrombus area in μ^2/mm, mean±SD: 22613 ± 12221 on Day 1 vs. 11930 ± 5348 on Day 14). Although there was a slight increase in thrombus area from Day 1 to Day 4 in Panel B (15894±10052 on Day 1 to 19414± 7870 on Day 4), by Day 10 a reduction was seen (14618±6312 on Day 10). When aspirin was added to rafigrelide in treatment period 2, a similar pattern of reduction in thrombus area was observed: Panel A: 25656±14324 on Day 1 to 14696±2446 on Day 14; Panel B: 24593±9401 on Day 1 to 14675±7331 on Day 10.There was no clear difference in high shear thrombus area between rafigrelide administered alone or in combination with aspirin in either panel (Figure 4.28, Figure 4.30).

Under low shear conditions, there were generally small reductions in mean thrombus area after rafigrelide treatment with or without aspirin in both panels but these reductions were not statistically significant.

There was a significant correlation between platelet count and high shear thrombus area (Figure 4.31).

Viscoelastic properties of thrombus assessed using Thromboelastography® (TEG) and Platelet Mapping™:

Administration of rafigrelide alone in Panel A increased time to initiate clot formation - R time (measured in min, mean±SD). In this group, the R time increased from 5.17 ± 0.80 min at baseline to 8.25 ± 2.33 min on Day 14. In Panel B, R time increased from

6.35 ± 1.29 min at baseline to 7.05 ± 2.09 min on Day 10. This increase was not statistically significant. After drug withdrawal, R time returned towards baseline to 6.07 ± 1.93 min on Day 21 for Panel A, but remained constant until Day 18 in Panel B (7.10 ± 2.24 min). A similar pattern was seen for Panel A when rafigrelide was co-administered with aspirin; R time increased from baseline to Day 14 and decreased after drug withdrawal. In Panel B, treatment with a combination of aspirin and rafigrelide had little effect on mean R time between Days 1 and 10 (8.48 ± 1.09 min on Day 1; 8.20 ± 1.78 min on Day 10) but there was a small reduction in R time by Day 18 in these participants (7.02 ± 2.65 min). Similar patterns were seen for K time.

On assessment of the effects of rafigrelide on MA (a measure of clot strength), in Panel A, MA decreased with time after initiation of treatment in Period 1 (mm, mean±SD, 68.8±2.3 on Day 1 to 63.4±5.8 on Day 14, $p<0.05$) and Period 2 (67.4±2.6 on Day 1 to 61.6±0.8 on Day 14, $p<0.05$) and increased again after treatment cessation indicating a reduction in clot strength with drug treatment that recovers after drug withdrawal. In Panel B, treatment with rafigrelide, both alone (Period 1) and when co-administered with aspirin (Period 2), demonstrated a similar effect on MA up to Day 18 of the study. Other parameters such as G Kdynes/sec (shear elastic force of the thrombus) and Clot index (CI) also demonstrated a similar trend like MA, when treated with rafigrelide, both alone and when co-administered with aspirin.

Rafigrelide with or without aspirin had no discernible effect on lysis as measured by clot lysis 30, clot lysis 60 and estimated plasma lysis.

Platelet mapping™ tests were performed alongside baseline TEG®. Maximum viscoelastic strength of thrombus as measured by maximum amplitude upon stimulation with 10µl of arachidonic acid (MA-AA, in mm) demonstrated a numerical reduction when rafigrelide was co-administered with aspirin (Period 2). Panel A: 56.8±7.5 on Day 1 to 47.2±6.3 on Day 14; Panel B: 62.5±3.7 on Day 1 to 38.4±17.8 on Day 10, $p<0.01$. Percentage inhibition to arachidonic acid (% inhibition to AA, %) also increased proportionately when aspirin was co-administered with rafigrelide (Table 4.84, Table 4.85). No significant change in MA-AA or percentage inhibition to AA was observed when rafigrelide was administered alone (Figure 4.32, Figure 4.33, Figure 4.34 and Figure 4.35).

Pharmacokinetics:

Plasma concentrations of rafigrelide were generally similar in both panels when administered alone (Period 1) or in combination with aspirin (Period 2) (Table 4.86).

Tolerability, adverse events and safety:

Most of the AEs during both treatment periods were mild to moderate in severity, and were considered to be related to the IMP (rafigrelide), but rarely led to drug withdrawal. Although the overall incidence and number of AEs was slightly higher when rafigrelide was administered in combination with aspirin, this was primarily due to single event instances, in different volunteers, of palpitations, nasopharyngitis, contusion, migraine, cough, nasal congestion and four instances (in one participant) of mild photophobia. The most common AEs (occurring in at least three individuals during any period) for rafigrelide both as monotherapy and in combination with aspirin were thrombocytopenia (defined as a platelet count $<150x10^9$/L; 9 events and 10 events, respectively) and headache (11 events and 14 events, respectively).

One participant developed severe thrombocytopenia with platelet count falling to $82x10^9$/L on Day 14 (last dosing day).The platelet count continued to fall to below the clinically important level ($75x10^9$/L) to $50x10^9$/L on Day 17 and to $39x10^9$/L on Day 18. This was reported as a serious AE although the participant remained asymptomatic. Without intervention his platelet count increased to $43x10^9$/L on Day 19, $76x10^9$/L on Day 20, $144x10^9$/L on Day 21 and $410x10^9$/L on Day 27. In another participant, rafigrelide was withdrawn on Day 10 because his reduced platelet count ($140x10^9$/L) met the protocol-defined level for cessation of the IMP (platelet count below $150x10^9$/L or reduction of $\geq 60x10^9$/L over 3 days). Subsequently, his platelet count recovered to baseline levels without intervention.

Demographic data Mean ± SD	Treatment period 1 Panel A (n=6)	Treatment period 2 Panel A (n=7)	Panel B (n=6)
Age (years)	34.2 ± 5.4	30.9 ± 4.8	34.3 ± 7.7
Body Mass Index (Kg / m2)	25.7 ± 2.7	25.2 ± 7.4	25.3 ± 2.4
Systolic B.P. (mmHg)	126 ± 7.7	125 ± 7.4	125 ±12.3
Diastolic B.P. (mmHg)	80 ± 4.5	78 ± 5.3	82 ± 9.4
Platelet count (10^9/L)	283 ± 16.8	271 ± 27.2	250 ± 24.6
Mean platelet volume (fL)	10.7 ± 0.9	11.7 ± 0.7	10.1 ± 0.6
Glucose (mmol/L)	4.8 ± 0.5	4.9 ± 0.6	4.6 ± 0.7
Creatinine (µmol/L)	85 ± 9.0	85 ± 6.2	83 ± 13.2

Table 4.80 Baseline characteristics of healthy volunteers in Rafigrelide study

Mean±SD	Day 1	Day 4	Day 7	Day 10	Day 14	Day 18	Day 21
Treatment Period 1 – Rafigrelide alone Panel A							
Platelet count x 10^9/L	283±16.8	276±32.1	244±14.5 *	183±16.5 *	125±46.9 *		206±47.8 *
MPV, fL	10.7±0.9	10.6±1.1	10.7±0.8	11.3±0.7 *	12.2±0.8 *		12.0±0.9 *
Panel B							
Platelet count x 10^9/L	250±24.6	252±27.4	226±18.3 *	196±37.4 *	160±50.6 *	160±57.5 *	
MPV, fL	10.1±0.6	10.2±0.7	10.3±0.7	10.8±0.9 *	11.5±1.1 *	11.6±1.0 *	
Treatment Period 2 – Rafigrelide and aspirin Panel A							
Platelet count x 10^9/L	280±35.9	275±52.1	254±45.6	191±32.5	134±27.0		239±41.1
MPV, fL	10.5±0.5	10.4±0.6	10.9±0.5	11.1±0.5	12.2±0.9		11.5±0.9
Panel B							
Platelet count x 10^9/L	244±16.2	248±28.4	235±29.7	198±29.6	166±46.9	171±48.9	
MPV, fL	10.1±0.8	10.4±0.9	10.3±1.0	10.7±0.8	11.3±1.0	11.6±1.2	

Table 4.81 Effect of Rafigrelide on platelet count and MPV - treatment period 1 and 2; *p<0.05

Mean±SD	Day 1	Day 7	Day 14	Day 21
Treatment Period 1 – Rafigrelide alone Panel A				
High shear thrombus area, μ^2/mm	22613± 12221	17784± 11045 *	11930± 5348	15518± 3728
Low shear thrombus area, μ^2/mm	8233± 1471	7444± 1018	6997± 2586	6821± 1683
Treatment Period 2 – Rafigrelide and aspirin Panel A				
High shear thrombus area, μ^2/mm	25656± 14324	16321± 6598	14696± 2446	17785± 7707
Low shear thrombus area, μ^2/mm	8188± 2555	8286± 1398	7670± 1353	7504± 3013

Table 4.82 Effect of Rafigrelide on thrombus area - treatment period 1 and 2 in panel A

*p<0.05

Mean±SD	Day 1	Day 4	Day 10	Day 18
Treatment Period 1 – Rafigrelide alone Panel B				
High shear thrombus area, μ^2/mm	15894± 10052	19414± 7870	14618± 6312	12526± 5590
Low shear thrombus area, μ^2/mm	7268± 2727	7658± 3143	6040± 1991	6192± 1733
Treatment Period 2 – Rafigrelide and aspirin Panel B				
High shear thrombus area, μ^2/mm	24593± 9401	18834± 7738	14675± 7331 *	12850± 4239 *
Low shear thrombus area, μ^2/mm	9438± 2286	8324± 2183	7733± 1309	7980± 2074

Table 4.83 Effect of Rafigrelide on thrombus area - treatment period 1 and 2 in panel B

*p<0.05

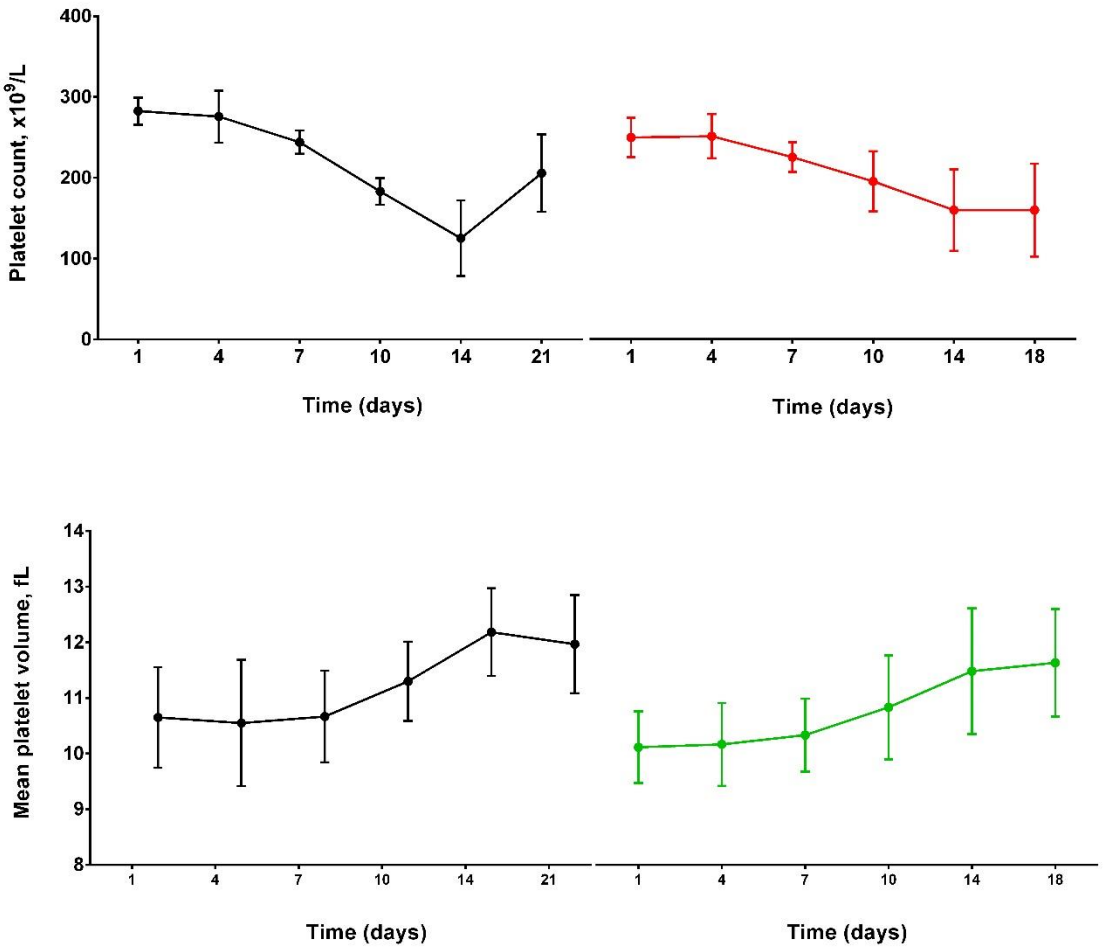


Figure 4.27 Effect of rafigrelide on platelet count and MPV - treatment period 1. Panel A and B

Treatment with rafigrelide was stopped on Day 14 for both panels

Panel A (top left and bottom left graphs) and B (top right and bottom right graphs)

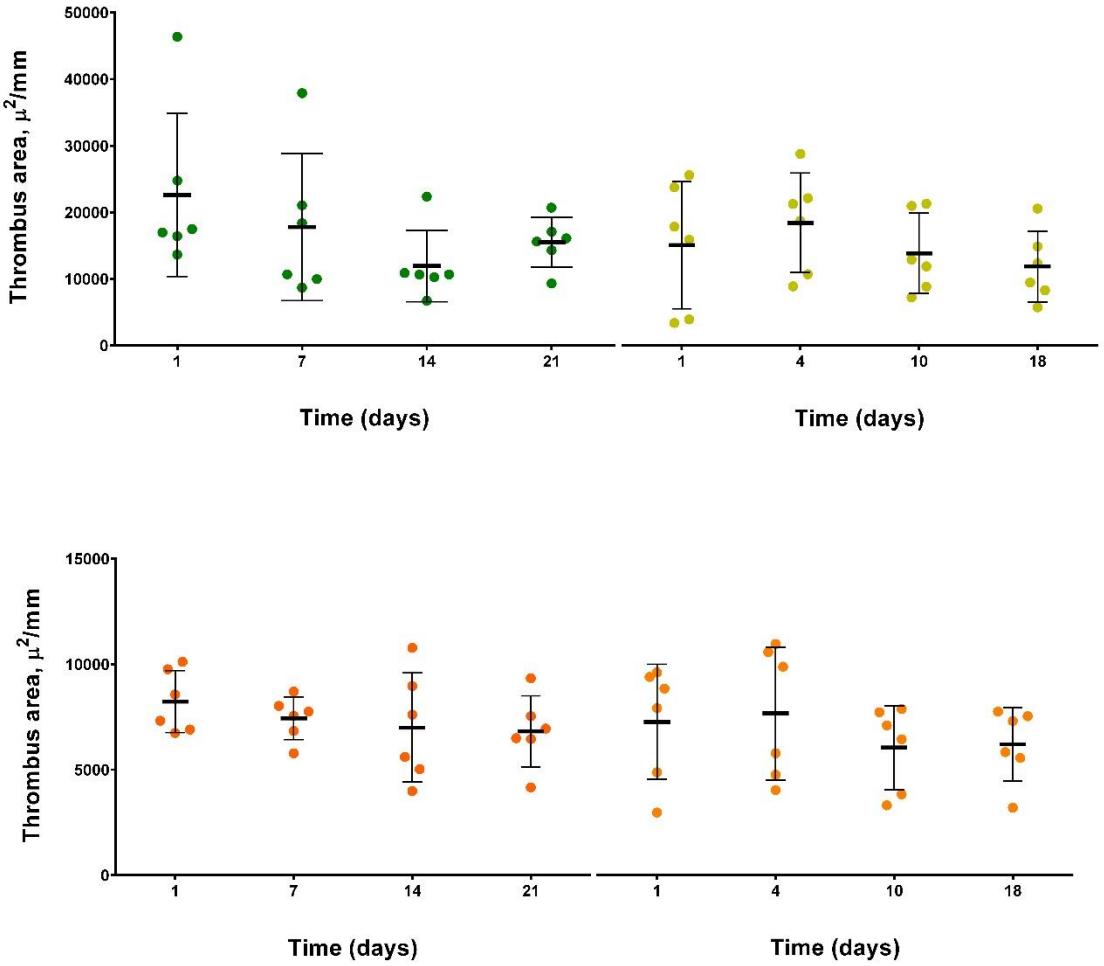


Figure 4.28 Effect of rafigrelide on high and low shear thrombus - treatment period 1. Panel A & B

Treatment with rafigrelide was stopped on Day 14 for both panels

Panel A (top left and bottom left graphs) and B (top right and bottom right graphs)

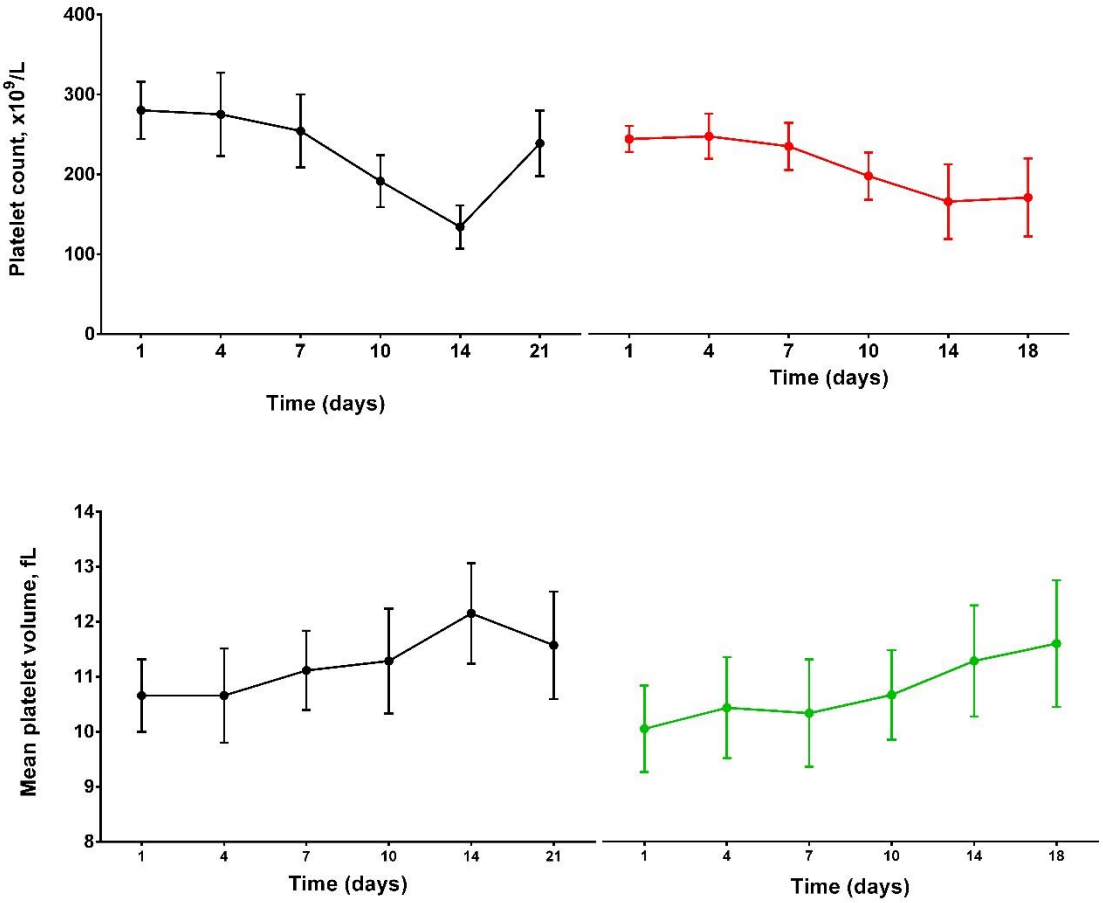


Figure 4.29 Effect of rafigrelide + aspirin on platelet count & MPV - treatment period 2. Panel A & B

Treatment with rafigrelide was stopped on Day 14 in both the panels

Aspirin administration was stopped on Day 20 for Panel A and Day 17 for Panel B during treatment period 2

Panel A (top left and bottom left graphs) and B (top right and bottom right graphs)

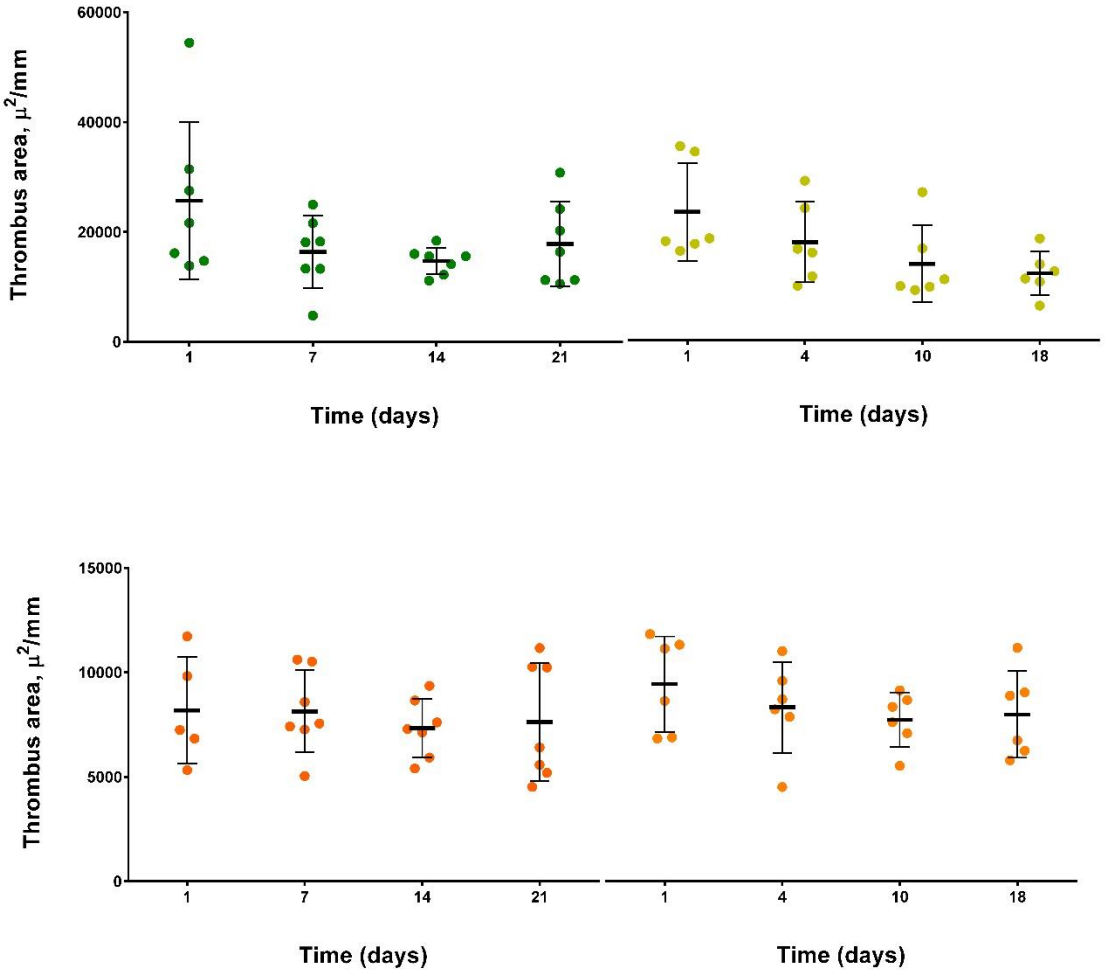


Figure 4.30 Effect of rafigrelide on high and low shear thrombus - treatment period 2. Panel A and B

Treatment with rafigrelide was stopped on Day 14 for both panels

Aspirin administration was stopped on Day 20 for Panel A and Day 17 for Panel B during treatment period 2

Panel A (top left and bottom left graphs) and B (top right and bottom right graphs)

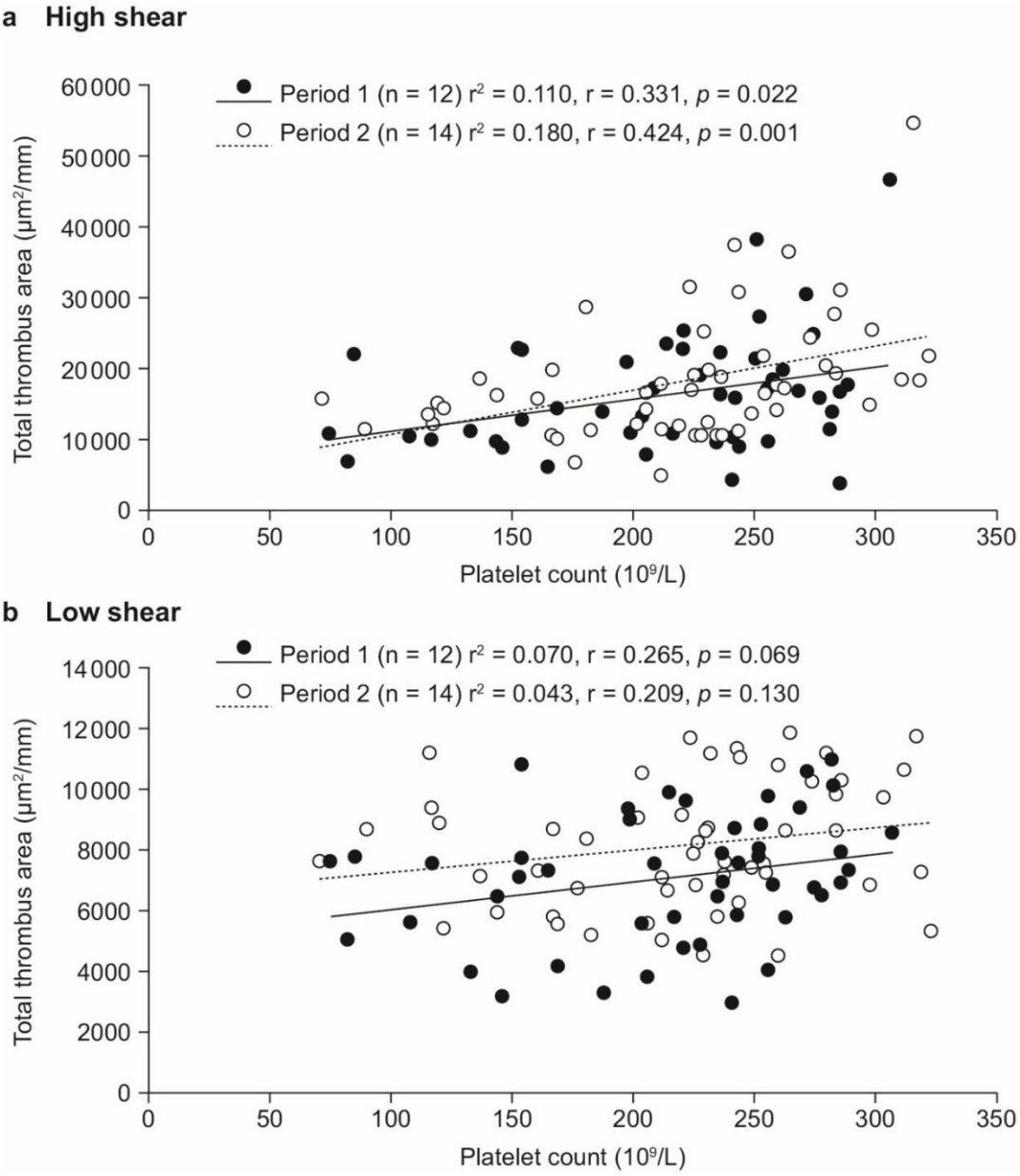


Figure 4.31 Correlation between thrombus area and platelet count

Relationship between the total thrombus area and platelet count under high shear (A) and low shear (B) conditions when platelet levels were lowered with rafigrelide (Period 1) and rafigrelide in combination with aspirin (Period 2)

Data shown are measurements provided by all participants at each time point in their respective panels (Panel A: Days 1, 7, 14 and 21; Panel B: Days 1, 4, 10 and 18).

Regression lines are shown with correlation coefficients (r, r^2) and *p*-values

Mean±SD	Day 1	Day 7	Day 14	Day 21
Treatment Period 1 – Rafigrelide alone; Panel A				
R, min	5.2±0.8	6.5±2.1	8.2±2.3 *	6.1±1.9
K, min	1.7±0.3	2.2±0.7	2.8±1.0	1.8±0.6
MA, mm	68.8±2.3	66.3±4.2	63.4±5.8 *	69.3±3.4
G Kdynes/sec	11.1±1.2	10.1±2.1	9.0±2.3 *	11.4±1.8
CI	1.2±1.1	-0.2±2.5	-2.3±3.2 *	1.0±2.0
MA-A, mm	5.0±1.6	5.8±3.5	10.8±13.9	7.8±5.8
MA-AA, mm	61.0±6.4	57.3±9.8	54.0±8.9 *	60.6±8.5
MA-ADP, mm	56.9±9.7	55.2±11.5	50.2±11.7*	60.2±3.9
% inhibition to AA	12.3±7.3	16.0±13.0	15.3±8.8	13.9±8.9
% inhibition to ADP	18.8±12.5	18.3±4.8	23.6±12.9	14.9±4.5
Treatment Period 2 – Rafigrelide and aspirin; Panel A				
R, min	6.0±1.7	6.7±2.0	7.8±1.5	7.0±1.6
K, min	2.2±0.4	2.7±1.0	3.1±0.4	2.5±0.6
MA, mm	67.4±2.6	64.4±5.8	61.6±0.8	66.1±4.6
G Kdynes/sec	10.4±1.3	9.3±2.1	8.0±0.3	10.0±2.4
CI	0.3±1.5	-1.1±1.7	-2.6±1.4	-0.8±2.0
MA-A, mm	8.3±8.8	4.6±1.5	10.1±13.8	13.2±13.9
MA-AA, mm	56.8±7.5	43.3±8.9	47.2±6.3	54.3±14.6
MA-ADP, mm	59.0±9.5	57.4±5.7	55.1±6.4	62.2±5.0
% inhibition to AA	14.6±12.9	11.6±4.4	9.4±9.8	7.6±4.5
% inhibition to ADP	18.5±9.9	34.6±17.8	27.6±8.5	22.8±20.2

Table 4.84 Changes to TEG-PM parameters - Panel A, treatment period 1 & 2

*p<0.05

Mean±SD	Day 1	Day 4	Day 10	Day 18
Treatment Period 1 – Rafigrelide alone; Panel B				
R, min	6.4±1.3	6.4±2.0	7.1±2.1	7.1±2.2
K, min	1.9±0.4	1.9±0.4	2.3±0.8	2.3±0.8
MA, mm	67.4±3.6	68.5±2.9	65.9±4.2	66.6±2.9
G Kdynes/sec	10.5±1.7	11.0±1.5	9.8±1.8	10.1±1.3
CI	0.4±1.7	0.4±1.8	-0.7±2.7	-0.9±2.7
MA-A, mm	6.7±3.2	6.2±1.9	8.3±6.9	9.0±5.9
MA-AA, mm	60.1±5.6	58.7±4.0	58.3±6.2	60.8±5.1
MA-ADP, mm	58.5±4.4	58.8±5.1	56.3±7.9	58.2±6.7
% inhibition to AA	12.4±8.3	15.7±6.7	14.2±9.3	10.1±9.1
% inhibition to ADP	14.7±6.3	15.5±6.9	17.1±9.5	14.5±9.4
Treatment Period 2 – Rafigrelide and aspirin; Panel B				
R, min	8.5±1.1	8.6±2.0	8.2±1.8	7.0±2.6
K, min	2.6±0.5	2.7±0.6	2.5±0.7	2.8±1.0
MA, mm	66.6±3.1	66.3±2.4	66.5±2.3	66.0±3.3
G Kdynes/sec	10.1±1.3	9.9±1.1	9.9±1.0	9.8±1.3
CI	-1.9±1.5	-2.0±2.1	-1.6±2.1	-1.2±2.9
MA-A, mm	6.5±2.4	7.3±2.7	8.1±5.1	10.9±12.8
MA-AA, mm	62.5±3.7	45.6±19.3	38.4±17.8 *	41.5±20.0
MA-ADP, mm	60.5±2.3	59.4±.3.2	57.3±4.3	57.8±4.0
% inhibition to AA	8.3±6.4	37.8±27.4	48.0±27.1 *	42.0±29.4
% inhibition to ADP	9.8±5.3	11.6±6.1	15.8±7.6	15.0±6.8

Table 4.85 Changes to TEG-PM parameters - Panel B, treatment period 1 & 2

*p<0.05

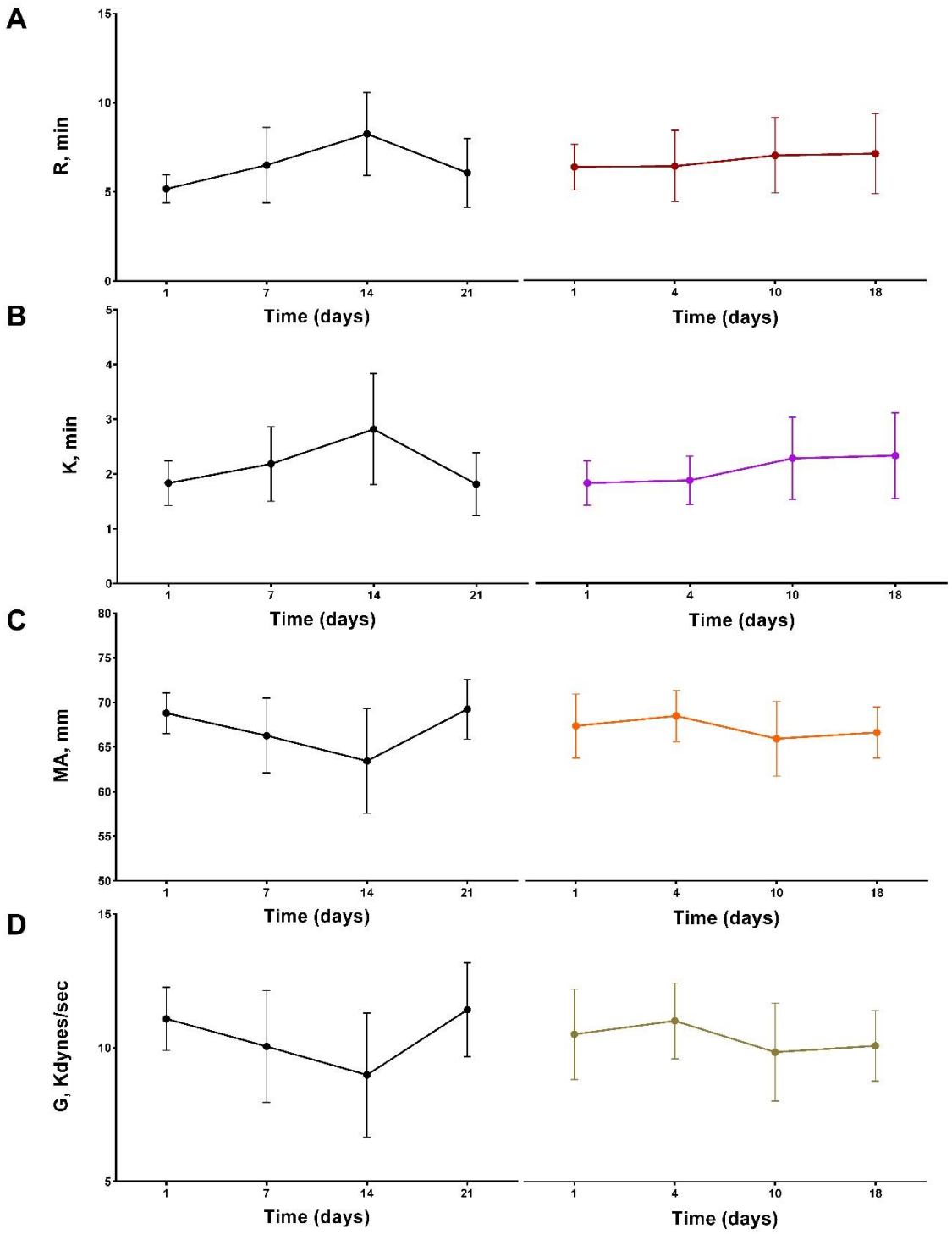


Figure 4.32 Effect of rafigrelide alone on TEG® parameters in panel A & B

Treatment with rafigrelide was stopped on Day 14 for both panels

Panel A (all graphs on the left) and B (all graphs on the right)

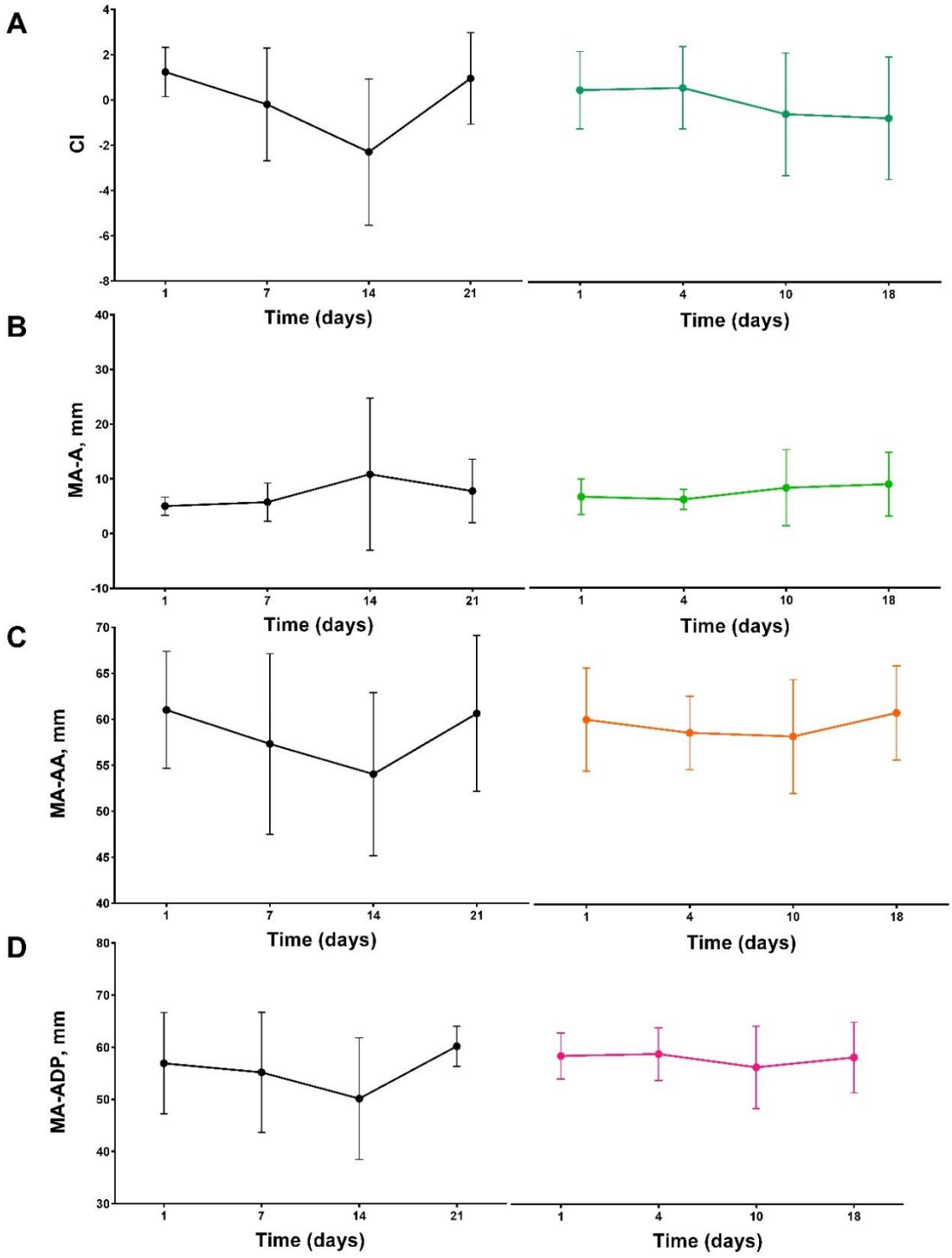


Figure 4.33 Effect of rafigrelide alone on TEG®-PM™ indices in panel A & B

Treatment with rafigrelide was stopped on Day 14 for both panels

Panel A (all graphs on the left) and B (all graphs on the right)

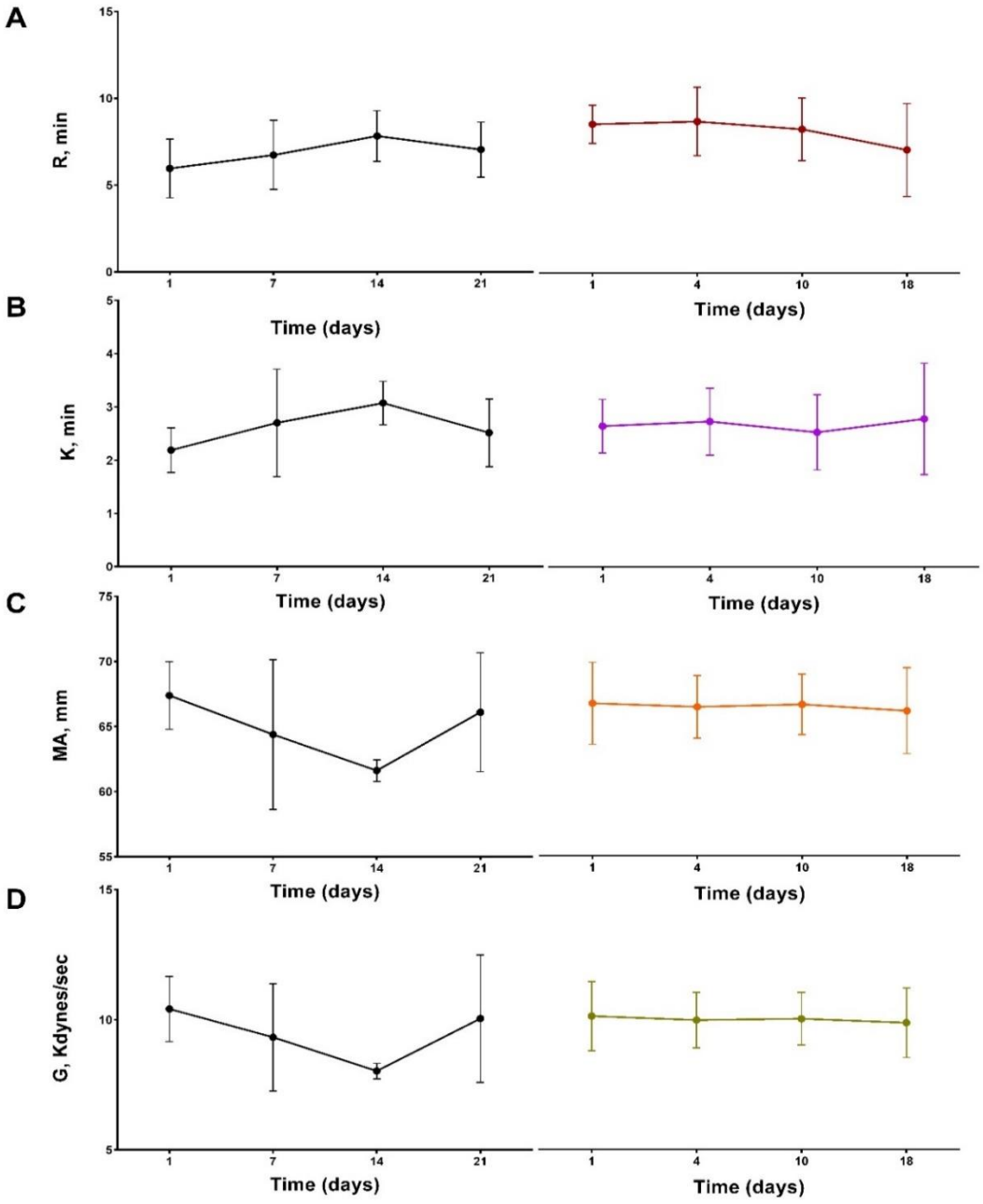


Figure 4.34 Effect of rafigrelide and aspirin on TEG® parameters in panel A & B

Treatment with rafigrelide was stopped on Day 14 for both panels

Aspirin administration was stopped on Day 20 for Panel A and Day 17 for Panel B during treatment period 2. Panel A (all graphs on the left) and B (all graphs on the right)

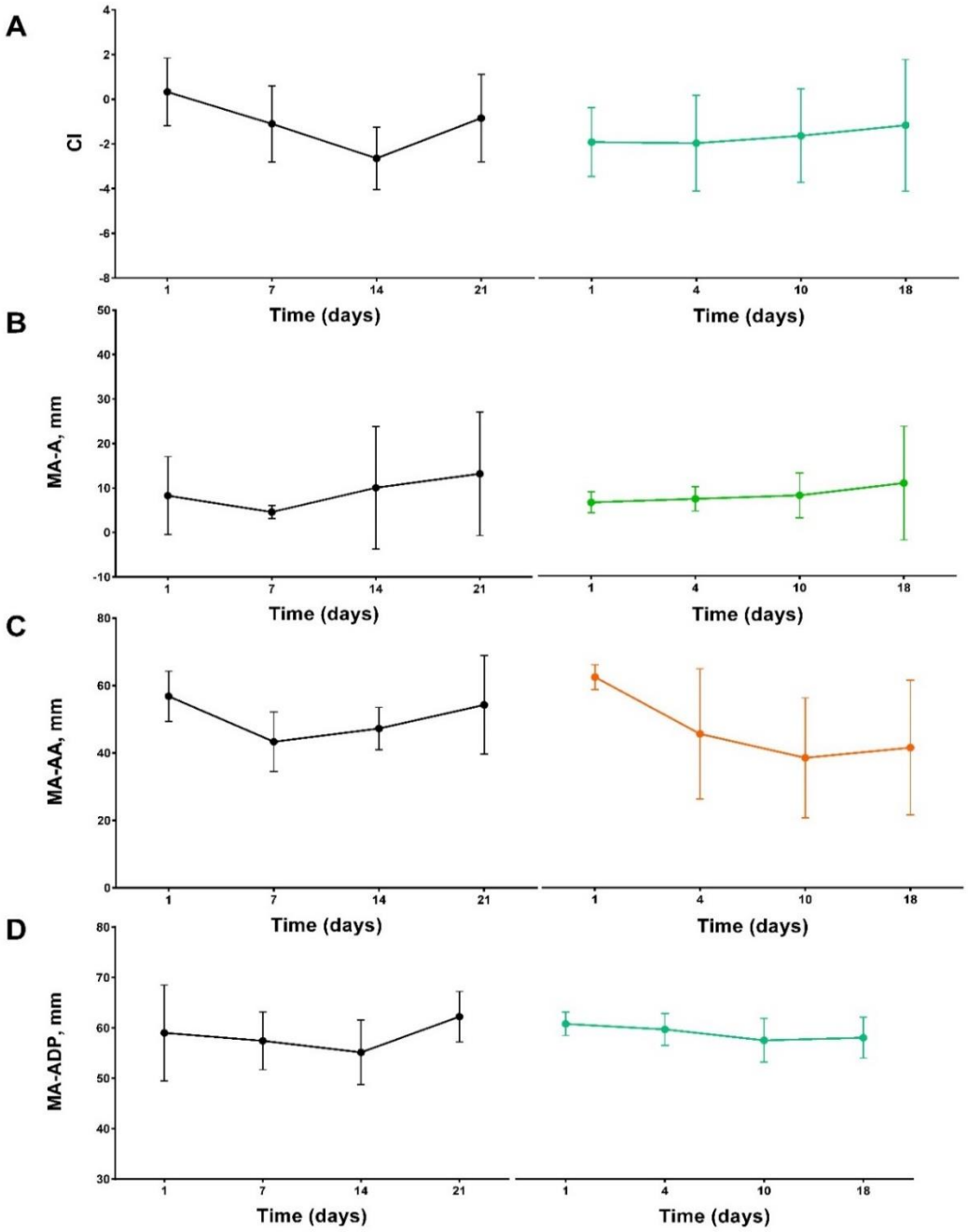


Figure 4.35 Effect of rafigrelide and aspirin on TEG®-PM™ indices in panel

A & B

Treatment with rafigrelide was stopped on Day 14 for both panels

Aspirin administration was stopped on Day 20 for Panel A and Day 17 for Panel B during treatment period 2. Panel A (all graphs on the left) and B (all graphs on the right)

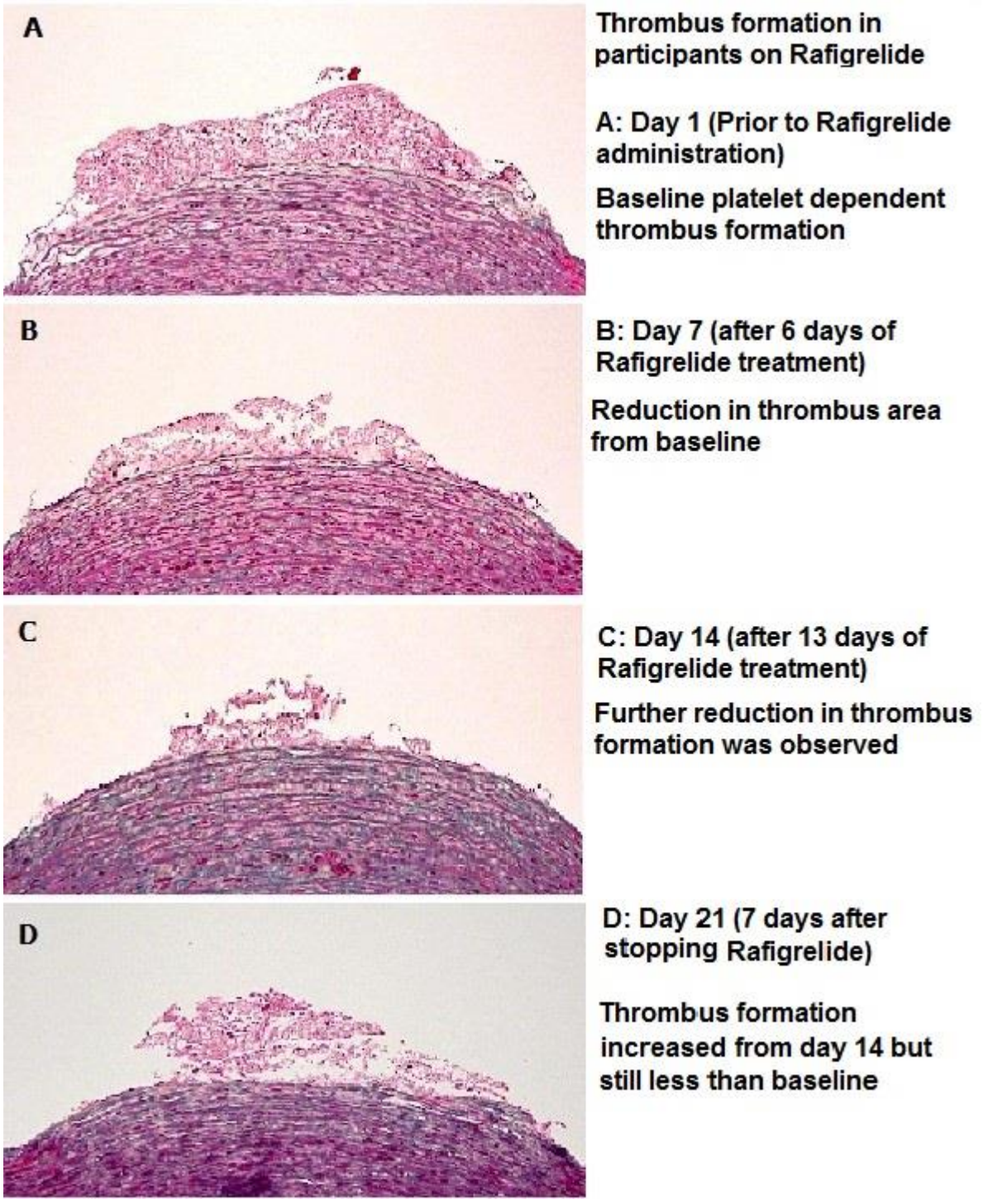


Figure 4.36 Reduction in high shear thrombus area with rafigrelide (Period 1, Panel A).

There is a stepwise reduction in thrombus formation with Rafigrelide administration until day 14. After stopping the IMP on day 14, thrombus formation increased on day 21 but was still less than baseline. These are sample images from one of the volunteers in the study.

		Plasma rafigrelide concentration (mean±SD, in ng/mL)			
		Treatment day			
		1	7	14	21
Panel A	n	6	6	6	6
	Period 1	0.00±0.00	64.83±20.91	67.02±20.01	3.08±2.05
	n	8	8	7	7
	Period 2	6.21±17.57	56.01±19.81	44.21±22.42	1.79±1.94
		Treatment day			
		1	4	10	18
	n	6	6	6	6
Panel B	Period 1	0.00±0.00	36.05±12.72	40.47±17.32	5.85±4.29
	n	6	6	6	6
	Period 2	0.00±0.00	26.90±16.59	41.35±13.74	5.57±4.95

Table 4.86 Pharmacokinetics of rafigrelide

Plasma concentrations of rafigrelide throughout the study

4.8.2 Discussion

In this early phase study of rafigrelide, a novel platelet lowering agent, we report a reduction in circulating platelets and thrombus size together with delayed initiation of clot formation and reduced clot strength. The addition of aspirin to rafigrelide did not affect thrombus area or standard TEG parameters. Platelet mapping demonstrated a reduction in MA-AA and percentage inhibition to AA in Panel B from Day 1 to Day 10. But these parameters did not change in Panel A despite addition of aspirin in treatment period 2 (Balasubramaniam *et al.*, 2014).

The Badimon perfusion chamber system is a validated *ex vivo* model of vessel injury and thrombosis as explained before (Badimon *et al.*, 1986; Badimon *et al.*, 1987) and has been used to evaluate the effects of novel antithrombotic agents in human participants in different disease states (Lev *et al.*, 2001; Osende *et al.*, 2001; Sarich *et al.*, 2003). The model enables the measurement of thrombus formation in native (non-anticoagulated) whole blood triggered by exposure to physiologically relevant substrate (collagen in tunica media), and under different rheological conditions mimicking flow in stenosed coronary arteries. Thromboelastography is a point of care assay that assesses visco-elastic forces in whole blood under low shear conditions during coagulation *in vitro*. It measures the strength of the thrombus (fibrin-platelet binding) as it forms and is widely used in different clinical areas (Kang *et al.*, 1985; Gorbet and Sefton, 2004; Reikvam *et al.*, 2009).

The role of platelets in thrombus formation is well established and has been explained in detail earlier. Briefly, platelets become activated when they come into contact with a thrombogenic surface such as injured endothelium or an artificial surface such as a stent, vascular graft, or cardiopulmonary or haemodialysis equipment (Gorbet and Sefton, 2004). This in turn triggers a complex cascade involving cellular and non-cellular components leading to thrombus formation at the site of injury (Blockmans *et al.*, 1995). Platelet count has been associated with thrombosis and the role of platelets as mediators and regulators of inflammation in thrombotic events has been well established (Hamad *et al.*, 2012). The contribution of P-selectin-mediated platelet-leukocyte aggregates and tissue-factor-positive platelet-derived microparticles in atherothrombosis, has also been studied

extensively (Zarbock *et al.*, 2007; Morel *et al.*, 2008).

Successful antithrombotic therapy balances improvements in ischaemic outcomes whilst minimising bleeding risk. Aspirin and clopidogrel exert significant but relatively weak antiplatelet effects when compared to other P2Y12 inhibitors (ticagrelor and prasugrel) and GP IIb/IIIa inhibitors like abciximab. However, this weak antiplatelet effect is balanced with an acceptable bleeding risk and this is partly the reason behind their success as standard antiplatelet agents (Jernberg *et al.*, 2006; Wiviott *et al.*, 2007b). Despite optimal therapy with modern antiplatelet and antithrombotic agents, patients presenting with atherothrombotic disease typically continue to have high rates of recurrent events up to one year after initiating therapy. Hence there is a need for improvement in the currently available antithrombotic therapies. Newer therapies such as factor Xa inhibitors, direct thrombin inhibitors, nitric oxide donors, and collagen- and ristocetin-mediated platelet aggregation inhibitors are in various stages of development (Cannon *et al.*, 2007; Zafar *et al.*, 2007a; Zafar *et al.*, 2007b; Buller *et al.*, 2008; Zafar *et al.*, 2010).

Our data show rafigrelide to have platelet-lowering properties and that these translate into a reduction in platelet-dependent thrombosis. Although mean platelet counts were similar to baseline at Day 4, steady reductions were observed thereafter until treatment cessation. Following abrupt cessation of rafigrelide treatment on Day 14, the platelet count remained low for 3 to 4 days before increasing toward baseline levels. Platelet counts had generally returned to baseline levels at the final follow-up visit.

The reduction in platelet count was associated with a reduction in mean thrombus area in both low and high shear environments, mimicking rheologies of arterial and venous flow conditions, respectively. Under high shear conditions, the reduction in thrombus area was more pronounced and there was a moderate correlation between platelet count and thrombus area, as would be expected given the greater role of platelets in arterial thrombosis (platelet rich) compared with thrombus formation in veins (fibrin rich) under low shear conditions.

Seven days after the last dose of rafigrelide, reversal of the antithrombotic effect was evident. With the addition of aspirin, there were no discernible additional

effects on platelet count or TTA beyond that seen with rafigrelide alone. Rafigrelide delayed initiation of clot formation (R time), time taken to form a firm thrombus (K time) and provided small reductions in MA (a measure of clot strength), G (shear elastic force) and CI (over all measure of thrombus strength).

The adverse events reported with rafigrelide over each 2-week treatment period were mild to moderate in nature, although one serious case of thrombocytopenia was observed, which resolved after IMP cessation and without any additional intervention. The platelet count fell below the protocol-defined threshold for study termination in only two individuals. There were no bleeding complications in any of the participants during the treatment or follow-up periods. The occurrence of photophobia, reported in one individual in this study, is another AE that may be of interest in future studies. Overall, in this early phase study of the pharmacodynamic and pharmacokinetic effects of rafigrelide on healthy volunteers, the product was found to be well tolerated.

In conclusion, this new platelet-lowering agent rafigrelide showed antithrombotic properties during a 2-week treatment period in healthy male volunteers. Importantly, the reductions in thrombus formation were seen in surrogate models of both high and low shear rates suggesting drug efficacy in different arterial conditions. The antithrombotic effect of rafigrelide in this early phase study suggests that further clinical studies involving different doses of rafigrelide, longer therapy duration and measuring other elements of coagulation system to confirm its mechanism of action are warranted. Lowering platelet count, especially in patients with thrombocytosis or platelet counts in the higher end of normal might be an option to further reduce future adverse thrombotic events following acute coronary syndrome alongside antiplatelet therapy. Possible adverse reaction such as thrombocytopenia and bleeding should be regularly checked for.

Limitations

In addition to platelets, a number of cellular and non-cellular components of the coagulation system are also involved in thrombus formation and these were not measured in our study. Though we measured platelet numbers and MPV, neither platelet reactivity nor fibrinogen were measured.

Chapter 5 Conclusion

5.1 General discussion

The main objective of my first study was to determine the effect of age and age plus T2DM on platelet-dependent thrombus (PDT) formation in patients with stable CAD and to compare the effects of dual antiplatelet therapy on PDT. My hypothesis was that dual antiplatelet therapy (DAPT) with aspirin and clopidogrel inhibits PDT formation more powerfully in subjects aged ≥75 years than in younger individuals but in the presence of T2DM, the effect of age is reduced.

My study did not demonstrate any difference in thrombus area between elderly and young and between patients with and without T2DM at baseline (on aspirin monotherapy). After one week of DAPT, there was a significant reduction in PDT formation in both elderly and young, and in those with and without T2DM. Thrombus area was again similar between the groups after one week of DAPT, as was the change in thrombus area with DAPT. Thus my hypothesis was disproved.

The VerifyNow® point of care assay demonstrated a higher proportion of hyporesponders (high platelet reactivity) to aspirin in the elderly cohort and in those with T2DM at baseline. After one week of DAPT, the mean platelet reactivity to clopidogrel remained high (≥240 cut-off) in the elderly and in those with T2DM suggesting platelet hyporesponsiveness to clopidogrel. An interesting observation was that even among these hyporesponders there was a significant reduction in PDT after one week of DAPT. Addition of clopidogrel appeared to potentiate the antiplatelet effect of aspirin in the young cohort but this effect was not demonstrated in the elderly patients.

Standard thromboelastography (TEG®) parameters were similar between the groups both at baseline and after one week of DAPT. The elderly non-diabetic cohort took longer to initiate clot formation and they demonstrated less over-all clot strength after the addition of clopidogrel. Platelet Mapping™ data demonstrated much better platelet inhibition to aspirin at baseline in the elderly cohort, a finding different to that from VerifyNow® data. The antiplatelet response to clopidogrel was similar between the groups using platelet mapping indices.

The Multiplate® point of care assay demonstrated that the highest percentage of clopidogrel hyporesponders was in the elderly T2DM cohort. After addition of clopidogrel, significant platelet inhibition occurred to a similar degree in all the groups but the mean ADP AU remained ≥460 in the elderly T2DM cohort.

All three above mentioned point of care assays are platelet centric, mainly assessing platelet response to various agonists, whereas the Badimon chamber thrombus area gives a composite measure of all cellular and non-cellular components involved in thrombus formation and thus forms a more holistic assessment of blood thrombogenicity. This might be one reason why I was not able to demonstrate a significant difference in thrombus area between the groups, despite high platelet reactivity in the elderly and T2DM patients. A sample size of around forty stable CAD patients on appropriate secondary prevention therapy in each of the four groups might not be adequate enough to demonstrate effects of age or T2DM on thrombus area. However, this sample size was adequate to demonstrate a significant reduction in thrombus area with one week of DAPT when compared to aspirin monotherapy, suggesting that platelet reactivity is one of the major contributing factors to blood thrombogenicity.

Despite reduction in thrombus area, platelet reactivity remained high after one week of DAPT in elderly and T2DM patients in the stable CAD setting. This high residual platelet activity due to constant platelet activation, might be one of the reasons for increased atherothrombotic events in these high risk population groups. A significant reduction in PDT after one week of DAPT even among hyporesponders, highlights the fact that platelet reactivity indices do not exactly correlate with thrombus burden and might not be useful in predicting future thrombotic events, at least in patients with stable CAD.

Coagulation biomarkers (P selectin, CD40L and PAI-1) were similar between elderly and young cohort, both with and without T2DM. CD40 ligand levels were reduced in elderly, young and in non-diabetic patients after addition of clopidogrel. This reduction was not seen in T2DM cohort. An interesting finding in my study was a significant increase in these biomarkers measured from post chamber “effluent” blood in all the groups, suggesting the release of these biomarkers from platelets granules within seconds after platelet activation within the chamber.

Inflammatory biomarkers (IFNy and IL-1) were similar between the groups. TNFα levels and IL-6 levels were higher in elderly T2DM compared to young and non DM individuals. These levels remained elevated in elderly T2DM cohort after DAPT for one week. Post chamber “effluent” blood demonstrated a reduction in IL-6 levels, possibly due to its consumption in the process of thrombus formation.

Scanning electron microscopic analysis of fibrin architecture demonstrated a reduction in platelet content of thrombus following one week of clopidogrel therapy. Significant increase in fibrin diameter, fibrin fibre density, number of hubs and spokes per square micron of thrombus were demonstrated following one week of clopidogrel. These changes favour fibrinolysis. Though not significant, the elderly cohort had thinner fibrin fibres than younger patients. This is the first study to assess fibrin ultrastructure in whole blood thrombus from elderly patients with stable CAD. Prospective, long term outcome and follow up studies are necessary to see if fibrin fibre parameters can assess the response to antiplatelet therapy and predict future adverse cardiovascular outcomes in elderly and T2DM. The association between viscoelastic properties of thrombus and fibrin fibre parameters should be studied further to explore opportunities for new therapeutic target in high risk populations (elderly and T2DM).

Overall, elderly patients compared to young have demonstrated similar thrombus area but high platelet reactivity using VerifyNow® and Multiplate® and more hyporesponders to aspirin and clopidogrel. Significant release of coagulation markers from platelet granules after activation were demonstrated in both elderly and young patients with and without T2DM. DAPT demonstrated a significant but similar reduction in PDT in all the four groups. Addition of clopidogrel for 7 days, resulted in similar changes to fibrin architecture in all the four groups. These changes included increase in fibrin diameter, fibrin fibre density and number of hubs and spokes, all favouring fibrinolysis. The reduction in thrombus formation was not associated with any increase in bleeding complications during and one week after the study period.

The elderly population with CAD has never been extensively studied as far as thrombus formation, platelet reactivity indices and antiplatelet therapies are

concerned. The findings from this elderly stable CAD study are important for the following reasons:

iv. This is the first study assessing and comparing platelet dependent thrombus formation using the Badimon chamber, in stable CAD patients with age<75 years and age≥75 years, with and without T2DM

v. All participants were on appropriate secondary prevention therapy including maintenance dose aspirin; clopidogrel was added for 1 week as dual antiplatelet therapy (DAPT)

vi. Participants were carefully chosen for the study with strict inclusion and exclusion criteria to try to minimise confounding variables

vii. To achieve a significant reduction of thrombus area with DAPT in all the 4 groups of patients with stable CAD and good secondary prevention therapy (good lipid control, good hypertension control and good glycaemic control) is clinically very important

viii. The data describe the effect of dual antiplatelet therapy on thrombus formation and platelet function in stable CAD patients with age≥75 years and age<75 years, with and without T2DM

With increasing age, patients are at increased risk of atherothrombosis and CAD. Those with established CAD are at increased risk of future adverse cardiovascular events. However, these patients also encounter an increased bleeding risk associated with antiplatelet therapy. Thus, we face a challenging treatment dilemma in these patients. Multiple factors contribute to higher blood thrombogenicity in elderly patients. Platelets in elderly are constantly kept activated by background chronic low grade inflammation. Increased platelet reactivity and endothelial dysfunction play a significant role in this increase thrombotic milieu. This platelet hyperactivation was demonstrated in my study by high platelet

reactivity indices and higher thrombus formation. Similar to in the elderly, platelets in patients with T2DM are also in a constant state of activation. Platelet dysfunction, inflammation, hyperglycaemic state and activation of coagulation markers all contribute to increased blood thrombogenicity in T2DM.

The role of inflammation in thrombogenicity has become a great area of interest since this area is not yet fully evaluated. My findings from post chamber “effluent” blood demonstrated the release of coagulation markers (P selectin, CD40 ligand and PAI-1) from platelet granules immediately after platelet activation. Elderly patients also demonstrated higher levels of TNFα and IL-6. Mediators of inflammation such as hsCRP and IL-6 were associated with PDT in elderly diabetic patients. All these data suggest that inflammation has a direct role in increased thrombogenicity in both the elderly and the T2DM population. This causal link needs confirmation from large scale studies, following which opportunities for newer therapeutic targets will arise. In ACS setting, along with dual antiplatelet and antithrombotic agents, there might be a role for anti-inflammatory agents (e.g. anti -TNFα antibodies).

In patients with stable CAD, the CHARISMA trial demonstrated a small but significant benefit in the subgroup of patients with known atherothrombosis, demonstrating a potential role for intensification of antithrombotic therapy beyond aspirin alone (long-term dual antiplatelet therapy) in certain high-risk groups such as elderly and T2DM. The recently published DAPT and PEGASUS trials, favour long-term use of DAPT following coronary stent insertion. The DAPT study demonstrated that patients taking DAPT at 30 months had lower risk of stent thrombosis, myocardial infarction (MI) and major ischaemic adverse events compared to those who stopped DAPT at 12 months, while patients with long-term DAPT had an increased risk of moderate or severe bleeding (Mauri *et al.*, 2014). The PEGASUS trial enrolled patients with acute coronary syndromes (STEMI and NSTEMI). Major findings of this study were that 3 years of DAPT (ticagrelor plus aspirin) after DES implantation was associated with significant less incidence of cardiac death, MI and stroke and a higher incidence of bleeding (Murphy *et al.*, 2015). However, fatal bleedings, or intracranial haemorrhage or haemorrhagic stroke was not significantly different in the 2 study arms. These studies indirectly

suggest, DAPT could be beneficial long-term atleast in high risk population such as elderly and T2DM (Elmariah *et al.*).

Clopidogrel has the least bleeding risk when compared to prasugrel or ticagrelor and is much more cost effective. With more and more data suggesting the need for extended DAPT following an acute coronary event and revascularisation as discussed above, this study reinforces the potential usage of clopidogrel in certain high risk groups, especially in the elderly and T2DM. My findings strengthen the argument that dual antiplatelet therapy might be able to provide better platelet inhibition without overtly increasing the bleeding risk in stable CAD patients in elderly and T2DM who are at high risk of future thrombotic events. Large scale studies are needed to further validate the role of long term dual antiplatelet therapy in providing clinical benefits with reduction in future thrombotic events without significant increase in bleeding in this population.

Change in platelet content of thrombus was associated with a reduction in thrombus area, suggesting the possibility that reducing the platelet count might reduce thrombus quantity. The hypothesis of my second study was that reduction in the platelet count will reduce platelet dependent thrombus formation and alter thrombus characteristics. Rafigrelide was the novel pharmacological agent which reduced the platelet count. This reduction in platelet count was associated with a reduction in both high shear and low shear thrombus area. Under high shear conditions, the reduction in thrombus area was more pronounced and there was a moderate correlation between platelet count and thrombus area, as would be expected given the greater role of platelets in arterial thrombosis (platelet rich) compared with thrombus formation in veins (fibrin rich) under low shear conditions. This study demonstrates that modification of platelet synthesis and reduction of platelet count might be a possible therapeutic option in patients with high normal platelet count and high risk factors such as elderly or T2DM.

5.2 Limitations

My study was a single centre study on a small cohort of patients (131 patients in three groups recruited by myself and data from 41 young T2DM patients used from a previous study from our group). The fact that all the patients in my study were

strictly selected with pre-defined inclusion / exclusion criteria suggests that these patients might not represent the “real world” population. All my study participants were Caucasians and hence the results cannot be extrapolated to global elderly / diabetic population. I used clopidogrel in my study for dual antiplatelet therapy alongside aspirin as it is the most widely used antiplatelet agent after aspirin especially in elderly population and in T2DM patients. Newer antiplatelet agents (P2Y12 inhibitors) such as prasugrel and ticagrelor which are superior to clopidogrel in platelet inhibition have been licensed for use in the UK, in acute coronary syndrome setting and in patients undergoing coronary intervention. Use of these newer agents has increased significantly in recent years, with a significant reduction in clopidogrel usage especially in the diabetic population. This is not a major limitation as far as elderly patients are concerned since my study suggests that clopidogrel still has a significant role at least in elderly cohort and in stable CAD setting. My study participants received clopidogrel at maintenance dose only for a week and hence my study does not address the effects of long term dual antiplatelet therapy. Various point of care platelet function assays were used to assess effect of antiplatelet agents and various serum and plasma assays were used to measure coagulation and inflammatory biomarkers. Flow cytometry methods would have been more accurate but much more time consuming. My study involved only two visits, day 1 and day 8 (after 7 days of clopidogrel), I did not record any long term clinical outcomes of my participants.

My second study recruited only healthy volunteers with very small sample size of 12 volunteers. In this study, platelet count, mean platelet volume, platelet-dependent thrombus and TEG® and Platelet Mapping™ were studied. Other than platelets, a number of cellular and non-cellular components of the coagulation system are involved in thrombus formation but these were not measured in our study. Other established point of care platelet function tests such as VerifyNow® and Multiplate® were not used in this study. The mechanism of rafigrelide is to reduce the platelet count and 14 days of rafigrelide demonstrated significant reduction in platelet count to pre-defined adverse event range in 2 healthy volunteers. IMP was stopped after 14 days and hence long term effect of this IMP on platelet count, thrombus formation and risk of bleeding secondary to significant thrombocytopenia were not addressed since this was not the aim of the study.

5.3 Future directions

My study has demonstrated a trend towards higher blood thrombogenicity in elderly and T2DM patients with stable CAD. Ageing is a well establish independent risk factor for future cardiovascular events especially in people with established CAD. T2DM is another most important risk factor for atherosclerosis, thrombosis and future adverse cardiovascular events in patients with established CAD.

In patients with STEMI, NSTE-ACS and those undergoing coronary intervention, newer and more potent antiplatelet agents have shown promising results by demonstrating significant reduction in adverse cardiac events compared to clopidogrel. Hence international societies have included these agents in their guidelines. However, in elderly population (age≥75 years), severe bleeding risk associated with prasugrel compensated for any improvement in adverse atherothrombotic events and the overall outcome was worse compared to clopidogrel. Hence prasugrel is not used in elderly patients (age≥75 years). A lowered maintenance dose of prasugrel (5mg daily) was tested in the elderly population. This study demonstrated similar outcome and bleeding risk compared to clopidogrel, without any additional benefit from prasugrel (Roe *et al.*, 2013). Hence it is cost-effective to use clopidogrel 75mg daily instead of prasugrel 5mg daily. Ticagrelor has shown improved benefits over clopidogrel without any substantial bleeding risk and hence is currently the recommended antiplatelet therapy of choice alongside aspirin. Patients with T2DM still have poor outcomes compared to non DM population despite the introduction of these newer more potent antiplatelet agents.

My thesis, to some extent has helped in understanding the complex pathophysiology of atherothrombosis in elderly population and in T2DM. My study has demonstrated heightened blood thrombogenicity and high platelet reactivity in elderly population alongside some unique information on coagulation and inflammation in this high risk population.

The following studies might be helpful in carrying forward this research further so as to understand pathophysiology and treatment options in the elderly in detail:

i. Study to assess the effect of long term dual antiplatelet therapy in elderly stable CAD patients in terms of adverse cardiovascular events, bleeding risk and overall long term benefits.

ii. Study evaluating platelet dependent thrombus and platelet reactivity comparing newer antiplatelet agents such as ticagrelor with clopidogrel in elderly patients with stable CAD.

iii. Study evaluating PDT, platelet reactivity, inflammation and coagulation biomarkers, viscoelastic properties and ultrastructural assessment of thrombus in elderly patients presenting with ACS.

iv. Study evaluating PDT, platelet reactivity, viscoelastic properties and ultrastructural assessment of thrombus in elderly patients presenting with ACS comparing the effects of clopidogrel vs. ticagrelor.

v. Interventional studies to assess the role of novel therapeutic agents such as anti-inflammatory agents (anti-TNFα antibodies), agents that reduce the platelet count by inhibiting platelet synthesis, fibrin antagonists and direct thrombin inhibitors in high risk patient groups such as elderly and T2DM using Badimon perfusion chamber

Appendices

Appendix 1: Publications

Peer reviewed original research article

Balasubramaniam K, Viswanathan G, Dragone J, Grose-Hodge R, Martin P, Troy S, Preston P, Zaman AG. (2014) 'Antithrombotic properties of rafigrelide: a phase 1, open-label, non-randomised, single-sequence, crossover study', Thromb Haemost, 112(1), pp. 205-15.

Review article

Karthik Balasubramaniam, Girish N. Viswanathan, Sally M. Marshall, and Azfar G. Zaman, “Increased Atherothrombotic Burden in Patients with Diabetes Mellitus and Acute Coronary Syndrome: A Review of Antiplatelet Therapy,” Cardiology Research and Practice, vol. 2012, Article ID 909154, 18 pages, 2012. doi:10.1155/2012/909154

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