The influence of vitamin E bonded haemodialysis membranes on erythropoiesis stimulating agent requirements, oxidative stress, inflammation, haemostasis and outcomes.

Simon Watson Lines

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Medicine

August 2013

The candidate confirms that the work submitted is his own work and that appropriate credit has been given where reference has been made to the work of others.

ACKNOWLEDGEMENTS

I would like to thank my lead supervisor, Dr Mark Wright, for giving me the opportunity to undertake this research project and for all the guidance and advice he provided throughout.

I am also indebted to my project co-supervisors, Dr Angela Carter and Dr Emma Dunn, for their help and input during my research tenure. I am particularly grateful to Dr Carter for her patience and help in the laboratory and for tolerating my incessant question asking.

I would like to thank the team of research nurses and assistants who helped to undertake the clinical aspects of the study: Ros Wheatley, Emma Giddings, Shyama Rughooputh, Stuart Turner and Frank Lee. I am particularly grateful to Ros who was the lead nurse for this project.

I am very grateful to Dr Victoria Richardson for teaching me how to do ELISAs, for undertaking a subset of the C3 ELISA and TBARS assays and, most of all, for helping me to maintain a sense of humour in the face of adversity.

I am grateful to Mrs May Boothby for teaching me basic laboratory techniques and the fibrin clot structure and function assays and for her encyclopaedic knowledge of the laboratories inventory.

I would like to thank Ms Jia-Ying Lee for her help in performing a subset of the C3 ELISAs and her help with the properdin optimisation ELISAs.

I am grateful to Asahi Kasei Medical Corporation Limited, the National Institute for Health Research and the Leeds Teachings Hospitals Renal Charitable Trustees for funding this project.

I would like to thank all the members of the LIGHT laboratories for their support, encouragement and ready distractions in the form of coffee breaks during my time in research.

I would like to thank the Leeds Teaching Hospitals NHS Trust dialysis staff for their assistance in conducting this research project. I am also indebted to the patients who agreed to participate and without whom this project would not have been possible.

Finally I am eternally grateful to my wife, Sam, and to my children, Sophie, Oliver and Gabriela, for their patience, love and support in helping me to undertake this ambitious and time consuming project.

ABSTRACT

Introduction: Haemodialysis (HD) patients have high rates of cardiovascular (CV) disease and mortality yet the reasons for this have not been fully elucidated. High doses of erythropoiesis stimulating agents (ESAs), increases in oxidative stress and inflammation, and alterations to the fibrin clot phenotype are all possible contributors. Vitamin E (VE)-bonded dialysis membranes are purported to have favourable effects on a number of these parameters which were tested here in the setting of a randomised controlled trial.

Methods: Patients were randomised to HD with VE-bonded polysulfone membranes or non-VE-bonded equivalents and followed for 12 months. Data on anaemia parameters were collected monthly and blood tests were performed at baseline, 6 and 12 months for the measurement of oxidative stress (oxidatively modified-low density lipoprotein, thiobarbituric acid reactive species), inflammation (C-reactive protein, complement components C3, SC5b-9, factor D, properdin) and fibrin clot properties. Contemporaneous data were collected on CV events and death.

Results: Of the 260 patients enrolled, 123 were randomised to dialysis with VE-bonded membranes. Analysis of the full dataset revealed no differential effects of the VE-membranes on ESA requirements, oxidative stress, inflammation, fibrin clot structure or clinical outcomes. Key findings included a potential ESA-sparing effect of the VE-membranes in ESA resistant patients, a progressive decline in C3 levels over 12 months and associations between the levels of C3 and SC5b-9 at baseline and the subsequent risks of dying or experiencing a CV event.

Conclusions: There were no benefits in switching prevalent HD patients to dialysis with VE-bonded dialysis membranes with the exception of possible utility in ESA-resistant patients. The novel finding suggesting a link between the complement system and poor outcomes in HD patients may provide further insights to explain the high rates of CV disease and mortality for this patient group and merits further study.

PUBLICATIONS ARISING FROM THIS THESIS

Journal Articles

- **Lines SW**, Carter AM, Dunn EJ, Lindley EJ, Tattersall JE, Wright MJ. A randomised controlled trial evaluating the erythropoiesis stimulating agent sparing potential of a vitamin E bonded polysulfone dialysis membrane. *Nephrology, Dialysis and Transplantation* 2013; epub ahead of print 28th November 2013

Oral Presentations

- **Lines SW**, Carter AM, Dunn EJ, Wright MJ. Examining the ESA-sparing potential of a Vitamin E bonded dialysis membrane: results of a 12-month randomised controlled trial. [Renal Association, Gateshead, 2012]

Conference Abstracts

- **Lines SW**, Carter AM, Dunn EJ, Wright MJ. A 12-month randomised controlled trial examining the ESA-sparing effects of a Vitamin E bonded dialysis membrane. [ERA/EDTA, Paris, 2012]

- **Lines SW**, Carter AM, Dunn EJ, Wright MJ. A 12 months randomized controlled trial evaluating the effects of a vitamin E bonded dialysis membrane on inflammation, oxidative stress, fibrin clot parameters, cardiovascular events and mortality. [American Society of Nephrology, San Diego, 2012]

- **Lines SW**, Carter AM, Dunn EJ, Wright MJ. Increased fibrin clot density is associated with cardiovascular disease in haemodialysis patients. [American Society of Nephrology, San Diego, 2012]

- **Lines SW**, Lee J-Y, Dunn EJ, Wright MJ, Carter AM. Complement C3 predicts cardiovascular events during 12 months of follow-up in haemodialysis patients and declines significantly with time on haemodialysis. [XXIV International Complement Workshop, Crete, 2012]

TABLE OF CONTENTS

CHAPTER 1 : INTRODUCTION 1

1.1 RENAL FAILURE 1
1.1.1 Chronic kidney disease and end-stage renal failure 1
1.1.2 Haemodialysis for end-stage renal failure 2
1.1.3 Medical management of dialysis patients 4
1.2 CARDIOVASCULAR DISEASE IN RENAL FAILURE 5
1.2.1 Epidemiology of cardiovascular disease in dialysis patients 5
1.2.2 Pathophysiology of cardiovascular disease 6
1.2.2.1 Atherosclerosis 6
1.2.2.2 Arterial calcification 7
1.2.3 Clinical spectrum of cardiovascular disease in dialysis patients 9
1.2.4 Summary of cardiovascular disease in dialysis patients 10
1.3 ANAEMIA IN DIALYSIS PATIENTS 11
1.3.1 Overview of anaemia in dialysis patients 11
1.3.2 Factors contributing to anaemia in haemodialysis patients 12
1.3.2.1 Impaired erythropoiesis and altered iron metabolism 12
1.3.2.2 Reduced erythrocyte lifespan and enhanced oxidative stress 15
1.3.3 Treatment of anaemia in haemodialysis patients 16
1.3.4 Anaemia and cardiovascular disease in haemodialysis patients 17
1.4 OXIDATIVE STRESS 21
1.4.1 Overview of oxidative stress 21
1.4.2 Oxidative stress in haemodialysis patients 22
1.4.2.1 Increased generation of reactive oxygen species 23
1.4.2.2 Reduced anti-oxidant defences 24
1.4.3 Oxidative stress and cardiovascular disease 25
1.4.4 Interplay of oxidative stress and inflammation in haemodialysis patients 26
1.5 INFLAMMATION 27
1.5.1 Overview of inflammation in haemodialysis patients 27
1.5.2 The complement system 29
1.5.2.1 Pathways of complement activation 29
1.5.2.2 Effector functions of complement 30
1.5.2.3 Regulators of the complement system 32
1.5.3 Inflammation and cardiovascular disease 32
1.6 HAEMOSTASIS AND FIBRINOLYSIS 36

1.6.1 The haemostatic system 36
1.6.1.1 Overview of the haemostatic system 36
1.6.1.2 Primary haemostasis 37
1.6.1.3 Secondary haemostasis 38
1.6.1.4 Control of haemostasis 40
1.6.2 The fibrinolytic system 41
1.6.2.1 Overview of the fibrinolytic system 41
1.6.2.2 Generation of plasmin 42
1.6.2.3 Control of fibrinolysis 42
1.6.3 Determinants of fibrin clot structure and function 44
1.6.4 Haemostatic factors in cardiovascular disease 47
1.6.4.1 Elevated fibrinogen levels and cardiovascular disease 47
1.6.4.2 Fibrin structure and function in relation to cardiovascular disease 47
1.6.4.3 Summary of haemostatic factors and cardiovascular disease 49
1.7 VITAMIN E AND CARDIOVASCULAR DISEASE 50
1.7.1 Overview of vitamin E 50
1.7.1.1 Anti-oxidant activity of Vitamin E 50
1.7.1.2 Anti-inflammatory activity of Vitamin E 50
1.7.1.3 Haemostatic effects of vitamin E 52
1.7.2 Human studies of vitamin E 52
1.7.2.1 Vitamin E supplementation in non-dialysis patients 52
1.7.2.2 Vitamin E supplementation in dialysis patients 53
1.7.3 Vitamin E coated dialysis membranes 55
1.7.3.1 Dialysis membrane composition and biocompatibility 55
1.7.3.2 Vitamin E coated modified cellulose membranes 57
1.7.3.3 Vitamin E coated polysulfone membranes 57
1.8 SUMMARY 58
CHAPTER 2 : AIMS 60
CHAPTER 3 : METHODS 61
3.1 STUDY DESIGN AND INTERVENTION 61
3.2 TRIAL APPROVALS 61
3.3 FUNDING 61
3.4 STUDY SETTING 61
3.5 RESEARCH TEAM 62
3.6 PATIENTS 62

3.6.1 Screening, recruitment and enrolment 62
3.6.2 Randomisation 63
3.7 DATA COLLECTION 64
3.7.1 Demographic, lifestyle and comorbidity data 64
3.7.2 Medications 65
3.7.3 Dialysis information 65
3.7.4 Blood sampling 66
3.7.5 Blood transfusions 67
3.7.6 Clinical events 67
3.8 ANAEMIA DATA 68
3.8.1 Anaemia management 68
3.8.2 Erythropoiesis stimulating agent (ESA) resistance index 69
3.9 ROUTINE BIOCHEMISTRY AND HAEMATOLOGY ASSAYS 69
3.10 OX-LDL ELISA 69
3.11 THIOBARBITURIC ACID REACTIVE SPECIES ASSAY (TBARS) 71
3.11.1 Development and optimisation of TBARS assay 71
3.11.2 Final TBARS assay protocol 74
3.12 C3 ELISA 74
3.13 PROPERDIN ELISA 76
3.13.1 Optimisation of properdin ELISA 76
3.13.2 Final properdin ELISA protocol 79
3.14 FACTOR D ELISA 80
3.14.1 Development and optimisation of factor D ELISA 80
3.14.2 Final factor D ELISA protocol 83
3.15 SC5B-9 ELISA 84
3.15.1 Development and optimisation of SC5b-9 ELISA 84
3.15.2 Final SC5b-9 ELISA protocol 89
3.16 TURBIDIMETRIC FIBRIN CLOT STRUCTURE AND FUNCTION ASSAYS 90
3.16.1 Turbidimetric clotting assay 91
3.16.2 Turbidimetric fibrinolysis assay 92
3.17 STATISTICS 94
3.17.1 Overview of statistical methods used 94
3.17.2 Power calculations 94
3.17.3 Baseline between-group comparisons 94
3.17.4 Analysis of baseline data 95
3.17.5 Analysis of longitudinal data 96

3.17.6 Analysis of clinical outcome data 97
CHAPTER 4 : PATIENT CHARACTERISTICS AND FOLLOW UP 98
4.1 PATIENT RECRUITMENT, FOLLOW-UP AND BASELINE CHARACTERISTICS 98
4.2 DIALYSER ADHERENCE 102
4.3 WATER QUALITY DATA 103
4.3.1 Microbiology data 103
4.3.2 Chlorine levels 103
4.4 DISCUSSION 105
CHAPTER 5 : ANAEMIA 107
5.1 DETERMINANTS OF BASELINE ESA RESISTANCE INDEX 107
5.2 TEMPORAL CHANGES IN ANAEMIA PARAMETERS AND THE EFFECTS OF VITAMIN E 110
5.2.1 12-month changes in haemoglobin and ferritin levels 110
5.2.2 12-month changes in ESA resistance index 112
5.3 DISCUSSION 118
5.3.1 Baseline determinants of ESA resistance index 118
5.3.2 The effects of vitamin E on anaemia 121
CHAPTER 6 : OXIDATIVE STRESS 129
6.1 DETERMINANTS OF BASELINE OXIDATIVE STRESS 129
6.1.1 Determinants of baseline Ox-LDL levels 129
6.1.2 Determinants of baseline TBARS levels 133
6.2 TEMPORAL CHANGES IN OXIDATIVE STRESS AND THE EFFECTS OF VITAMIN E 136
6.2.1 12-month changes in Ox-LDL levels 136
6.2.2 12-month changes in TBARS levels 137
6.3 OXIDATIVE STRESS AND ESA RESISTANCE INDEX 139
6.4 DISCUSSION 139
6.4.1 Baseline determinants of oxidative stress biomarkers 143
6.4.2 Changes in oxidative stress over time and the effects of vitamin E 146
CHAPTER 7 : INFLAMMATION 150
7.1 DETERMINANTS OF BASELINE INFLAMMATION 150
7.1.1 Determinants of baseline CRP levels 150
7.1.2 Determinants of baseline C3 levels 151
7.1.3 Determinants of baseline SC5b-9 levels 151
7.1.4 Determinants of baseline factor D levels 152

7.1.5 Determinants of baseline properdin levels 153
7.2 TEMPORAL CHANGES IN INFLAMMATION AND THE EFFECTS OF VITAMIN E..... 158
7.2.1 12-month changes in CRP levels..... 158
7.2.2 12-month changes in C3 levels..... 160
7.2.3 12-month changes in SC5b-9 levels 162
7.2.4 12-month changes in factor D levels..... 163
7.2.5 12-month changes in properdin levels 164
7.2.6 Inter-relationships of the 12-month changes in the inflammatory markers166
7.3 INFLAMMATION AND ESA RESISTANCE INDEX 166
7.4 DISCUSSION..... 168
7.4.1 Baseline determinants of inflammation biomarkers..... 168
7.4.2 Changes in inflammatory markers over time and the effects of vitamin E 171
CHAPTER 8 : FIBRIN CLOT STRUCTURE AND FUNCTION 177
8.1 ASSAY DATA USED FOR ANALYSES..... 177
8.2 DETERMINANTS OF BASELINE FIBRINOGEN LEVELS AND FIBRIN CLOT PARAMETERS 180
8.2.1 Determinants of baseline fibrinogen levels..... 181
8.2.2 Determinants of baseline lag times 185
8.2.3 Determinants of baseline maximum absorbance..... 185
8.2.4 Determinants of baseline $Lys50_{t0}$ times..... 185
8.3 TEMPORAL CHANGES IN FIBRINOGEN LEVELS AND FIBRIN CLOT PARAMETERS AND THE EFFECTS OF VITAMIN E 190
8.3.1 12-month changes in fibrinogen levels..... 192
8.3.2 12-month changes fibrin clot parameters 193
8.4 CHANGES IN FIBRINOGEN LEVELS AND FIBRIN CLOT PARAMETERS IN RELATION TO CHANGES IN OXIDATIVE STRESS 195
8.5 CHANGES IN FIBRINOGEN LEVELS AND FIBRIN CLOT PARAMETERS IN RELATION TO CHANGES IN INFLAMMATION 195
8.6 DISCUSSION..... 196
8.6.1 Fibrin clot variables selected for further analyses..... 198
8.6.2 Baseline determinants of fibrinogen levels and fibrin clot parameters 200
8.6.3 Changes in fibrin clot parameters over time and the effects of vitamin E 204
CHAPTER 9 : CLINICAL OUTCOMES AND MORTALITY 211
9.1 BASELINE PREDICTORS OF MORTALITY..... 211
9.2 BASELINE PREDICTORS OF CARDIOVASCULAR EVENTS 214

9.3 BASELINE PREDICTORS OF INFECTIVE EVENTS 217
9.4 BASELINE PREDICTORS OF NON-INFECTIVE DIALYSIS ACCESS EVENTS 219
9.5 DISCUSSION 222
9.5.1 Mortality and cardiovascular events 222
9.5.2 Infective events 227
9.5.3 Non-infective dialysis access events 227
CHAPTER 10 : CONCLUSIONS AND FUTURE PERSPECTIVES 231
REFERENCES 235
APPENDICES 297
APPENDIX A SUPPLEMENTARY DATA TABLES 297
APPENDIX B RESPONSIBILITIES OF CORE RESEARCH TEAM MEMBERS 303
APPENDIX C LEEDS TEACHING HOSPITALS LABORATORY ASSAYS 304
APPENDIX D ANAEMIA MANAGEMENT ALGORITHM 305
APPENDIX E BUFFERS 309
APPENDIX F ICH-GCP CERTIFICATE 310

LIST OF FIGURES

Figure 1 - Breakdown of renal replacement therapies by modality in the UK in 2010. 1
Figure 2 - Schematic representation of a dialyser. 2
Figure 3 - Schematics of a dialysis circuit..... 3
Figure 4 - Principal sites of arterial calcification..... 8
Figure 5 - Positive feedback loop concerning anaemia and oxidative stress. 15
Figure 6 - Causal diagram outlining the possible explanations for the higher mortality in ESA intervention trials for patients randomised to the higher haemoglobin target group. 18
Figure 7 - Overview of the pathways of complement activation. 31
Figure 8 - Current schema of haemostasis and the central role of thrombin generation. . 39
Figure 9 - The principal activators and inhibitors of fibrinolysis..... 43
Figure 10 – Role of α-tocopherol in interrupting free radical propagated lipid damage. . 51
Figure 11 - TBARS assay standard curves from 3 experiments to determine the effects of altering the volume of serum or standard in the final reaction mix. 72
Figure 12 - Effects of different heating times in TBARS assay. 73
Figure 13 - Properdin ELISA optimisation assay to determine the optimal dilutions of coating and detection antibodies..... 77
Figure 14 - Example standard curve for the properdin ELISA..... 78
Figure 15 - Factor D optimisation assay to determine the optimal dilutions of coating and detection antibodies.. 81
Figure 16 - Example standard curve for the factor D ELISA. 82
Figure 17 - Factor D optimisation assay examining the effects of sample dilution and increased incubation times with the antigen and detection antibody 83
Figure 18 - SC5b-9 optimisation assay to determine optimal concentration of coating and detection antibody using 1 hour incubations. 85
Figure 19 - SC5b-9 optimisation assay to determine optimal dilutions of coating and detection antibodies using 2 hour incubations..... 86
Figure 20 - Example standard curve using serially diluted zymogen-activated serum sample.. 87
Figure 21 - Graph of absorbance against concentration for the zymosan activated serum standard curve following incubation with the substrate for 30, 40, 50 and 60 minutes..... 88
Figure 22 - Clotting assay parameters. 91
Figure 23 - Fibrinolysis assay parameters..... 93
Figure 24 - Patient recruitment and screening..... 99

Figure 25 - Patients completing the study and reasons for discontinuation by treatment group.. 100
Figure 26 - Bacterial and endotoxin counts, Ox-LDL levels, ESA resistance index (ERI), C3 and CRP levels grouped by dialysis unit at the time of the baseline, 6- and 12-month study visits. 104
Figure 27 - Chloride levels, ESA resistance index (ERI), bilirubin and haemoglobin levels grouped by dialysis unit at the time of the baseline, 6- and 12-month study visits. 105
Figure 28 - Monthly mean haemoglobin concentrations for the two study groups..... 111
Figure 29 - Mean monthly ferritin concentrations for the two study groups. 112
Figure 30 - Box and whisker plot of the monthly ERI by study group. 113
Figure 31 - Comparison of the change in ERI over 12 months between groups showing no significant difference. 113
Figure 32 - Comparison of change in ERI between groups stratified by tertiles of ERI at baseline.. 115
Figure 33 - Scatter plot for the change in ERI against baseline ERI with best fit regression lines through the data points for each group.. 116
Figure 34 - Frequency distribution of Ox-LDL levels at baseline. 130
Figure 35 - Mean baseline TBARS levels by quartiles of Ox-LDL. 133
Figure 36 - Comparison of the Ox-LDL levels in patients randomised to the control or Vitamin E membrane. 136
Figure 37 - Comparison of the 12 month change in Ox-LDL levels for patients randomised to the control or Vitamin E membrane. 137
Figure 38 - A graph displaying the mean TBARS levels at each of the sampling points for the two groups.. 138
Figure 39 - Comparison of the 12 month change in TBARS levels between patients randomised to the control and vitamin E membranes..... 138
Figure 40 - CRP levels at baseline, 6 and 12 months in each study group. 159
Figure 41 - Comparison of 12-month change in CRP levels between study groups..... 160
Figure 42 - C3 levels at baseline, 6 and 12 months in each study group. 160
Figure 43 - Comparison of 12-month change in C3 levels between study groups. 161
Figure 44 - SC5b-9 levels at baseline, 6 and 12 months in each study group 162
Figure 45 - Comparison of 12-month change in SC5b-9 levels between study groups.. 162
Figure 46 - Factor D levels at baseline, 6 and 12 months in each study group. 163
Figure 47 - Comparison of 12-month change in factor D levels between study groups.. 164
Figure 48 - Properdin levels at baseline, 6 and 12 months in each study group.. 165

Figure 49 - Comparison of 12-month change in properdin levels between study groups.. 165
Figure 50 - Breakdown of fibrin clot structure samples assayed, excluded and subsequently used for analyses.. 179
Figure 51 - Scatter plots of lag times and maximum absorbancies derived from the clotting and fibrinolysis assays. 181
Figure 52 - Fibrinogen levels at baseline, 6 and 12 months..... 193
Figure 53 - Comparison of 12-month change in fibrinogen levels between study groups. 193
Figure 54 - Comparisons of the fibrin clot parameters between study groups across the three study visits and the change in each parameter after 12 months.. 194
Figure 55 - Patient mortality categorised by aetiology of renal failure.. 212
Figure 56 - Patient survival by baseline tertiles of SC5b-9..... 213
Figure 57 - Comparison of 12-month mortality between study groups. 214
Figure 58 - Breakdown of cardiovascular events by vascular territory. 214
Figure 59 - Cardiovascular event rates compared on the bases of a history of ischaemic heart disease or peripheral vascular disease at baseline 215
Figure 60 - Cardiovascular event free survival by baseline tertiles of SC5b-9.. 216
Figure 61 - Comparison of cardiovascular events between study groups. 217
Figure 62 - Primary sites of infections for study patients. 217
Figure 63 - Infective event rates compared by dialysis access at baseline. 218
Figure 64 - Comparison of infective events between study groups. 219
Figure 65 - Breakdown of non-infective dialysis access events. 220
Figure 66 - Non-infective dialysis events compared by dialysis access at baseline.. 220
Figure 67 - Comparison of non-infective dialysis access events between study groups. 221

LIST OF TABLES

Table 1 - Examples of commonly measured biomarkers of oxidative damage. 22
Table 2 - Studies examining effects of vitamin E bonded polysulfone dialysis membranes on oxidative stress, anaemia and inflammation. 58
Table 3 - Blood testing schedule 66
Table 4 - Comparison of baseline characteristics between study groups. 101
Table 5 - Baseline biochemistry and lipid profiles 102
Table 6 - Results of dialyser adherence monitoring 102
Table 7 – Determinants of ESA resistance index at baseline: continuous variables 108
Table 8 - Determinants of ESA resistance index at baseline: categorical variables 109
Table 9 - Comparison of the baseline anaemia parameters between study groups 110
Table 10 - Baseline and 12 month ESA resistance indices for study patients, stratified by ERI at baseline and study group. 114
Table 11 - Factors significantly related to baseline ERI compared between the tertiles of ERI at baseline. 117
Table 12 - Between group comparisons of change in each variable over 12 months for patients in the high ERI tertile. 118
Table 13 - Comparison of studies evaluating vitamin E bonded polysulfone membranes with a primary anaemia endpoint 125
Table 14 - Determinants of Ox-LDL levels at baseline: analysis of continuous variables between quartiles of Ox-LDL 131
Table 15 - Determinants of Ox-LDL levels at baseline: categorical variables 132
Table 16 - Determinants of TBARS at baseline: continuous variables 134
Table 17 - Determinants of TBARS at baseline: categorical variables 135
Table 18 - Determinants of CRP, C3, factor D and properdin levels at baseline: continuous variables. 154
Table 19 - Determinants of CRP, C3, SC5b-9, factor D and properdin levels at baseline: Patient factors and comorbidities 155
Table 20 - Determinants of CRP, C3, SC5b-9, factor D and properdin levels at baseline: Medications 156
Table 21 - Determinants of SC5b-9 levels at baseline: analysis of continuous variables across quartiles of SC5b-9. 157
Table 22 - Comparison of CRP, C3, SC5b-9, factor D and properdin levels between quartiles of Ox-LDL 158

Table 23 - Analyses of baseline and 12-month data for the inflammatory markers CRP, C3, SC5b-9, factor D and properdin. 159
Table 24 - Spearman's rank correlation coefficients for the 12-month change in C3 levels and the 12-month change in each of the variables significantly associated with C3 levels at baseline 161
Table 25 - Spearman's correlation coefficients for the 12-month change in each of the inflammatory markers 166
Table 26 - Spearman's rank correlation coefficients between baseline levels of ERI and CRP, C3, factor D and properdin 166
Table 27 - Correlation coefficients between the change in ERI and the change in each of the measured biomarkers of inflammation. 167
Table 28 - Clot characteristics of samples obtained from patients on warfarin 178
Table 29 - Clot characteristics of samples obtained via central venous catheters 178
Table 30 - Correlation matrix for shared clotting and fibrinolysis assay variables 180
Table 31 - Correlation matrix for fibrinolysis assay variables. 180
Table 32 - Determinants of baseline fibrinogen levels: analysis of continuous variables by quartiles of fibrinogen (patient and dialysis factors). 182
Table 33 - Determinants of baseline fibrinogen levels: analysis of continuous variables by quartiles of fibrinogen (laboratory parameters). 183
Table 34 - Determinants of baseline fibrinogen levels: categorical variables. 184
Table 35 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$ times at baseline: continuous variables. 187
Table 36 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$: categorical variables (Patient factors and Comorbidities) 188
Table 37 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$: categorical variables (Drugs) 189
Table 38 - Analysis of fibrin clot variables by quartiles of fibrinogen, Ox-LDL and SC5b-9 concentrations at baseline. 190
Table 39 - Comparison of baseline characteristics between study groups for the subset of patients with fibrin clot structure and function assays data 191
Table 40 - Comparison of the baseline biochemistry, oxidative stress and inflammation parameters between study groups for the subset of patients with fibrin clot structure and function assays data 192
Table 41 - Comparison of fibrinogen levels and fibrin clot parameters between study groups at baseline. 192

Table 42 - Spearman's rank correlation coefficients for changes in oxidative stress markers, fibrinogen and fibrin clot parameters. 195
Table 43 - Spearman's rank correlation coefficients for changes in inflammation markers, fibrinogen and fibrin clot parameters. 196
Table 44 - Breakdown of clinical events encountered by study patients 211
Table 45 - Univariate Cox-regression hazard ratios for patient characteristics and mortality 212
Table 46 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and mortality. 213
Table 47 - Univariate Cox-regression hazard ratios for patient characteristics and cardiovascular events 215
Table 48 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and cardiovascular events 216
Table 49 - Univariate Cox-regression hazard ratios for patient characteristics and infective events 218
Table 50 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and infective events. 219
Table 51 - Univariate Cox-regression hazard ratios for the baseline fibrin clot parameters and the subsequent risk of arteriovenous fistula thrombosis. 222
Table 52 - Studies of vitamin E supplementation in dialysis patients - effects on oxidative stress, inflammation, anaemia, lipids and cardiovascular outcomes 298
Table 53 - Studies examining the effects of vitamin E bonded modified cellulose dialysis membranes on oxidative stress, inflammation, anaemia, leukocyte function and complement activation 300
Table 54 - Definitions used in ESA dosing algorithm 305
Table 55 - Darbepoetin alfa algorithmic dose adjustments 306
Table 56 - Identification of iron deficiency by iron dosing decision support system 307
Table 57 - Summary of intravenous iron dosing algorithm 308

List of abbreviations

8-OhdG	8-hydroxy-2'-deoxyguanosine
A2RB	Angiotensin II receptor blocker
ACEi	Angiotensin converting enzyme inhibitor
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
ADPKD	Autosomal dominant polycystic kidney disease
AGE	Advanced glycation end products
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
AOPP	Advanced oxidation protein products
APC	Activated protein C
APTT	Activated partial thromboplastin time
ATIII	Anti-thrombin III
AVF	Arteriovenous fistula
BHT	Butylated hydroxytoluene
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
C	Complement component
CEHC	Carboxyethylhydroxychromanols
CI	Confidence interval
CKD	Chronic kidney disease
CKD-MBD	Chronic kidney disease - mineral bone disorder
CRP	C-reactive protein
CV	Cardiovascular
CoV	Coefficient of variance
CVC	Central venous catheter
DNA	Deoxyribonucleic acid
DRI	Direct renin inhibitor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERI	Erythropoiesis stimulating agent resistance index
ESA	Erythropoiesis stimulating agent
ESRF	End stage renal failure
EudraCT	European union drug regulating authorities clinical trials
F	(Clotting) Factor

GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSH	Glutathione
Hb	Haemoglobin
HD	Haemodialysis
HDL	High density lipoprotein
HIF	Hypoxia inducible factor
HR	Hazard ratio
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IHD	Ischaemic heart disease
IL	Interleukin
IQR	Interquartile range
ISRCTN	International standard randomised controlled trial number
L50	50% lysis point
LDL	Low density lipoprotein
LTHT	Leeds Teaching Hospitals Trust
LVH	Left ventricular hypertrophy
LV	Left ventricular
MASP	Mannose-binding lectin associated proteases
MBL	Mannose-binding lectin
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NHS	National Health Service
NICE	National institute for health and clinical excellence
NIHR	National institute for health research
NFκB	Nuclear factor-κB
Ox-LDL	Oxidatively modified low density lipoprotein
PAI	Plasminogen activator inhibitor
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween
PGF2α	Prostaglandin-$F_{2\alpha}$
PKC	Protein kinase C
PT	Prothrombin time
PTFE	Polytetrafluoroethylene

PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acid
PVD	Peripheral vascular disease
QC	Quality control
R&D	Research and development
RAS	Renin-angiotensin system
RBC	Red blood cell
rHuEPO	Recombinant human erythropoietin
ROS	Reactive oxygen species
RRT	Renal replacement therapy
RT	Room temperature
SEM	Standard error of the mean
TAC	Total antioxidant capacity
TAFI	Thrombin activatable fibrinolysis inhibitor
TBARS	Thiobarbituric acid reactive species
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TMP	1,1,3,3-tetramethoxypropane
TNF-α	Tumour necrosis factor-α
tPA	Tissue plasminogen activator
UK	United Kingdom
uPA	Urokinase plasminogen inhibitor
USRDS	United States Renal Data System
VCAM-1	Vascular adhesion molecule-1
VE	Vitamin E
VIF	Variance inflation factor
vWF	von Willebrand factor
ZAS	Zymogen activated serum

Chapter 1 : Introduction

1.1 Renal failure

1.1.1 Chronic kidney disease and end-stage renal failure

In health the kidneys perform a number of disparate roles including electrolyte, acid-base and blood volume homeostasis in addition to endocrine and metabolic functions. In patients with chronic kidney disease (CKD) the ability of the kidneys to perform some or all of these functions is reduced. Chronic kidney disease is common and, depending on the definition used, affects upwards of 10% of the population becoming more prevalent with advancing age [1-3]. End-stage renal failure (ESRF) generally refers to patients with severe renal impairment who require renal replacement therapy (RRT), either in the form of dialysis or renal transplantation, for the purpose of sustaining life.

The prevalence of patients requiring RRT in the United Kingdom (UK) is approximately 832 patients per million and this figure has been rising year on year [4]. Renal replacement therapy provision is associated with significant healthcare costs as, despite only affecting 0.08% of the UK population, it has been estimated to account for 1-2% of National Health Service (NHS) expenditure [5]. The most frequently identified causes of ESRF in the UK are diabetes and glomerulonephritis, although the diagnosis is uncertain in up to a fifth of patients [4]. The options for patients requiring RRT are dialysis, either in the form of haemodialysis (HD) or peritoneal dialysis, or renal transplantation. In the UK renal transplantation is the most prevalent form of RRT with HD the most commonly used dialytic therapy as illustrated in Figure 1 [4].

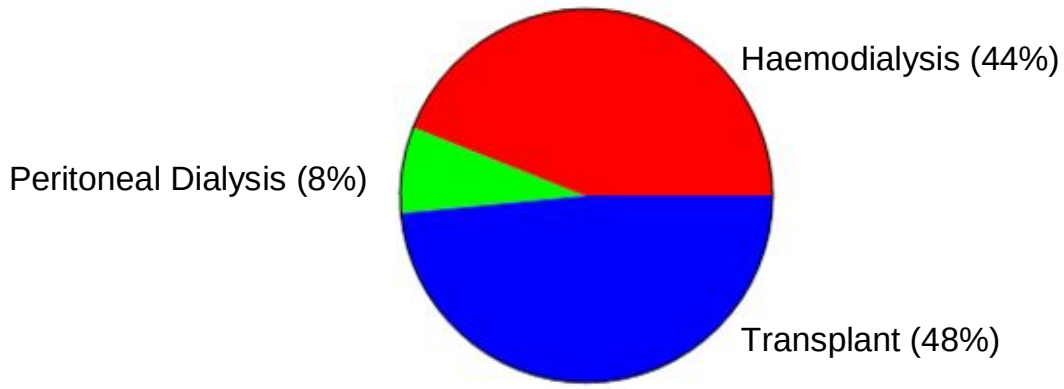


Figure 1 - Breakdown of renal replacement therapies by modality in the UK in 2010. (Data taken from UK Renal Registry Report 2011 [4]).

1.1.2 Haemodialysis for end-stage renal failure

Haemodialysis refers to the process whereby a patient's blood is passed through a dialyser, in which waste products and fluid are removed, before the blood is returned to the patient. Blood is passed through hollow fibres within the dialyser which are bathed in a fluid known as dialysate as shown in Figure 2. The walls of the fibres are semi-porous and represent a physical barrier between the blood and the dialysate and are referred to as the dialysis membrane or dialysing surface. The dialysis membrane has numerous small pores permitting the passage of molecules between the blood and dialysate along a diffusion gradient. There is a constant flow of fresh dialysate, of the order 300-800 mL/min, to maintain this concentration gradient thereby maximising diffusive transport. In addition to this diffusive transport of molecules, hydrostatic forces are used to generate a transmembrane pressure gradient resulting in the net movement of fluid from the blood into the dialysate. Fluid accumulation is one of the clinical consequences of renal failure and this latter mechanism is the principal way in which fluid is removed from patients.

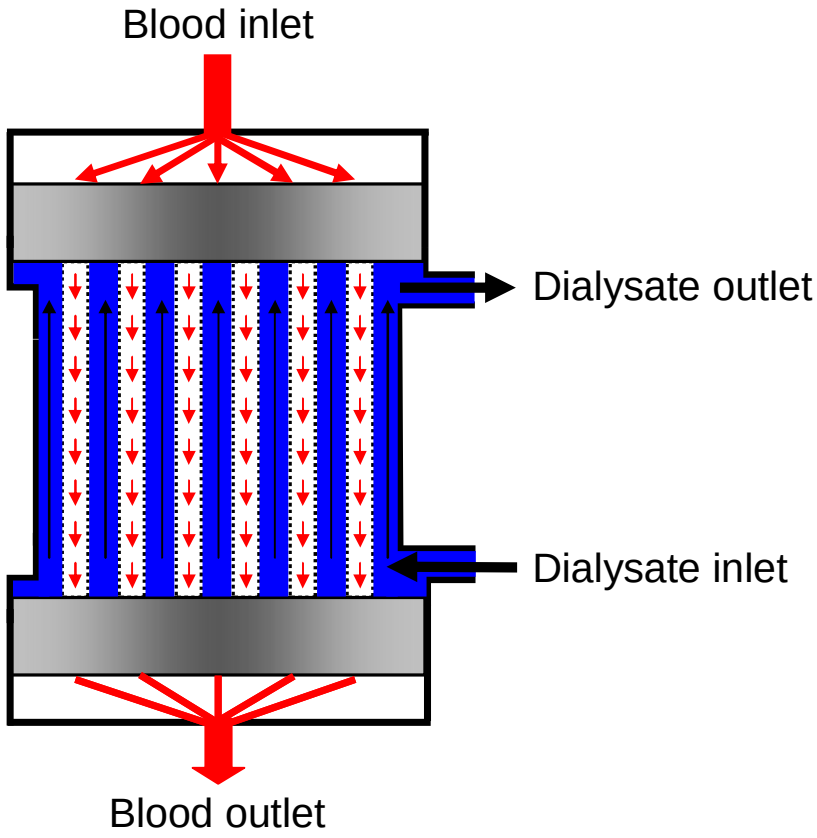


Figure 2 - Schematic representation of a dialyser. Blood (red arrows) is pumped through hollow fibres which are bathed in dialysate (blue) which flows around the fibres in the opposite direction to the blood (black arrows). The constant supply of fresh dialysate serves to maximise the diffusion gradient and hence the diffusive transport of solutes across the walls of the dialysis fibres.

In order to obtain and maintain a constant supply of blood from the patient at sufficient flow rates to permit meaningful solute removal, typically 250-450 mL/minute, a specialised form of what is termed ‘dialysis access’ is required. Dialysis access can take the form of either an arteriovenous fistula (AVF), graft or a central venous catheter (CVC). Fistulas are created by surgically anastomosing an artery and a vein - typically in the forearm of the patient’s non-dominant hand. Grafts again require a surgical procedure but in these instances an artery and a vein are connected by interposing a hollow segment of synthetic material such as polytetrafluoroethylene (PTFE); the upper thigh is a common anatomical site for graft insertion. Two needles are then inserted into the AVF or graft and connected to the dialyser with hollow tubing thereby establishing the dialysis circuit which is depicted graphically in Figure 3. Central venous catheters used for chronic dialysis usually comprise a dual lumen hollow tube which is sited in a large vein, most often the superior vena cava, and it is common practice to tunnel them under the skin in order to reduce the risk of bacteraemia. The lumen of the catheter is partitioned therefore blood can be withdrawn and returned via the same catheter without significant mixing of the blood in the afferent and efferent limbs of the dialysis circuit.

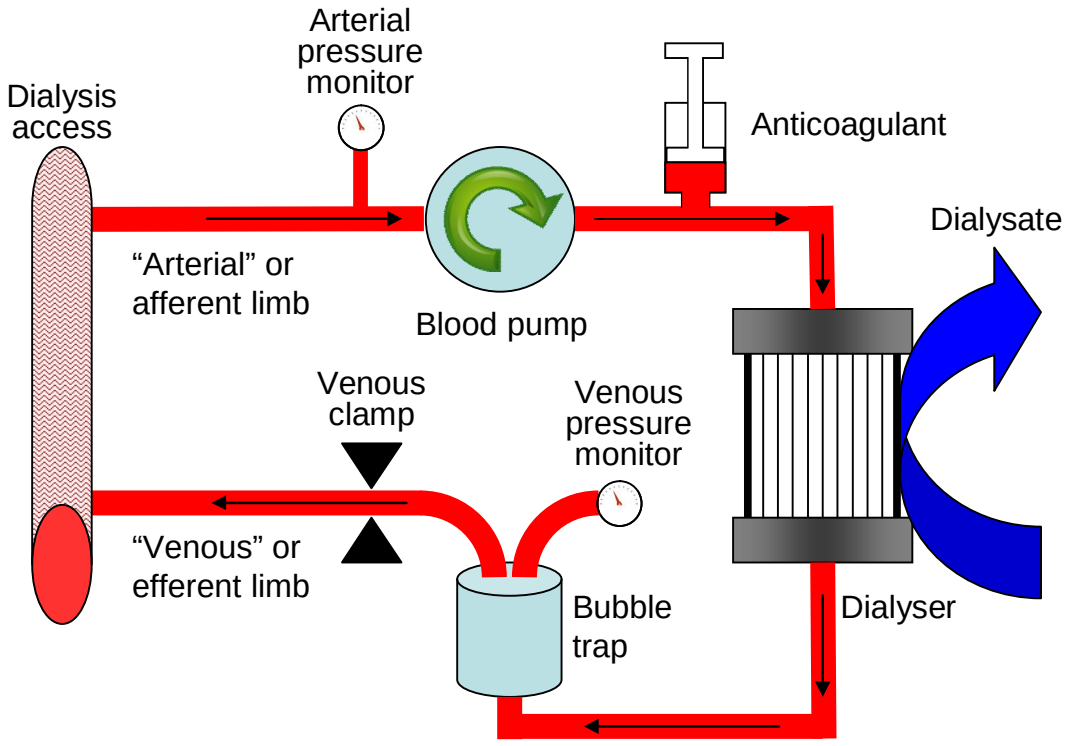


Figure 3 - Schematics of a dialysis circuit. Patients are connected to the dialyser via their dialysis access. Blood is pumped out of the patient via the “arterial” or afferent limb of the dialysis circuit and through the dialyser. Blood then passes through a bubble trap, removing any potential air emboli, before being returned to the patient via the “venous” or efferent limb.

In terms of limiting patient morbidity, principally from infective complications, the preferred type of dialysis access is an AVF followed by a graft and then CVCs [6, 7]. The use of CVCs, however, is unavoidable for a number of patients as there is an inherent delay following fistula or graft surgery before the access can be used and both are associated with appreciable failure rates. One of the principal advantages of CVCs is that they can be used immediately following placement. Patients almost universally require anticoagulation to facilitate HD given the potent pro-coagulant stimulus of passing blood through the dialyser and dialysis tubing. This is most often achieved by infusing heparin into the dialysis circuit for the duration of the HD session, or by administering a bolus of low-molecular weight heparin at the start of dialysis. Patients typically have HD three times per week with a usual treatment session lasting 4 hours; however alternative HD regimens such as nocturnal or short daily HD are followed by some patients.

1.1.3 Medical management of dialysis patients

A number of the complications of renal failure are only partially, or not at all, corrected by HD therapy. Examples of such complications include hypertension, renal osteodystrophy (also termed CKD-mineral bone disorder (CKD-MBD)) and anaemia. Elevated blood pressure is common in dialysis patients due to a combination of factors which include fluid retention, calcification of the vascular tree and, as is commonly the case, as a complicating factor of the medical condition which led to renal failure, such as diabetes or glomerulonephritis [8]. Treatment of hypertension is centred on accurate assessment of intravascular fluid status and the attainment of euvolaemia, by removing excess fluid during dialysis, and frequent recourse to anti-hypertensive drug therapy. A number of factors in HD patients conspire to cause alterations in the mineral-bone-kidney axis resulting in CKD-MBD - chief among which are phosphate retention, reduced activation of vitamin D in the kidney and the consequent development of secondary hyperparathyroidism [9]. The treatment of CKD-MBD is multifaceted and commonly involves dietary phosphate restriction, the administration of phosphate binders to limit intestinal phosphate absorption and supplementation with activated Vitamin D (1α-hydroxycholecalciferol) [9]. Anaemia is frequently encountered in HD patients and reduced renal production of erythropoietin (EPO) is the main driver. The mainstay of anaemia management is the provision of erythropoiesis stimulating agents (ESAs), such as recombinant human EPO (rHuEPO) or one of its derivatives. A detailed overview of anaemia in HD patients is provided in section 1.3.

1.2 Cardiovascular disease in renal failure

1.2.1 Epidemiology of cardiovascular disease in dialysis patients

Cardiovascular (CV) disease is the single largest cause of death in the general population [10], therefore it is perhaps not surprising that it is also the leading cause of death in chronic dialysis patients [11]. One striking difference, however, is the magnitude of CV complications among patients receiving dialysis with patients in their thirties having similar rates of fatal CV events as non-dialysis patients in their eighties [12]. Cardiovascular complications accelerate as renal function declines and a high percentage of incident dialysis patients already have a substantial burden of CV disease [13, 14]. Findings such as these have prompted investigators to try and determine which aspects of renal insufficiency contribute to the increased CV risk.

One possible explanation was thought to be the high incidence of “traditional” CV risk factors such as hypertension, dyslipidaemia, obesity, diabetes mellitus and left ventricular hypertrophy among dialysis patients [15]. Interestingly, however, a number of these factors appear to behave differently in dialysis patients. For example, there is an approximately linear relationship between increasing blood pressure and the risk of CV events in the general population [16]. This contrasts with data from dialysis populations in which the relationship appears to be U-shaped with lower blood pressures, of the order associated with good outcomes in the general population (i.e. 110 mmHg systolic blood pressure), seemingly associated with an increased risk of death in HD patients [17-20]. One explanation for this observation may be that low blood pressure is a proxy for co-morbid diseases, such as heart failure [21], as studies which specifically look at late mortality, particularly in dialysis cohorts without other significant comorbidities, have reported a more linear relationship between blood pressure and mortality akin to that observed in the general population [22, 23]. Similarly, there is a wealth of evidence for the general population supporting the use of cholesterol lowering therapy to reduce CV events both in terms of secondary prevention [24, 25] and primary prevention in high risk groups [26-28]. Studies performed in dialysis patients [29-31] have failed to demonstrate any significant mortality benefit from lipid lowering therapy and, furthermore, observational data suggest that hypocholesterolaemia may be linked to increased mortality [32, 33]. The correlation between hypocholesterolaemia and mortality may be a reflection of nutritional status as, for example, an elevated body mass index (BMI) is a well established CV risk factor among the general population [34-36] but is associated with a lower risk of death in

dialysis patients [37-40]. In line with all of these findings, standard risk factor algorithms based on data gathered from non-dialysis patients fail to predict the higher observed rates of CV disease among patients on dialysis [15, 41]. Consequently investigators have had to look for alternative explanations for the high rates of CV disease among dialysis patients.

Epidemiological studies, examining both renal and non-renal patient cohorts, have identified a number of "non-traditional" CV risk factors such as inflammation [42], oxidative stress [43], hyperfibrinogenaemia [44, 45], malnutrition and hypoalbuminaemia [46-48] and hyperhomocysteinaemia [49]. As with many of the traditional risk factors, these "non-traditional" risk factors are over-represented in dialysis populations. In addition to these risk factors some of the therapies used to treat the complications of renal failure, such as ESAs for the treatment of renal anaemia [50-52], may also contribute to the development of CV complications. Prior to examining how some of these factors may be involved in the development of CV disease in HD patients an overview of CV disease, and particularly how it differs in renal and non-renal patients, is provided in section 1.2.2.

1.2.2 Pathophysiology of cardiovascular disease

Cardiovascular disease is an umbrella term incorporating diseases relating to the heart and blood vessels. Such diseases include myocardial infarction, stroke and critical limb ischaemia. Common pathological processes underlie these disease states with the different clinical syndromes arising from similar processes occurring in different vascular beds. The two main pathological processes which give rise to CV disease are atherosclerosis and vascular calcification. The relative contributions of each of these processes differ in renal and non-renal patients.

1.2.2.1 Atherosclerosis

Atherosclerosis is the pathological process whereby lipid-rich material accumulates within arterial walls forming plaques, leading to progressive luminal narrowing and eventual arterial occlusion or thrombosis. The initial step in the development of an atherosclerotic lesion involves changes to the vascular endothelium which are thought to arise primarily as a response to injury [53]. A number of injurious mechanisms have been identified including hyperlipidaemia, hypertension, diabetes mellitus, free radicals from cigarette smoking, infectious micro-organisms (e.g. herpes viruses, *Chlamydia* pneumoniae) and genetic alterations [53]. These changes to the endothelium result in increased vascular permeability, altered expression of cell surface adhesion molecules and the release of cytokines and growth factors. Circulating monocytes are then able to migrate into the

vascular wall, differentiate into macrophages, and take up oxidised low density lipoprotein (Ox-LDL) to form foam cells. Activated macrophages and foam cells, in conjunction with activated platelets, lymphocytes sequestered at the site of injury and the overlying endothelial cells, secrete a variety of growth factors (e.g. granulocyte-macrophage colony stimulating factor (GM-CSF), platelet derived growth factor B), pro-inflammatory cytokines (e.g. interleukin-1 (IL-1), IL-8, tumour necrosis factor-α (TNF-α) and chemokines (e.g. monocyte chemotactic protein-1(MCP-1)), as well as induce endothelial cell expression of cellular adhesion molecules (e.g. intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), E-selectin, P-selectin) . The net effects of these mediators are smooth muscle cell proliferation and further cellular recruitment. This results in the release of more mediators, thereby establishing a positive feedback loop, leading to intimal thickening and luminal narrowing with the development of an atherosclerotic plaque. Over time, plaques can become unstable and ultimately may rupture exposing the thrombogenic lipid rich core to the circulation resulting in thrombus formation with the potential for vessel occlusion or distal clot embolisation.

As early as 1974 observations of accelerated atherosclerosis in HD patients were reported [54]. Subsequently several post mortem studies have demonstrated more advanced, widespread atherosclerotic disease in dialysis patients compared to patients not on dialysis [55-58]. Part of the explanation for these findings is likely to be the clustering of factors known to be associated with the development of atherosclerosis in dialysis populations, e.g. diabetes and hypertension, however these do not fully explain the discordant rates of CV disease in dialysis and non-dialysis patients as already highlighted. In addition to an increased atherosclerotic burden, arterial calcification is an important contributor to the development of CV disease in HD patients.

1.2.2.2 Arterial calcification

Arterial calcification can be sub classified on the basis of whether the deposits are found principally within the intimal or medial layers of arteries (see Figure 4). Calcification at each of these sites represents a different disease process although both contribute to the development of CV disease but via different mechanisms. Intimal calcification chiefly occurs in conjunction with atherosclerosis, i.e. in the vicinity of lipid deposits, and interferes with the conduit function of arteries [59]. This contrasts with medial calcification, also known as Monckeberg's arteriosclerosis, which is characterised histologically by diffuse fibroelastic intimal thickening, increases in collagen and medial ground substance with fragmentation of the elastic lamellae and secondary fibrosis and calcification of the media,

the end result of this process is increased arterial stiffness [60, 61]. Arterial stiffness has physiological implications because the elastic properties of healthy arteries serve as a cushion for the pulsations generated by intermittent ventricular contractions. This permits storage of part of the volume of blood ejected during systole to be returned during diastole thereby maintaining organ and tissue perfusion throughout the cardiac cycle [62]. Arterial stiffening, and the consequent loss of cushioning ability, can ultimately lead to adverse arterial and cardiac remodelling [63-65], manifesting as left ventricular hypertrophy (LVH), reduced ventricular ejection fraction, diastolic dysfunction, systolic hypertension and an increase in myocardial oxygen consumption with reduced subendocardial blood flow [63, 65-68]. Non-invasive tests available for assessing calcification, such as electron beam computed tomography and ultrasonography, are unable to reliably differentiate between intimal and medial calcification [69].

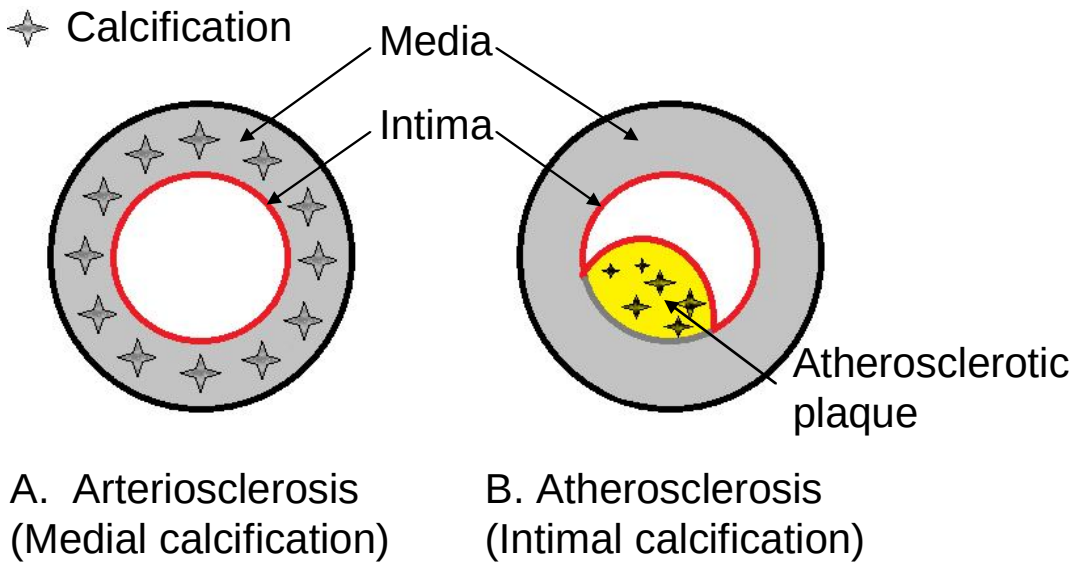


Figure 4 - Principal sites of arterial calcification. A. Arteriosclerosis (medial calcification) and B. Atherosclerosis (intimal calcification).

Dialysis patients have increased vascular calcification compared to non-renal patients [55-58, 70, 71] with more extensive vascular calcification being associated with increased mortality [72, 73]. The clinical consequences of enhanced intimal or plaque calcification in HD patients are not clear. Data from non-renal patients suggest that calcification of carotid artery plaques is associated with a lower stroke risk [74-77] and calcification of coronary artery plaques is associated with more stable disease [78]. The few studies examining coronary calcification measured using electron-beam computerised tomography in small numbers of HD patients have produced mixed results with one study reporting an

association between the degree of coronary artery calcification and the severity of atherosclerosis at angiography [79] and another reporting no such association [80]. A retrospective series of dialysis patients undergoing coronary angioplasty reported improved outcomes in patients with more extensive vascular calcification measured at angiography [81]. Putting these findings together, it seems unlikely that enhanced intimal or plaque calcification is solely responsible for the increased rates of CV disease in HD patients.

Perhaps more relevant to the high rates of CV disease in HD patients is medial calcification. Medial calcification is commonly associated with advanced age, diabetes and renal failure and, when present, is associated with worse outcomes [73, 82]. Patients with renal disease are subject to a number of factors which increase the propensity for vascular calcification, chief among which is altered mineral metabolism, i.e. CKD-MBD. The main drivers of this process are phosphate retention [71, 83], and the consequent development of secondary hyperparathyroidism [9], and the therapeutic options used in their treatment such as Vitamin D therapy [84-86] and calcium containing phosphate binders [87-89]. It can therefore be seen that the pathological spectrum of CV disease in HD patients differs from non-HD patients in that it tends to be more widespread and advanced with more extensive vascular calcification, particularly medial calcification. These pathological differences may underlie the different spectrum of clinical disease observed in HD patients.

1.2.3 Clinical spectrum of cardiovascular disease in dialysis patients

In addition to the discordant rates of CV events in dialysis compared to non-dialysis populations, the nature of these events also appears to differ. A large observational series in the USA of patients presenting with acute coronary syndromes (ACS) found that the clinical characteristics and management of dialysis patients presenting with ACS differed significantly from non-dialysis patients in terms of their demographics (dialysis patients tended to be younger), established cardiac risk factor profiles and the treatments they received [90]. In particular, the authors reported that there was a lower index of clinical suspicion of ACS in dialysis patients with twice as many dialysis patients presenting with acute myocardial infarction (AMI) misdiagnosed on admission, possibly due to a lower proportion of patients presenting with chest pain as has been similarly reported in CKD patients presenting with AMI [91]. This suggests HD patients may present with different

symptomatology compared to non-HD patients. When looking at causes of death in dialysis patients, sudden cardiac deaths account for approximately a quarter of deaths [92-94] contrasting starkly with reports of only a few cases per thousand in the general population [95, 96]. Furthermore, data from post-mortem studies suggest that approximately 60% of sudden death cases in the general population have evidence of coronary atherosclerosis, with the majority having an identifiable acute ischaemic lesion [97, 98]. In a similar report of sudden cardiac death in Japanese dialysis patients, less than 6% of cases had post mortem evidence of an acute coronary event [99]. In addition, differences in the aetiological or contributory factors for sudden cardiac death in general and dialysis populations have also been reported. For example, evidence of left ventricular dysfunction appears to be an important risk factor in the general population [100] but a study in dialysis patients found no difference in the incidence of sudden death when patients with ejection fractions of greater and less than 40% were compared [101]. Similarly LVH appears to be associated with a greater risk of sudden cardiac death in the general population [102] but not in patients on dialysis [101]. Part of the explanation for these differences may be that dialysis patients are subject to factors which are themselves associated with a greater risk of death and which are much less frequently encountered in non-dialysis patients such as rapid electrolyte shifts, hyperkalaemia and perturbations in fluid balance [93, 101, 103, 104]. With reference to section 1.2.2.2, vascular calcification may contribute to sudden cardiac death in HD patients with one study, for example, showing an association between cardiac calcification and pro-arrhythmogenic electrocardiographic features [105].

1.2.4 Summary of cardiovascular disease in dialysis patients

It can be seen that CV disease in HD patients differs from that seen in non-dialysis populations in terms of its incidence, prevalence, pathogenesis, clinical spectrum of disease, relationship to emerging and established risk factors and outcomes. It is important to recognise these differences as CV intervention studies showing benefit in the general population may not necessarily translate into similar benefits for patients on dialysis, as exemplified by the largely negative studies of lipid lowering therapies in HD patients with respect to mortality [29-31]. This underscores the need to conduct clinical trials in HD patients with interventions aimed at improving factors known to contribute to the development of CV disease in this group of patients. The main aetiological factors contributing to the elevated rates of CV disease in HD patients can be grouped under three broad headings: (i) clustering of patients at high risk of developing CV complications by nature of their comorbidities (e.g. diabetes, hypertension), (ii) factors attributable to loss

of renal function (and the therapies used in their treatment) and (iii) factors related to dialysis therapy. In the following sections, several factors which are known or thought to contribute to CV disease in HD patients and which are relevant to this thesis are discussed in more detail.

1.3 Anaemia in dialysis patients

1.3.1 Overview of anaemia in dialysis patients

Anaemia is a common feature of CKD and its incidence increases as glomerular filtration rate (GFR) declines [1, 106, 107]. Anaemia can give rise to disabling symptoms such as lethargy, dyspnoea, loss of appetite, poor memory and concentration. Studies looking at global measures of wellbeing and disability have shown a reduced quality of life for anaemic versus non-anaemic dialysis patients [108], a reduced capacity for exercise [109] and improvements in these parameters with anaemia correction [110-112]. Observational studies in dialysis patients have consistently shown anaemia to be associated with increased morbidity and mortality [113-119].

Longstanding anaemia results in marked compensatory CV changes. There is an increase in the cardiac output and a drop in peripheral resistance due to tissue hypoxia-induced vasodilation [120]. There is also a tendency for worsening of myocardial ischaemia owing to the reduced oxygen carrying capacity of the blood in conjunction with the high prevalence of coronary artery disease in dialysis patients. Prolonged anaemia contributes to LVH [121, 122] which has been shown to increase the mortality of dialysis patients independent of age, diabetes, hypertension, hyperlipidaemia and smoking, even when it is asymptomatic [123, 124]. In CKD patients, LVH develops rapidly as GFR declines and consequently LVH is already present in approximately 75% of patients starting RRT and is strongly predicative of late (i.e. >2 years) mortality on dialysis [124]. This time lag is important as it may represent an opportunity to implement interventions directed at slowing the progression of LVH and therefore potentially improving patient outcomes. One such intervention appears to be the improvement of haemoglobin levels with ESAs, such as the use of synthetic rHuEPO, which has been shown to reduce LVH [118, 125] and improve CV mortality [118]. In addition to reducing mortality and the requirements for blood transfusions, the treatment of renal anaemia in dialysis patients has been associated with improvements in quality of life [110-112, 126] and cognitive function [127, 128], as well as enhancing immune functioning [129, 130] and improving the bleeding diathesis associated with renal insufficiency [131-133].

1.3.2 Factors contributing to anaemia in haemodialysis patients

Several mechanisms have been implicated in the development of anaemia in HD patients. These are important to recognise as they highlight potential ways of minimising or correcting the anaemia; some of the key contributory factors are presented here.

1.3.2.1 Impaired erythropoiesis and altered iron metabolism

The hormone EPO consists of a 165-amino acid protein combined with four complex carbohydrate chains and is intimately involved in the control of erythropoiesis [134, 135]. Erythropoietin stimulates red blood cell (RBC) production by binding to EPO-receptors located primarily on early erythroid progenitor cells, burst-forming unit erythroid cells and colony-forming unit erythroid cells in the bone marrow [134, 135]. The binding of EPO to these receptors prevents the apoptosis of these early erythroblast cells which are then able to divide and develop into mature erythrocytes [136]. In health, the secretion of EPO reflects the oxygen content of blood with a reduction in oxygen delivery to the tissues, as occurs in anaemia, representing a powerful stimulus for secretion. The synthesis of EPO is principally mediated via the oxygen-dependent expression of a number of genes, central to which is a family of hypoxia-inducible transcription factors (HIFs) [135, 137, 138]. The two most important molecules in this family are HIF-1 and HIF-2 which comprise an oxygen-regulated α-subunit and a structural β-subunit [139]. Production of the α-subunits occurs largely independently of oxygen concentrations however oxygen mediated hydroxylation of residues present on the α-subunit increases the rate of proteosomal destruction, thus modifying the transcriptional activity of the molecules [139]. In the presence of reduced oxygen delivery this results in potentiation of HIF activity and an increase in EPO synthesis [140].

Erythropoietin is normally synthesised by interstitial fibroblasts in the renal cortex and directly stimulates the erythroid progenitor cells [135, 141]. In foetal and early postnatal life the liver produces significant amounts of EPO although this soon diminishes after which time the kidneys are the primary source of EPO synthesis [134]. Although the liver cells retain the ability to produce EPO, they are unable to compensate for the loss of renal production in patients with significant renal impairment [142] and only low levels are present in anephric adult patients [143]. The progressive loss of renal mass with declining renal function leads to a reduction in circulating EPO and a fall in haemoglobin levels [144]. However, the relationship between EPO levels and anaemia is not clear cut with many anaemic CKD patients having similar EPO levels to non-anaemic non-uraemic

controls [145] and patients with anaemia and renal impairment are often able to increase EPO production in response to a hypoxic stimulus [141]. This suggests that there is more underlying the pathophysiology of anaemia in CKD patients besides simply EPO deficiency.

An adequate supply of iron is essential for erythropoiesis as iron is a key constituent of haem, the oxygen carrying component of mature erythrocytes. Effective erythropoiesis requires the transfer of large amounts of iron from its storage sites, mainly in the liver and spleen, to the bone marrow for haemoglobin synthesis. Iron deficiency can arise due to depletion of iron stores, termed true iron deficiency, or there may be sufficient stores of iron but problems with its mobilisation or utilisation which has been termed functional iron deficiency [146]. Both factors are important in the development of anaemia in HD patients. Absolute iron deficiency is not uncommon among dialysis patients due to occult or non-occult blood loss coupled with poor dietary intake and impaired intestinal absorption [146, 147]. Functional iron deficiency is also frequently encountered in HD patients, although detecting it is not straightforward using routinely available clinical laboratory tests such as ferritin determination [148]. Measurement of the percentage of hypochromic red blood cells or the transferrin saturation provide an indirect measure of iron utilisation and are useful for diagnosing functional iron deficiency in this setting [146, 149]; published guidelines of iron therapy in HD patients are based on these parameters [150, 151].

Inflammation is an important contributor to functional iron deficiency. It can lead to increased levels of the peptide hepcidin [152], a negative regulator of iron metabolism (the levels of which are already increased in patients with renal impairment [153-155]), reduced uptake of iron from the gut [147] and enhanced uptake of lactoferrin (a molecule involved in iron trafficking) by activated macrophages expressing lactoferrin receptors [156, 157]. In addition to its effects on iron metabolism, several inflammatory mediators, such as TNF-α, IL-1 and IFN-γ, have been shown to exert a suppressive effect on erythroid progenitor cells in the bone marrow [158, 159] and blunt the secretion of EPO in response to falling haemoglobin levels [160]. Several markers of malnutrition, such as BMI, albumin levels and the protein catabolic rate, have been linked to anaemia severity [161-163], although inflammation and malnutrition frequently co-exist in HD patients [164] making it hard to separate out their individual effects.

Besides inflammation, several other aspects of renal insufficiency and dialysis therapy contribute to impaired erythropoiesis. A link between hyperparathyroidism and anaemia

has been known for some time [165, 166]. The most likely mechanism behind this association is a pro-fibrotic effect on the bone marrow [167-169], although a direct inhibitory effect of PTH on early erythroid cells has been reported by some [170], but not all [171, 172], investigators with the differing findings possibly related to the preparations of PTH used [173]. As yet unidentified factors retained in the plasma of dialysis patients, so called uraemic inhibitors of erythropoiesis, may also play a role in anaemia. Evidence for their existence comes from observations that HD patients often experience a rise in haemoglobin levels following the initiation of dialysis [174-176] or an increase in dialysis dose [177-180]. Early *in vitro* work using animal cells lines demonstrated the ability of uraemic serum to inhibit erythropoiesis [181-183] although subsequent work using an autologous human model failed to corroborate these findings [184]. Therefore proof of the existence of uraemic inhibitors of erythropoiesis, their nature and mechanism of action remains enigmatic.

The renin-angiotensin system (RAS) may also be relevant to the development of anaemia in dialysis patients. Activation of the RAS is common in dialysis patients, particularly if they are hypertensive [185-188], and pharmacological blockade of the RAS is frequently employed owing to the anti-hypertensive and cardioprotective properties of these medications [189]. Elevated haematocrits have been reported in patients with activation of the RAS secondary to renal artery stenosis of both native [190] and transplanted [191] kidneys. Similarly blood pressure trials in non-dialysis patients have reported lower haemoglobins in patients taking angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor antagonists [192-195]. In HD patients, the use of drugs which antagonise the RAS appear to increase ESA requirements [196, 197] with improvements in the ESA requirements observed following drug cessation [198]. Receptors for angiotensin-II, one of the effector molecules of the RAS, have been identified on erythroid progenitor cells and the formation of blood forming units *in vitro* is increased in the presence of angiotensin-II and suppressed by pharmacological receptor blockade [199, 200]. Data from rat models [201, 202] and human studies [203, 204] point to a role for angiotensin-II as an EPO secretagogue. An endogenous haematopoiesis inhibitor, N-acetyl-seryl-aspartyl-lysl-proline, has been identified which is almost exclusively degraded by ACE, providing another mechanism for anaemia in patients treated with ACE inhibitors [205, 206].

1.3.2.2 Reduced erythrocyte lifespan and enhanced oxidative stress

The haemoglobin concentration at any one time reflects the balance between RBC production and destruction. It has been known for some time that erythrocyte lifespan is reduced in uraemic patients [207-211] by approximately 20% compared to healthy controls [212]. Corpuscular factors appear to be important in this setting as erythrocytes from uraemic individuals have different physical properties when compared to erthrocytes obtained from non-uraemic individuals. They have a more rigid structure [213-218], rendering them more susceptible to splenic sequestration and destruction [216, 218, 219], resulting in a shortened lifespan. This notion is supported by anecdotal evidence of improvements in anaemia for dialysis patients who underwent splenectomy in the pre-modern treatment era [216, 219].

Oxidative stress is thought to be an important contributor to shortened RBC survival owing to increased membrane rigidity as a consequence of erythrocyte membrane lipid peroxidation [218, 220]. In support of this, several studies have reported an association between increased levels of oxidative stress and lower haemoglobin levels [221-224] and higher ESA requirements [225, 226]. Importantly, however, the link between oxidative stress and renal anaemia is not clear cut and is probably bi-directional because RBCs, or more specifically the anti-oxidants within RBCs (e.g. glutathione reductase, superoxide dismutase), form an important part of the host defence against oxidative stress. In other words, oxidative stress begets anaemia and anaemia begets increased oxidative stress as shown in Figure 5. A detailed account of the factors predisposing to increased oxidative stress in HD patients and its association with CV disease is provided in section 1.4.

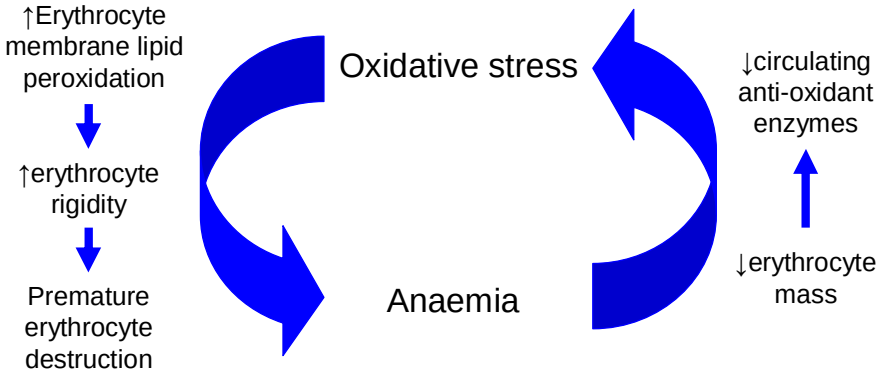


Figure 5 - Positive feedback loop concerning anaemia and oxidative stress. Oxidative modification of erythrocyte membranes leads to increased erythrocyte rigidity and premature destruction. Anaemic patients have lowered anti-oxidant defences by virtue of a decreased mass of circulating erythrocytes and the anti-oxidant defence mechanisms contained therein.

Besides oxidative stress, a number of other factors can contribute to premature erythrocyte destruction in HD patients. Erythrocytes isolated from HD patients appear to be particularly susceptible to complement mediated cell lysis [227] and HD patients are both primed for complement activation [228, 229] and frequently exposed to factors known to promote activation of the complement system, such as the dialysis membrane [230-234]. Detailed accounts of the complement system, complement activation in HD patients and the implications for the development of CV disease are provided in section 1.5. Dialysate contaminants, such as chloramines [235, 236] or heavy metals [237, 238], can result in haemolysis, with a consequent reduction in the eryrthrocyte lifespan, underscoring the need to maintain high standards of water quality in HD units. Blood loss is also common in HD patients, particularly from the gastrointestinal tract [239-242], owing to the presence of a number of risk factors for bleeding including the effects of uraemic toxins on platelet function, the regular administration of anti-coagulants to facilitate dialysis and anaemia itself [243, 244].

In summary, it can be seen that a number of factors conspire to cause anaemia in HD patients. These can influence both the ability to generate new red blood cells, erythropoiesis, and shorten the lifespan of circulating erythrocytes. Effective management of renal anaemia, therefore, requires a multifaceted approach in order to address these different factors. An overview of the strategies used to treat renal anaemia in chronic HD patients is provided in the next section.

1.3.3 Treatment of anaemia in haemodialysis patients

Prior to the 1980's, patients on regular HD frequently required blood transfusions to maintain their haemoglobin levels. This presented significant risks in terms of viral transmission, iron overload and allosensitisation; the latter being important in limiting organ availability for renal transplantation. Other measures to improve anaemia included the use of cobalt [245] or androgens [246] to stimulate erythropoiesis. Early studies on sheep had shown that administration of EPO rich plasma could correct the anaemia association with renal failure [247]. In 1977 human EPO was purified [248] and 8 years later the gene was identified and cloned [249, 250] enabling the large scale production of rHuEPO. Shortly after this, successful trials of rHuEPO being used to treat anaemia in dialysis patients were published [251, 252]. Since this time, a number of different agents capable of stimulating erythropoiesis, collectively termed ESAs, have been developed (e.g. HIF stabilisers, peglyated rHuEPO [253]). The use of ESAs is now commonplace with over 90% of UK dialysis patients receiving an ESA [4]. In 2001 darbepoetin-alfa, the ESA used in the

present study, was licensed for the treatment of renal anaemia. It is a modified rHuEPO, differing in its amino acid sequence and carbohydrate content, resulting in a lower binding affinity for the EPO receptor but a longer half-life and greater duration of biological activity [254, 255]. The advantages of this being reduced frequency of administration enabling weekly, fortnightly or even monthly dosing in stable patients [256], compared to the initial three times per week dosing schedule with rHuEPO. The ESA requirements differ significantly between patients and, for an individual patient, change over time necessitating regular monitoring of the haemoglobin levels with ESA dose adjustments in order to maintain haemoglobin levels within a desired range. This variation is in part due to the large number of factors known to influence ESA responsiveness, such as inflammation, the availability of iron stores, dialysis adequacy and intercurrent clinical events such as haemorrhage or dialysis circuit clotting [257]. As previously discussed, iron deficiency is common in HD patients and iron supplementation is frequently employed in the treatment of anaemia for patients on HD [258]. Trials of iron preparations in dialysis patients have demonstrated that oral preparations are frequently inadequate [259, 260] and that intravenous preparations can be effective in the treatment of anaemia [261-263]. Intravenous iron is frequently co-prescribed with ESAs and it has been shown to reduce ESA requirements [264-268], even in patients with high ferritin levels [264, 269]. Treatments for anaemia, particularly the provision of ESAs, are associated with significant healthcare costs which are attributable both to the procurement of the drugs and the infrastructure required to administer and monitor them [270]. In addition to cost considerations, anaemia and the use of ESA are closely linked to the development of CV disease in HD patients.

1.3.4 Anaemia and cardiovascular disease in haemodialysis patients

In the first published reports of ESA administration for the treatment of renal anaemia, investigators were able to demonstrate dose-dependant increases in haemoglobin levels and the avoidance of blood transfusions, but at the expense of higher blood pressures and rates of vascular thrombosis [251, 252, 271]. Similar findings were reported in subsequent placebo-controlled trials [272, 273] along with observational reports of improved quality of life scores [108, 112, 126] and patient outcomes [274, 275]. Since these early studies, a large number of observational [52, 113, 114, 116, 274-283] and interventional studies [284] in dialysis patients, and meta-analyses and systematic reviews of CKD patients (including patients on dialysis) [285-289] have tried to determine the optimal haemoglobin

target for ESA treated dialysis patients. The message from these studies appeared to be that targeting higher haemoglobin levels, usually above 13 g/dL (i.e. near normalisation), was associated with a greater incidence of CV events, mortality, hypertension and vascular thrombosis (particularly in patients receiving large doses of ESAs [50-52]) compared to aiming for a lower haemoglobin target of around 11 g/dL (i.e. partial correction), with no apparent benefits in terms of patient reported quality of life outcome measures. What is less clear is whether the excess harm was attributable to higher haemoglobin levels, higher ESA doses or a confounding effect of one or more of the factors known to contribute to ESA resistance. Three potential causal mechanisms to explain the findings of these studies are depicted graphically in Figure 6 and discussed further here.

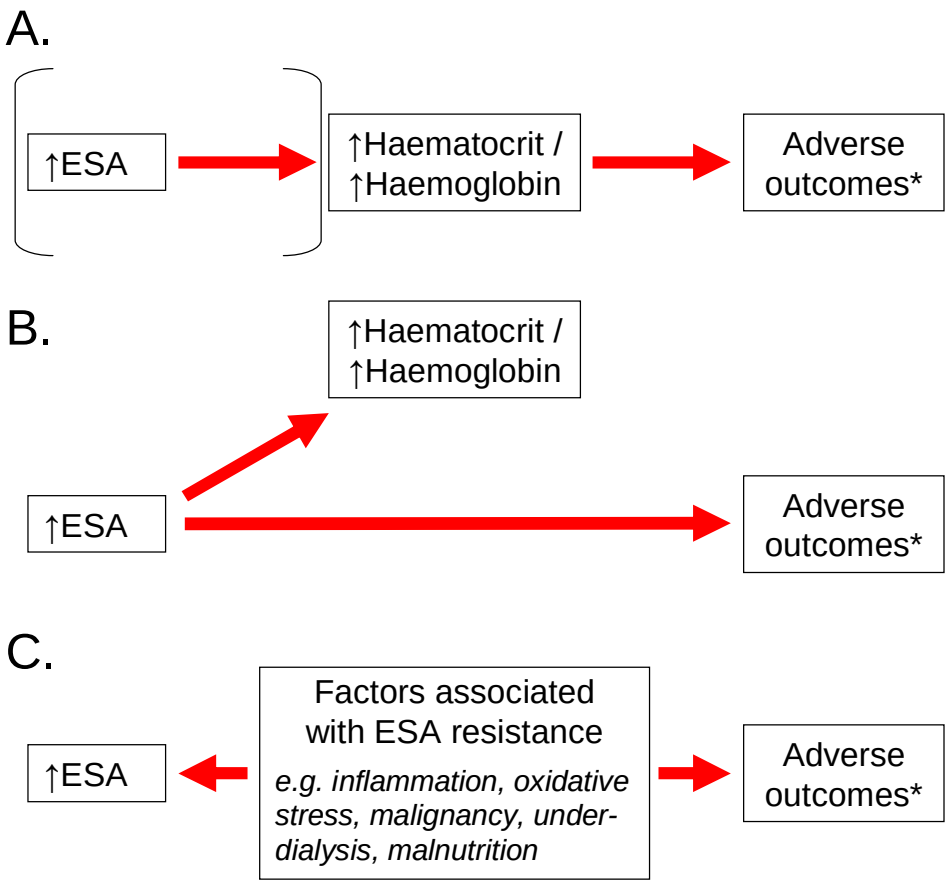


Figure 6 - Causal diagram outlining the possible explanations for the higher mortality in ESA intervention trials for patients randomised to the higher haemoglobin target group. A. Increased haemoglobin levels / haematocrit leads to adverse outcomes. B. Higher ESA doses are responsible for the increased rates of adverse outcomes, increased haemoglobin levels / haematocrit are an epiphenomenon. C. The factors which render patients ESA resistant, hence requiring of larger ESA doses, are also associated with adverse outcomes. (*Adverse outcomes: mortality, cardiovascular events, hypertension and vascular thrombosis; ESA: Erythropoiesis stimulating agent)

Higher haemoglobin levels could plausibly explain some of the association between the use of ESAs and the worsening of hypertension or the occurrence of vascular thromboses or CV events in the aforementioned studies [251, 252, 271, 273, 284]. Maintenance of blood volume requires a reduction in plasma volume for higher haemoglobin levels and the resultant higher haematocrit will increase the propensity for thrombosis, as well as increasing vascular resistance and blood pressure [290]. However, observational data from dialysis patients who maintain haemoglobin levels above 12 g/dL without the need for ESAs identified no excess mortality for these patients [291]. Additionally, a large observational study of 58,058 HD patients reported that higher ESA doses were associated with increased mortality, irrespective of haemoglobin levels and, in contrast to the previously cited intervention studies, a haemoglobin level in the range 12-13 g/dL was associated with the greatest survival [52]. Similarly, a secondary analysis of a study comparing higher and lower haemoglobin targets in CKD patients not on dialysis [50] found that both higher ESA doses and failure to achieve the target haemoglobin, which was much more prevalent in the higher target haemoglobin group, rather than the absolute haemoglobin levels were associated with higher mortality. Taken together, these findings suggest that higher ESAs doses, rather than higher haemoglobin levels or haematocrit, may be directly responsible for the development of CV complications.

A number of mechanisms have been put forward to explain the potential for harm with high ESA doses. The intermittent ESA dosing schedules, with the resultant swings in blood levels, are very different to the continuous low levels of EPO produced in the healthy state and the blood levels achieved with ESA administration are supra-physiological [292]. Many non-erythroid cells express the EPO-receptor, including vascular endothelial cells, smooth muscle cells and cardiac myocytes [293], where receptor activation generally promotes cellular survival. The supra- and non-physiological stimulation of these receptors, and the subsequent effects on cell survival and apoptosis, for example potentially resulting in adverse myocardial modelling, could explain some of the increased risks associated with ESA usage although no direct evidence for this exists [294]. Furthermore, studies have reported ESAs to increase endothelial and vascular smooth muscle cell proliferation [295-297], the expression of MCP-1 [297, 298] and markers of endothelial damage such as tissue-type plasminogen activator (tPA) and thrombomodulin [299], all of which have been linked to the development of atherosclerosis. Hypertension can develop in ESA treated patients due to the associated hypervolaemia, in addition to ESA-mediated up-regulation of vasoconstrictors, such as endothelin-1 and thromboxane A_2 [300, 301], and the increased expression of angiotensin II receptors in vascular smooth muscle [302].

Elevated levels of pro-inflammatory cytokines, such as TNF-α, have been reported in renal patients treated with ESAs [303, 304] and these in turn have been linked with an increased risk of CV disease in the general population [305, 306]. There are therefore a number of mechanisms through which ESAs may cause CV disease.

Prospective studies of both incident [307] and prevalent [308] dialysis patients have reported significant associations between ESA dose and mortality which became non-significant when the analyses were adjusted for confounders such as inflammation and comorbidity. Therefore, perhaps a more compelling explanation for the greater mortality in patients receiving higher ESA doses is that patients receiving the higher doses are often ESA resistant, i.e. requiring of higher ESA doses to achieve a given haemoglobin level, and the factors which contribute to this ESA resistance actually mediate the higher mortality rates. The effects of further ESA dose increases on haemoglobin levels in patients already taking large doses appear to be minimal [309], suggesting that in many cases failure to respond to an ESA is due to ESA resistance rather than under-dosing. A number of factors which contribute to ESA resistance, such as inflammation, oxidative stress, malignancy, under-dialysis and malnutrition, are themselves associated with an increased risk of mortality [162, 257]. The idea that ESA resistance, rather than high doses of ESAs *per se*, is associated with worse outcomes has been borne out by several large observational series [51, 310, 311] and a prospective study which specifically measured ESA-responsiveness [312]. It therefore seems likely that the factors which contribute to ESA resistance, and hence necessitate the usage of higher ESA doses, are the same factors which portend the higher mortality rates rather than it being directly attributable to the ESAs. In other words, in the studies which showed an association between ESA dose and adverse outcomes, the ESA dose may in fact be a proxy for the factors driving ESA resistance, rather than the ESA being mechanistically involved in the development of CV disease. In an attempt to better understand the relationship between ESA dose, ESA-resistance and adverse outcomes prospective studies are being undertaken comparing fixed high and low dose ESA administration, as opposed to haemoglobin target driven therapy, on patient mortality and CV outcomes [313].

From the data presented here it can be seen that a number of the factors which are implicated in the high rates of CV disease in HD patients also contribute to renal anaemia and ESA resistance. Therefore targeting these factors may lead to improved patient outcomes as well as improvements in anaemia. Additionally, such approaches may limit some of the potential harm and high costs associated with increased ESA usage. Two

such factors are oxidative stress and inflammation, both of which are commonly encountered in HD patients and are discussed in more detail here.

1.4 Oxidative stress

1.4.1 Overview of oxidative stress

Oxidative stress is a term which refers to an imbalance between the production of potentially harmful reactive oxygen species (ROS) and the normal anti-oxidant protective mechanisms present to guard against tissue damage. Reactive oxygen species include superoxide anions, hydrogen peroxide and the hydroxyl radical and they are formed from a wide variety of enzymatic and non-enzymatic reactions. Important sites of oxidative activity in mammals are the mitochondrial respiratory chain and phagocytes in the host defence against pathogens [43]. The mitochondrial enzyme cytochrome oxidase is responsible for much of the oxygen metabolised by humans and it catalyses the transfer of electrons to molecular oxygen reducing it to water [314]. If the oxygen is only partially reduced this can lead to the formation of ROS, such as the superoxide anion resulting from the transfer of a single electron to molecular oxygen [315]. Additionally, a number of different sources of ROS exist in vascular and other tissues including the nicotinamide dinucleotide phosphate (NADPH) oxidase enzyme complex, xanthine oxidase, lipoxygenases, cyclooxygenases [316] and nitric oxide synthase [317].

Mammalian systems have evolved a number of protective anti-oxidant mechanisms to try and limit the damage caused by ROS. These comprise enzymes, such as superoxide dismutase and glutathione peroxidise, as well as anti-oxidants such as reduced glutathione, transition metal ions, tocopherols and ascorbic acid [318, 319]. Oxidative stress, or ROS-mediated damage, occurs when these protective mechanisms become overwhelmed such that there is an excess of ROS. The ROS are then able to react with a number of different macromolecules, such as deoxyribonucleic acid (DNA), proteins, lipids or carbohydrates, leading to alterations in their structure and / or function.

The *in vivo* detection of ROS is technically difficult owing to the small quantities involved and their highly reactive nature with consequent short half-lives. To circumvent this problem, investigators have identified a number of end-products of oxidative damage, formed through different reaction pathways, which are stable and easier to measure [43, 320-323]. Some of the commonly measured markers of oxidative stress are listed in Table 1. Measurement of oxidative stress, however, is not restricted to the use of biomarkers as

various other methodologies have been employed including measurement of total anti-oxidant capacity (TAC), evaluation of anti-oxidant enzyme activity, such as erythrocyte superoxide dismutase, glutathione (GSH) or plasma glutathione peroxidase, or measuring the levels of antioxidants such as vitamins E and C [324, 325].

Table 1 - Examples of commonly measured biomarkers of oxidative damage.

Lipids
Malondialdehyde
Thiobarbituric acid reactive species (TBARS)
Oxidised low-density lipoprotein (Ox-LDL)
Exhaled alkenes
Advanced lipoxidation end products
4-hydroxynonaneal
$F_{2\alpha}$-isoprostane
Proteins
Thiol oxidation
Advanced oxidation protein products (AOPPs)
Carbonyl formation
Deoxyribonucleic acid
8-hydroxy 2' deoxyguanine
Carbohydrates
Advanced glycation end products (AGEs)
Reactive aldehydes

1.4.2 Oxidative stress in haemodialysis patients

A number of studies have demonstrated greater levels of oxidative stress in HD patients compared to healthy controls by measuring a variety of biomarkers including F2-isoprostanes, advanced oxidation protein products (AOPP), malondialdehyde (MDA) and Ox-LDL [326-337]. Interestingly, markers of oxidative stress appear to increase as renal function declines [338, 339] and several factors are thought contribute to the high oxidative burden observed in renal populations. These factors include the higher prevalence in renal populations of conditions which are themselves associated with enhanced oxidative stress such as hypertension [316], diabetes mellitus [340, 341] and increased age [342, 343]. Additionally, a number of different aspects of renal failure and HD therapy may directly contribute to oxidative damage both through the increased generation of ROS and reduced anti-oxidant defences.

1.4.2.1 Increased generation of reactive oxygen species

It has been known for some time that even a single session of HD can increase markers of lipid peroxidation [344, 345] and reduce the plasma antioxidant capacity [346]. An increase in the respiratory burst activity of neutrophils plays an important role in the enhanced oxidative stress observed in these patients. Neutrophils obtained from HD patients immediately prior to a dialysis session have a higher spontaneous rate of ROS production compared to those obtained from healthy controls [330, 347] and they are primed for an enhanced respiratory burst following stimulation [347, 348], as are neutrophils obtained from patients with advanced renal insufficiency not yet on HD [349, 350]. It therefore appears likely that there is an as yet unidentified factor (or factors) retained in the plasma of patients with renal insufficiency which mediates this phenomenon. In support of this theory, studies have demonstrated the ability of plasma from patients on HD, or with advanced renal insufficiency, to stimulate ROS generation by neutrophils [347, 350, 351]. This effect is partially corrected by high-flux dialysis or haemodiafiltration [351, 352] and reversed by renal transplantation [347] suggesting the presence of a circulating factor.

Another factor which is important in HD related oxidative stress is the dialysis membrane. Dialysis with bio-incompatible membranes, such as cuprophane, has been shown to greatly enhance oxidative stress [353, 354]. More recent studies looking at markers of lipid peroxidation have shown lower levels in patients dialysing with more biocompatible polysulfone compared to modified cellulose membranes [355]. These effects may be mediated through complement activation as the complement fragment C5a has the ability to stimulate, or at lower concentrations prime, the respiratory burst activity in neutrophils [356, 357]. (In the context of this thesis, an overview of the complement system is provided in section 1.5.2 and the biological consequences of membrane biocompatibility in section 1.7.3.1.)

Endotoxaemia may also contribute to oxidative stress through activation of NADPH oxidase in phagocytes [358, 359]. Trace amounts of endotoxin in dialysate have the potential to enter the bloodstream of patients dialysing with high-flux membranes [360, 361] although many centres, including the centre where the present study was conducted, use endotoxin filters reducing the potential for this to occur. The translocation of endotoxins from the bowel has also been reported in HD patients, particularly following periods of intra-dialytic hypotension [362], providing another mechanism of endotoxin exposure. Blood-membrane interactions and endotoxin exposure, however, can only partly

explain the increased oxidative burden in HD patients as evidence of enhanced oxidative stress is also present in peritoneal dialysis patients [334, 363] who are not exposed to these factors.

Intravenous iron preparations are frequently administered to dialysis patients for the treatment of anaemia (see section 1.3.3) and may be another important contributor to the oxidative burden [364-369], owing to the ability of free iron to act as a catalyst for free radical generation [370, 371]. However, current data would suggest that with modern intravenous iron preparations and dosing regimens aimed at the avoidance of iron overload, the toxicity attributable to free iron is minimal [372-374]. There is also data that ESAs may contribute to the oxidative burden in HD patients by increasing superoxide production by stimulated PMNs [375] and depleting anti-oxidant enzymes in erythrocytes [376, 377].

1.4.2.2 Reduced anti-oxidant defences

In addition to the pro-oxidant factors present among dialysis patients discussed above, there is also a reduced antioxidant capacity. The enzymes superoxide dismutase, catalase and the glutathione system are important in the host defence against ROS and are primarily intracellular. The activity of these enzymes in HD patients appears to be varied. The activity of superoxide dismutase and glutathione reductase in erythrocytes has been reported to be normal or decreased and glutathione peroxidase activity to be normal [332, 378, 379]. The whole blood concentration of glutathione appears to be reduced [379] despite preservation of normal levels within erythrocytes [380] and there also appears to be defects in the hexose monophosphate shunt within RBCs resulting in a reduced ability to detoxify oxidising free radicals [381].

Plasma proteins are also important in the host defence against oxidative stress [382] as the sulphydryl groups are capable of being oxidised through the formation of a disulfide bond with low molecular weight aminothiols, such as cysteine and homocysteine, present in the plasma. Levels of cysteine and homocysteine are significantly elevated in dialysis patients [383, 384] and fall post-dialysis [383, 385]. The removal of free aminothiols during dialysis may result in the dissociation of the protein bound portion thus freeing up sulphydryl groups and improving the antioxidant capacity of the plasma proteins. Owing to its abundance in plasma, albumin is a particularly important antioxidant [386, 387] and patients with hypoalbuminaemia have greater erythrocyte membrane lipid peroxidation than patients with normal albumin levels [388]. Levels of the most biologically active form

of Vitamin E, α-tocopherol, appear to be similar in HD patients and healthy controls [389-392], whereas Vitamin C levels tend to be lower in dialysis patients likely owing to dialytic losses of this water soluble vitamin and avoidance of vitamin C rich foods which are often high in potassium [390, 393, 394]. Anaemia in HD patients, as discussed in section 1.3.2.2, also contributes to lowered anti-oxidant defences, which improve following ESA therapy [395], owing to the abundance of anti-oxidant enzymes, such as glutathione reductase and superoxide dismutase, within erythrocytes. It can be seen, therefore, that renal insufficiency and HD contribute to oxidative stress both through factors which increase ROS production and impair anti-oxidant defence mechanisms.

1.4.3 Oxidative stress and cardiovascular disease

Observational studies have reported on the association between markers of oxidative stress and CV disease both in dialysis patients [396-399] and non-renal populations [400]. Some of this association may be due to the presence of factors which are themselves associated with increased oxidative stress and the development of CV disease, such as diabetes [340, 341], hypercholesterolaemia [401], advancing age [342, 343], tobacco consumption [402, 403] and hypertension [316]. However, there is observational and laboratory data pointing to a functional role for oxidative stress in the development of CV disease.

Enhanced oxidative stress is thought to favour the development of atherosclerosis and a number of markers of oxidative stress have been found to correlate with the degree of carotid intima-media thickness in HD patients [404-406]. Endothelial dysfunction is an important initiating event in the development of atherosclerotic lesions [53] (see section 1.2.2) and is associated with increased CV events, even in the absence of obstructive arterial disease [407]. Oxidative stress may promote endothelial dysfunction through interactions with the nitric oxide system, leading to reduced bioavailability of nitric oxide, and impaired vascular relaxation [408, 409]. Furthermore, superoxide anions (O_2^-) can react with nitric oxide forming cytotoxic nitric oxide derivatives, such as peroxynitrite, which promote platelet aggregation [410, 411] and therefore increase the propensity for thrombosis. Oxidative stress also leads to alterations in lipoprotein function and the formation of highly reactive lipid peroxidation products. These are thought to have a role in the development of CV disease as set out in the “oxidative modification hypothesis” of atherosclerosis [412] (see section 1.2.2.1) in which the the oxidative modification of LDL to form Ox-LDL prior to uptake by macrophages is one of the key steps in the pathogenesis of atherosclerotic lesions [413-415]. Greater levels of lipid peroxidation products [416],

including Ox-LDL [392, 417, 418], have been found in patients with renal failure along with increased expression of macrophage scavenger receptors leading to increased Ox-LDL uptake [419]. Certain transcription factors, including nuclear factor-κB (NFκB) and activator protein-1, appear to be up regulated in response to oxidative stress resulting in the increased expression of adhesion molecules involved in atherogenesis, such as VCAM-1, ICAM-1 and E-selectin, and the promotion of smooth muscle cell proliferation [420]. In addition to playing a role in atherosclerotic CV disease, there is emerging evidence that oxidative stress may promote vascular calcification through up regulation of bone morphogenetic protein-2 [421] and induction of Runx2 [422], key transcription factors for osteogenic differentiation of vascular smooth muscle cells, suggesting oxidative stress may contribute to CV disease through enhancing vascular calcification.

It can therefore be seen that the levels of oxidative stress are increased in HD patients owing to factors which both increase the generation of ROS and impair anti-oxidant defences. These heightened levels of oxidative stress, through the promotion of endothelial dysfunction, the enhanced atherogenicity of oxidatively modified lipids, effects on vascular calcification and worsening of anaemia, may in turn contribute to the increased rates of CV disease observed in HD patients.

1.4.4 Interplay of oxidative stress and inflammation in haemodialysis patients

A number of studies have reported on the positive correlation between markers of oxidative stress and inflammation in HD patients [327, 332, 423-425] and, furthermore, it appears that these process are mechanistically linked. One way in which these processes are linked is through the enzyme myeloperoxidase, an abundant haem protein present in phagocytes, which converts chloride and hydrogen peroxide to hypocholorous acid [426]. Neutrophil activation during HD is well recognised and elevated biomarkers of myeloperoxidase-catalysed oxidation following an HD treatment have been reported [333]. Thiol groups on proteins, such as albumin, are an important part of the host defence against oxidative damage [382, 386, 427] as discussed in section 1.4.2.2, and the levels of albumin fall in response to inflammation [428, 429] resulting in diminished anti-oxidant defences and a greater susceptibility to oxidative stress in the setting of inflammation. The complement system is also linked to oxidative stress as Ox-LDL has been shown to bind C3a *in vivo* [430] and ROS, such as hydrogen peroxide, have been shown to directly activate C5 via a non-enzymatic mechanism [431]. Further evidence of a synergistic

relationship between complement and oxidative stress comes from animal models of ischaemia-reperfusion injury whereby complement activation is attenuated by the addition of anti-oxidants [432, 433].

Oxidative stress and inflammation are frequently encountered in HD patients, are mechanistically linked through a variety of mechanisms and both are implicated in the pathogenesis of anaemia and CV disease in HD patients. An overview of inflammation in HD patients and the implications for the development of CV disease is provided in the following section.

1.5 Inflammation

1.5.1 Overview of inflammation in haemodialysis patients

Increased inflammation in HD patients is both frequently encountered and associated with increased mortality, principally from CV disease [163, 429, 434-442]. It is therefore a reasonable line of enquiry to investigate whether interventions which reduce inflammation translate into improved outcomes for patients. In order to do this, it is first necessary to understand the factors which contribute to inflammation in HD patients. Inflammation in HD patients is multifactorial. Some of the patient specific factors which have been shown to be important include the dialysis access [443], particularly thrombosed grafts [444], persistent subclinical infections, such as *Chlamydia pneumoniae* [445-447] or dental infections [448, 449], failing transplants [450] or comorbidities such as heart failure [451]. Vascular access appears to be particularly important with respect to inflammation and the type of dialysis access has been linked to patient mortality, being lowest with native vessel AVFs and highest with CVCs [7, 443, 452]. This association with mortality may be mediated, at least in part, through effects on inflammation as lower levels of the negative acute phase reactant albumin are found in patients dialysing with CVCs or synthetic grafts, compared to AVFs [453], and low albumin levels are a strong predictor of mortality in dialysis patients [47, 429, 435]. These associations may represent selection bias, with the healthiest patients tending to undergoing AVF formation [454], although albumin levels fall following CVC insertion [455] and rise following removal [456] suggesting a causal association between dialysis access and inflammation. The link between dialysis access and inflammation may also be mediated through infection as the incidence of infections, a powerful stimulus for inflammation, is increased in patients with CVCs [6, 457] and infective episodes are associated with an elevated risk of subsequent CV events in dialysis patients [457, 458]. Additionally CVCs, even in the absence of overt infection, may

contribute to systemic inflammation through the formation of biofilms and bacterial colonisation [459-461].

Renal insufficiency itself predisposes to inflammation through the reduced renal clearance of inflammatory cytokines [462-464] and components of the complement system, such as factors D [228] and B [465], in addition to the accumulation of advanced glycation end-products (AGEs) [466-468] which are able to trigger an inflammatory response *in vitro* [469, 470]. However the levels of inflammatory biomarkers and cytokines do not appear to be correlated with glomerular filtration rate in pre-dialysis patients [471, 472] suggesting reduced renal clearance is not the only factor. The dialysis process itself contributes to inflammation [473] through blood-membrane interactions promoting cytokine release [474] and complement activation [230-233], in addition to the potential additive effects of non-sterile dialysate or back-filtration [475, 476] and endotoxin exposure [360-362]. Optimised HD therapy using ultrapure dialysate and biocompatible membranes reduces markers of inflammation, such as C-reactive protein (CRP), but does not normalise them [477], suggesting that refinements to the dialysis technique can only go so far in reducing the inflammatory burden for HD patients [478].

The complement system plays an important role in the increased inflammation observed in HD patients. The ability of dialysis membranes to activate the complement cascade has been well established [230-233]. This is largely attributable to nucelophilic binding sites afforded by hydroxyl groups on the membrane surface binding C3b and triggering the alternative complement pathway [479, 480]. The adsorption of C3 onto polymer surfaces has also been shown to induce conformational changes, such that the molecule resembles C3b, providing another mechanism of alternative complement pathway activation in HD patients [234]. The majority of the complement proteins are synthesised in the liver and are acute phase reactants [481] and thus HD patients, with heightened levels of inflammation and the reduced renal clearance of complement proteins [228, 465], may be primed for complement activation. The elevated CRP levels frequently encountered in HD patients may also contribute to complement activation as CRP can form complexes with polysaccharides and C1q [482, 483], leading to activation of the classical complement pathway, and interact with factor H enhancing alternative pathway activation [484]. There are, therefore, a number of factors conspiring to enhance complement activation in HD patients, both in terms of patients being primed for activation and being subjected to multiple complement activating stimuli. Given these observations and the focus of this thesis, an overview of the complement system is provided in the following section.

1.5.2 The complement system

The complement system is an important component of the innate immune system and comprises a number of plasma and membrane bound proteins involved in pathways of cascading enzymatic reactions.

1.5.2.1 Pathways of complement activation

There are three major activation pathways for the complement system termed: (i) the classical pathway, (ii) the lectin pathway and (iii) the alternative pathway (see Figure 7).

(i) Classical pathway

The classical pathway is triggered by the binding of C1q to specific structures on microbial or apoptotic cells, or to endogenous pattern recognition molecules such as immunoglobulins (IgM or IgG) or pentraxins (e.g. CRP) [482, 485]. When the C1q subunit of the C1 complex binds to a target, the C1r subunit undergoes an auto-activation cleavage process which in turn cleaves and activates the C1s subunit. The C1s subunit then cleaves C4 and C2 resulting in the formation of the C3 convertase complex C4b2a. The C4b2a complex then combines with C3b, generated from the cleavage of C3, to form the C5 convertase complex C4b2a3b [486]. The C5 convertase cleaves C5 to form the anaphylotoxin C5a and the fragment C5b. The C5b fragment associates with C6 and C7, forming the C5b-7 complex, which becomes incorporated into the target cell membrane. Membrane bound C5b-7 acts as a receptor for C8 and the resultant C5b-8 complex binds and polymerises C9 to form the lytic terminal complement complex C5b-9 [487].

(ii) Lectin pathway

The lectin pathway is similar to the classical pathway with mannose-binding lectin (MBL) fulfilling a similar role to C1q and the MBL-associated serine proteases (MASPs) having structural and functional homology with the classical pathway C1r and C1s molecules. The MBL molecule binds specific carbohydrates on the surface of pathogens leading to the activation of MASPs which in turn cleave C2 and C4 to form the C3 convertase complex C4b2a as in the classical pathway [485].

(iii) Alternative pathway

The alternative pathway of complement activation is distinct from the other two pathways. Under normal physiological conditions, the C3 molecule undergoes low-grade spontaneous hydrolysis of an unstable internal thioester forming C3(H_2O), so called “C3

tick over" [488]. The C3(H_2O) then combines with factor B, which is subsequently cleaved by factor D, forming a soluble C3 convertase C3(H_2O)Bb. The formation of this initial C3 convertase of the alternative pathway is thought to be the mechanism through which the initial C3b molecules are generated [489]. These C3b molecules are important in immune surveillance as contact with a target cell leads to opsonisation by C3b and accelerated complement activity. This is achieved by the bound C3b combining with factor B which, after cleavage by factor D, generates the alternative pathway C3 convertase C3bBb [485]. This C3 convertase is subsequently stabilised by combining with properdin, to form C3bBbP [490], and leads to the generation of yet more C3b thereby establishing a positive feedback loop. This process is inhibited on healthy host cells but amplification occurs rapidly on damaged or target cells. More recently it has been discovered that the alternative pathway can be activated by properdin acting as a pattern recognition molecule [485, 491] in addition to the roles of properdin in amplifying the complement response by attracting C3b [492] and stabilising the C3 convertase complex (C3bBbP). The deposition of C3b generated by the classical or lectin pathways can serve as a catalyst for amplification of the complement response by the alternative pathway. This may be particularly important as up to 80% of the C5a and C5b-9 formed following complement activation via the classical pathway may be generated by the alternative pathway [493], highlighting the role of the alternative pathway in amplifying the complement response.

1.5.2.2 Effector functions of complement

There are several effector mechanisms of the complement system. The opsonisation of target cells by C3b facilitates immune clearance, principally by phagocytes bearing complement receptors, and amplification of the immune response. The anaphylatoxins C3a and C5a are produced in high quantities at sites of complement activation and act as chemoattractants for inflammatory cells in addition to exerting a wide range of biological effects through receptor interactions, including effects on vascular tone and permeability and upregulation of other inflammatory mediators such as IL-6 and TNF-α [494]. Complement activation also results in generation of the membrane attack complex C5b-9. This is initialised following C5b formation by the action of C5 convertases on C5 and results in target cell lysis by forming pores in the cell membranes [485].

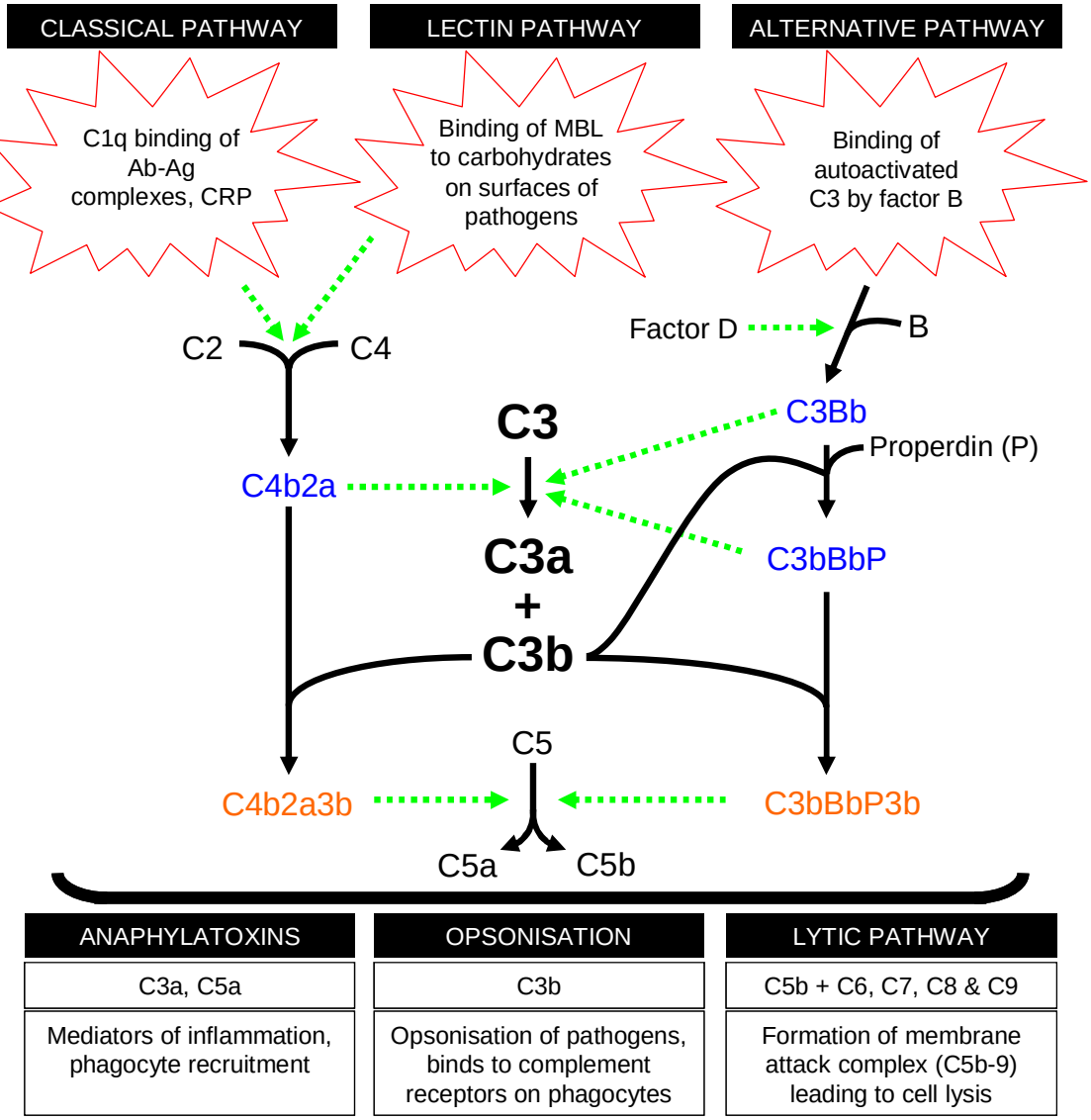


Figure 7 - Overview of the pathways of complement activation. The classical pathway is usually triggered by the binding of the C1q subunit to antibody-antigen complexes. The C1s subunit then cleaves C4 and C2 leading to the assembly of the classical C3 convertase, C4b2a, on the target cell membrane. The lectin pathway is similar except that initiation is by the binding of mannose-binding lectin (MBL) to sugar residues, with subsequent complexing of MBL-associated serine proteases (MASPs) which then cleave C4 and C2. The alternative pathway is activated by the binding of autoactivated C3, which is constitutively present, or C3b to factor B, which is cleaved in the presence of factor D, to form C3Bb; this complex is stabilised by properdin forming the alterative pathway C3 convertase C3bBbP. C3 convertases cleave C3 forming C3a and C3b, the latter of which combines with the C3 convertases shifting the substrate specificity to C5 i.e. forming C5 convertases. The effector mechanisms of the complement system are the production of the anaphylatoxins C3a and C5a, the opsonisation of target cells by C3b and the generation of the membrane attack complex C5b-9. (Figure adapted from Janeway *et al*. [495]).

1.5.2.3 Regulators of the complement system

Tight regulation of the complement system is important in order to maximise the damage to any invading pathogens whilst minimising damage to host cells. This regulation is achieved by a vast array of soluble and membrane-bound regulatory proteins which are able to interact with the complement cascade at different stages. Some of the key inhibitory molecules are C1-inhibitor, factor H, factor I, vitronectin, clusterin, CD55 (also known as decay accelerating factor) and CD59. The C1 inhibitor is a serine protease which binds and blocks C1r, C1s and the MASPs [496] effectively limiting complement activation at an early stage. The main actions of factor H are the removal of Bb from the alternative pathway C3 convertase, C3bBb, resulting in loss of activity known as decay acceleration, and acting as a cofactor for factor I mediated degradation of C3b [497]. Vitronectin (also known as protein S) and clusterin (also known as apolipoprotein J) are important in limiting formation of the membrane attack complex. Clusterin binds to C5b-7 interfering with complex assembly and vitronectin inhibits the polymerisation of C9 [485, 498, 499]. The combination of vitronectin with the terminal complement components results in the formation of the soluble SC5b-9 complex which can be detected in biological fluids and provides a marker of complement activation [498, 500]. Decay accelerating factor is a membrane bound protein found on a large number of cell types which prevents the assembly of the alternative pathway C3-convertase and accelerates the breakdown of formed convertases, effectively limiting complement activation on host cells [501]. The CD59 molecule is another membrane bound protein important in limiting complement mediated host cell damage and functions by binding C8 in the C5b-8 complex, impeding the subsequent binding of C9, and by binding to C9 in the C5b-9 complex preventing the binding of further C9 molecules necessary for membrane attack complex formation [502]. Of particular relevance to this thesis is a potential role for complement in the pathogenesis of CV disease as discussed in the next section.

1.5.3 Inflammation and cardiovascular disease

A large number of observational studies, both in HD [163, 429, 434-442, 503-506] and non-HD [42, 507] populations, have reported the link between elevated inflammatory markers (most commonly CRP) and adverse outcomes, particularly CV disease. Despite this well documented relationship it remains unclear whether the inflammatory process is itself driving atherosclerosis or merely an epiphenomenon accompanying established atherosclerotic disease.

One line of inquiry has been whether the acute phase reactants themselves are involved in the initiation or progression of atherosclerosis. Much of the published work in this area has been undertaken examining CRP, an acute phase protein synthesised in the liver, which is an objective, although non-specific, marker of inflammation. In addition to the associations between CRP levels and adverse outcomes already cited, the levels of CRP have also been shown to be an independent predictor of the number of carotid artery plaques in dialysis patients [447] and correlate closely with other markers of atherosclerosis in pre-dialysis patients, such as carotid intimal thickening [48]. There is also evidence that CRP may itself be pathogenic as it has been found within atherosclerotic lesions of infarcted myocardium [508-511], as well as potentially increasing LDL uptake by macrophages [512] and aiding monocyte recruitment during atherogenesis [513]. Studies using cultured endothelial cells have also demonstrated the ability of CRP to attenuate nitric oxide production [514], induce adhesion molecule expression (e.g. ICAM-1, VCAM-1 and E-selectin) [515] and increase chemokine production (e.g. MCP-1) [516], all processes involved in atherosclerosis. Similarly in animal models, treatment with human CRP has been shown to increase aortic plaque size [517] as well as cerebral [518] and myocardial [519] infarct size following arterial ligation. These findings all point to a role for CRP in the development of atherosclerosis.

Contrary to these findings, some commentators believe that the association between elevated CRP levels and CV disease is merely an epiphenomenon [520] and that the CRP levels simply reflect inflammation which increases CV risk through effects on endothelial dysfunction, insulin resistance and oxidative stress, for example, rather than having a mechanistic role. In support of this, several large genetic studies looking at polymorphisms in the CRP gene, which are known to be associated with higher CRP levels, have reported no association between elevated CRP levels and CV disease [521-524] thus strengthening the case that elevated CRP levels are merely an epiphenomenon rather than being mechanistically involved in the development of CV disease. Furthermore, some of the data demonstrating pro-atherosclerotic effects of CRP *in vitro* were subsequently attributed to bacterial contamination of the recombinant CRP used [525, 526]. The relevance of some of the studies cited so far has been criticised because of the non-specific nature of elevated CRP levels. In addition to CRP, other acute phase reactants may have a pathogenic role in the development of CV disease. The role of one such molecule, fibrinogen, is discussed further in this context in section 1.6.4. Pro-inflammatory cytokines may also be involved as, for example, TNF-α has been shown to mediate endothelial dysfunction [527] and promote vascular cell calcification *in vitro* [528] and IL-6 exacerbates

early atherosclerosis in rat models [529]. The levels of IL-6 also appear to predict CV risk in renal [429, 439, 505] and non-renal [530, 531] populations. In addition to the inflammatory mediators discussed so far, the role of complement in the pathogenesis of CV disease is particularly relevant to this thesis.

The role of complement in the development of CV disease has received little attention in the renal literature to date. This is a potential oversight given the multiple mechanisms of enhanced complement activation in HD patients and the emerging link between complement and CV disease. A number of observational series in non-renal patients have reported positive associations between C3 levels and both prevalent [532-536] and incident [537-540] CV disease, even after correction for a number of traditional CV risk factors. Furthermore, levels of SC5b-9 appear to predict the risk of subsequent CV events in patients with diabetes following AMI [541] and C5a levels are predictive of CV events in patients with peripheral vascular disease [542]. Studies have also demonstrated increased complement activation during acute myocardial infarction [543-545] and ischaemic stroke [546]. In addition to this observational data, there is evidence complement may have a functional role in the development of CV disease. Analyses of atherosclerotic lesions has consistently demonstrated the presence of activated complement components [511, 547-552], and isolated lipid extracts from human atherosclerotic lesions appear to be potent activators of the complement system [553]. Analyses of atherosclerotic lesions have also demonstrated co-localisation of CRP and activated complement components [509-511] which may represent a mechanistic role both for CRP and complement activation in atherosclerosis given the ability of CRP to bind C1q and activate complement via the classical pathway [482]. Studies on rabbits fed a cholesterol-rich diet have shown that the extent of atherosclerosis is greatly reduced in C6-deficient, compared to non-C6-deficient, animals suggesting a functional role for the terminal complement components in the progression of atherosclerosis [554]. Animal models of cardiac ischaemia-reperfusion injury have also demonstrated reduced infarct size with various inhibitors of the complement system (e.g. C1 esterase [555] and C5a [556, 557] inhibitors). A number of the complement system effector mechanisms may also have a role in the development of CV disease. Many of the molecules formed, such as C3a, C3b and C5a, are able to act as ligands for receptors on leukocytes triggering inflammation and the release of pro-inflammatory cytokines, such as IL-6 and TNF-α, and chemokines, such as IL-8, as well as acting as chemoattractants recruiting monocytes and lymphocytes [485, 558]. There is therefore a wealth of laboratory data supporting a mechanistic role for various components of the complement system in the development and progression of atherosclerotic lesions.

There is also emerging evidence of a role for complement in reducing vascular compliance of elastic arteries which, as discussed in section 1.2, is important in the pathogenesis of CV disease in renal patients. Fibromodulin and adiponectin have been shown to bind collagen fibres in vessel walls [559, 560] and also to bind C1q [561, 562] thereby activating the classical complement pathway leading to complement deposition. In mice, extensive binding of C3 and C4 to collagen and elastin fibres in the adventitia has been demonstrated [563] and a case report of a chronic HD patient with a congenital C4 deficiency found no evidence of arterial stiffness after 28 years on dialysis [564]. Furthermore, in a mouse model the development of ventricular stiffening in the setting of hyperglycaemia was dependent on the mannose-binding lectin complement pathway [565] and blocking the actions of C5a in a hypertensive rat model attenuated ventricular collagen deposition and stiffness [566]. Taken together, these findings suggest that complement might have a functional role in the development of vascular stiffness.

There also appears to be haemostatic consequences of complement activation which are relevant in the setting of CV disease. For example, there is evidence of enhanced complement activation in ruptured compared to stable coronary plaques [547] and the membrane attack complex is able to stimulate platelet degranulation and increase endothelial expression and release of von Willebrand Factor and tissue factor [567, 568] suggesting complement activation may influence thrombosis. The C3a molecule has also been shown to induce platelet aggregation and potentiate the activity of ADP *in vitro* [569]. There is evidence of cross-talk between the complement and haemostatic cascades. For example, MASP-2 is able to cleave thrombin from prothrombin [570] and thrombin generation is a key step in haemostasis as discussed in detail in section 1.6.1. Thrombin provides an important link between coagulation and complement as thrombin has been shown to have C5 convertase activity [571], be an agonist for the protein kinase C (PKC)-dependent pathway of decay accelerating factor regulation [572] and to cleave factor H resulting in the formation of a monocyte chemotactic factor [573]. A number of coagulation factors (F) have been found to activate components of the complement system; FXa, FXIa and plasmin activate C3 and C5 [574], thrombin, plasmin and kallikrein activate C3 and C4 [575] and FXIIa activates C1 [576]. Activation of the coagulation system results in increased C3a generation which can be counteracted by thrombin inhibition [577]. There also appears to be cross-talk at the inhibitor level as C1-inhibitor, in addition to inhibiting C1r, C1s, MASP1 and MASP2 in the complement cascade, also appears to inhibit FXIa and FXIIa of the coagulation cascade [578, 579]. Thrombin activatable fibrinolysis inhibitor

(TAFI), a key molecule involved in the regulation of thrombolysis (discussed in more detail in section 1.6.2.3), is also able to inactivate C5a [580].

It can be seen, therefore, that there is considerable observational and laboratory data suggesting an important role for several inflammatory process in the development of CV disease. In particular, the complement cascade appears to have important roles both in the development of atheromatous plaques and in promoting a pro-inflammatory and pro-thrombotic microenvironment [533]. Despite the high rates of CV disease and the multiple stimuli for complement activation in HD patients, the relationship between these two entities in this patient group has received little attention to date and is one of the foci of the present study.

As discussed so far, the increased levels of oxidative stress and inflammation observed in HD patients may contribute to their enhanced risk of developing CV disease. A number of mechanisms may underpin this association including effects on endothelial function and the development of atherosclerotic lesions. Another potential mechanism may be through effects on haemostasis and thrombosis or, more specifically, alterations in fibrin clot structure. In the following section an overview of the haemostatic and fibrinolytic systems is provided in addition to outlining the links between fibrin clot structure and CV disease.

1.6 Haemostasis and fibrinolysis

1.6.1 The haemostatic system

1.6.1.1 Overview of the haemostatic system

When the integrity of the vasculature is breached a clot, derived from components circulating in the blood stream, is formed in an attempt to minimise blood loss by a process termed haemostasis. Thrombosis, unlike haemostasis, is a pathological phenomenon in which the clotting system is activated erroneously resulting in clot formation within the lumen of a blood vessel impeding or obstructing the flow of blood. Blood clots comprise a mass of platelets, red blood cells and leukocytes bound in a mesh of interlocking fibrin fibres. Their formation requires tight control to restrict clot formation to the site of injury and prevent dissemination throughout the vascular tree. Haemostasis proceeds in two phases, termed primary and secondary haemostasis. Over time clots are broken down, by a process termed fibrinolysis, and replaced by new tissue to restore vascular integrity.

1.6.1.2 Primary haemostasis

Primary haemostasis refers to the processes culminating in the formation of a haemostatic plug made up of a mass of activated platelets tethered to the site of vascular injury. The key steps involved are platelet adhesion, platelet activation and platelet aggregation. The initial trigger for haemostasis is injury to the endothelium exposing components of the subendothelial matrix to the blood. Circulating platelets then adhere to the subendothelial matrix via receptor interactions leading to platelet activation. Of the exposed components of the subendothelial matrix, von Willebrand factor (vWF) and collagen appear to be the most important for primary haemostasis. Von Willebrand factor is a large multimeric protein secreted by endothelial cells which circulates in the plasma and is present in the subendothelial matrix bound to collagen. Following a breach in endothelial integrity, collagen and vWF in the subendothelial matrix are exposed to the circulation. Platelets then bind the immobilised vWF via the receptor GpIb-IX-V complex [581], which only has a low affinity for circulating vWF [582], and to exposed collagen via the GpVI receptor [583]. This binding leads to platelet adhesion and platelet activation. A number of receptors on the surface of platelets, such as members of the integrin family αIIbβ3 and α2β1, are constitutively expressed in their inactive form but undergo conformational changes following platelet activation exposing new binding sites. Several ligands for these receptors have been identified including fibrinogen, vWF, collagen, fibronectin, vitronectin and laminin [584-587]. Activated platelets release granules containing agonists, such as adenosine di-phosphate (ADP), which then bind to receptors (in the case of ADP, receptors $P2Y_1$ and $P2Y_{12}$ [588]) on nearby platelets leading to further platelet activation and the establishment of a positive feedback loop. Other mediators of this process include thromboxane A_2, serotonin, and collagen [589]. Thrombin, the terminal serine protease of the coagulation cascade, is important for platelet activation and provides a link between primary and secondary haemostasis. Thrombin cleaves two G protein-coupled protease activated receptors (PARs) on platelets, PAR1 and PAR4, exposing a new N-terminus which acts as a tethered ligand to activate the receptor [590, 591]. The net effects of ligands binding to these platelet receptors are platelet degranulation and increased integin expression leading to further platelet activation and platelet aggregation. The activated platelets undergo cytoskeletal remodelling, in the presence of calcium, to form the haemostatic plug. The haemostatic plug is comprised largely of a mass of activated platelets tethered to the subendothelial matrix and, as such, is relatively friable and liable to being dislodged from the site of injury. This initial platelet plug is then stabilised by processes collectively termed secondary haemostasis.

1.6.1.3 Secondary haemostasis

Secondary haemostasis refers to the processes which result in the formation of a network of cross-linked fibrin molecules, providing stability to the haemostatic plug, and ultimately a mature blood clot. It involves a number of serine proteases which circulate as zymogens and become proteolytically activated when stimulated and, in the presence of specific activators and co-factors, result in the cleavage of thrombin from prothrombin. This series of reactions is referred to as the coagulation cascade and it was first described in 1964 [592, 593]. Thrombin is the key effector enzyme of the coagulation system and has a number of important biological functions including the conversion of soluble fibrinogen to fibrin, platelet activation and amplification of the coagulation cascade [594]. Current understanding of secondary haemostasis is that it takes place in three phases termed the (i) initiation phase, (ii) amplification phase and (iii) propagation phase. An overview of the key steps in secondary haemostasis is provided in Figure 8.

(i) Initiation phase: exposure of tissue factor to coagulation factors

The initiating step in secondary haemostasis is the exposure of tissue factor (TF) to the circulation [595]. Tissue factor is a transmembrane glycoprotein constitutively expressed on a number of extravascular cells, particularly fibroblasts and pericytes in the vascular wall, but is absent from cells that are in contact with the circulation, such as endothelial cells [596]. Injury to the vessel wall, leading to endothelial damage, results in TF coming into contact with blood which then forms a complex with FVII, the extrinsic factor tenase complex TF:VIIa, on the phospholipid surface of the cell membrane and this represents the first stage of clot formation [597]. The TF:VIIa complex activates the zymogens FIX and FX and the FXa formed is capable of generating small amounts of thrombin from prothrombin. These small quantities of thrombin, formed in the vicinity of the TF-bearing cell, act as a primer for clot formation by causing platelet activation, the dissociation of FVIII from vWF, and the activation of FV, FVIII and FXI [598].

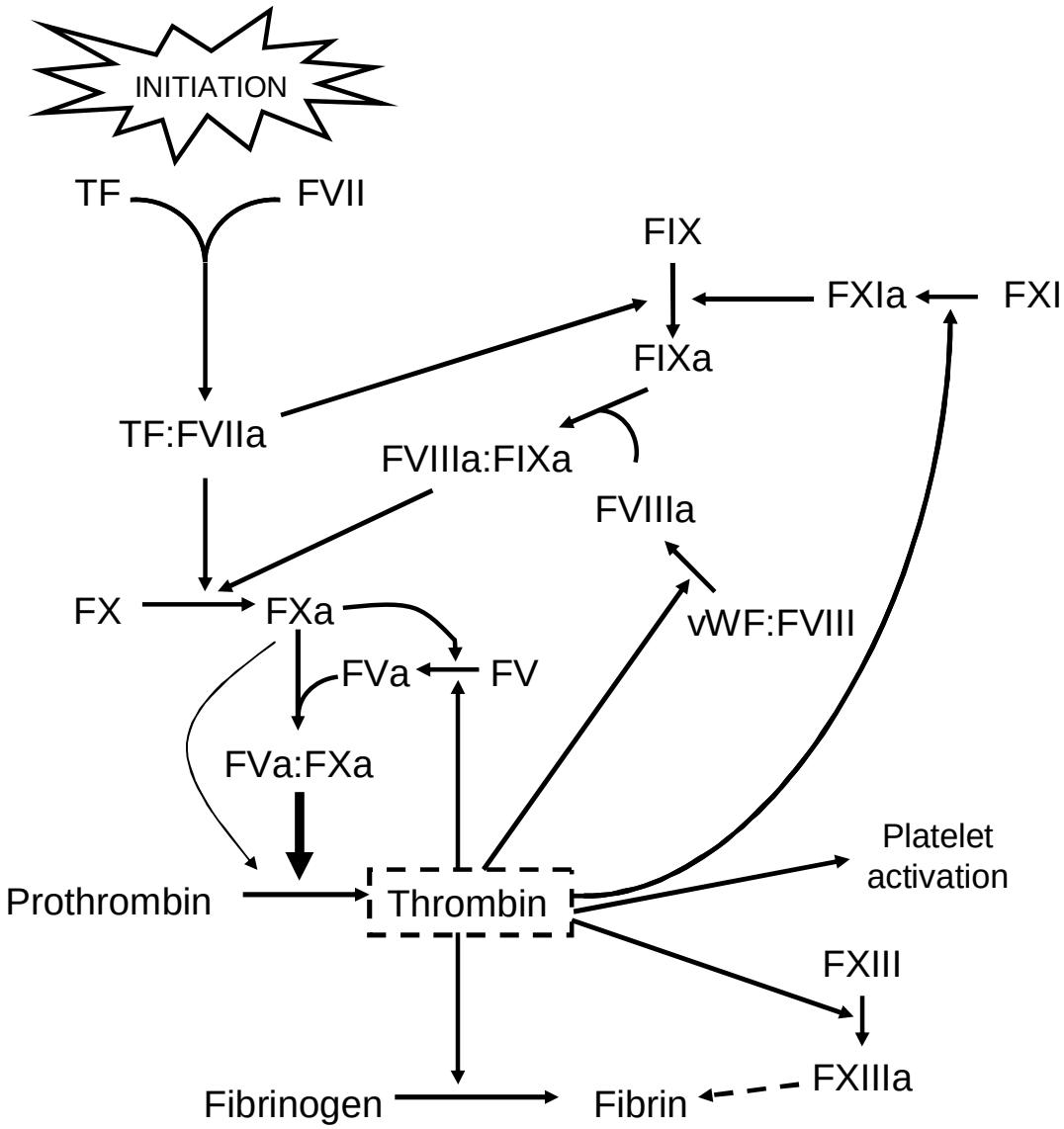


Figure 8 - Current schema of haemostasis and the central role of thrombin generation. The initiating event in clot formation is the exposure of tissue factor (TF) to the circulation and the formation of the TF:VIIa complex. This generates small amounts of thrombin through the activation of FX. The TF:VIIa complex also activates FIX and the formed FIXa complexes with FVIIIa (cleaved by thrombin from von Willebrand factor (vWF)), increasing FXa generation. FXa then associates with its cofactor FVa, again generated by thrombin, forming the FVa:FXa complex which is able to rapidly increase the generation of thrombin, far outweighing that generated by FXa alone. The final step involves the proteolytic cleavage of fibrinogen to form a fibrin clot, the stability of which is greatly enhanced by FXIIIa covalent cross-linking. (F: Factor, TF: Tissue factor, vWF: von Willebrand factor.)

(ii) Amplification phase: increased thrombin generation

Following the generation of small amounts of thrombin during the initiation phase the next step, termed the amplification phase, results in a rapid increase in the generation of

thrombin. The FIXa formed by the TF:VIIa complex associates with FVIIIa which, in the presence of calcium, forms the intrinsic tenase complex FVIIIa:FIXa on the membrane surface predominantly of platelets but also on activated endothelium and phospholipid microparticles [599]. The formation of the FVIIIa:FIXa complex is essential for amplifying the generation of thrombin by increasing the formation of FXa which far exceeds that generated by the TF:FVIIa complex [599-601]. Factor Xa then associates with its cofactor FVa on the phospholipid surface in the presence of calcium, forming the prothrombinase complex FVa:FXa, leading to the rapid generation of thrombin. The mass of activated platelets at the site of vascular injury is the primary site for thrombin generation which, in turn, is highly dependent on the amount of FIXa formed by the TF:FVIIa bearing cells and, to a lesser extent, FXIa generation [602]. In addition, thrombin interacts with platelets via the platelet receptor GpIb-IX-V which serves as scaffolding facilitating interactions with other platelet membrane components, such PAR-1 and PAR-4 [603]. This interaction results in further platelet activation, FVa membrane expression and activation of the GpIIb/IIIa platelet receptor [604, 605] further enhancing platelet aggregation and thrombin generation.

(iii) Propagation phase: formation of fibrin clot

The ensuing rapid generation of thrombin results in the formation of a stable fibrin clot. The first step is the cleavage of fibrinopeptide A and fibrinopeptide B from the fibrinogen molecule by thrombin, forming soluble fibrin monomers [606]. These soluble fibrin monomers then undergo spontaneous polymerisation, by forming side-to-side and end-to-side connections, leading to the formation of protofibrils which coalesce to form a fibrin polymer gel composed of thicker, branching fibres [595, 602]. The final stage involves XIIIa, itself activated by thrombin, covalently linking the fibrin strands to form a stable fibrin network [607, 608].

1.6.1.4 Control of haemostasis

Owing to the presence of a number of positive feedback loops in the haemostatic process, necessary for signal amplification, several mechanisms are in place to confine active clotting to the site of vascular injury and prevent widespread dissemination. Some of the principal molecules involved in controlling coagulation are detailed here.

(i) Antithrombin III

Antithrombin III (ATIII) is a circulating serine protease inhibitor synthesised in the liver. It inhibits coagulation by binding and forming complexes with thrombin and the activated factors FIXa, FXa and FXIa, both inhibiting their activity and facilitating their removal from the circulation [609]. The activity of ATIII is increased by binding to its cofactor heparin, present as heparin sulphate on the vascular endothelium, thus helping to maintain vascular patency and limit intravascular thrombosis in the physiological state [610].

(ii) Tissue factor pathway inhibitor (TFPI)

Tissue factor pathway inhibitor (TFPI) is synthesised by endothelial cells and circulates bound to plasma lipoproteins, with some TFPI being stored within platelets and some bound to proteoglycans on the vessel wall [602]. It is a serine protease which inhibits both FXa and the TF:FVIIa complex [611]. Inhibition of TF:FVIIa requires the presence of FXa [612] so TFPI dampens, but does not prevent, coagulation in the presence of ongoing TF:FVIIa generation and this ensures that coagulation is initiated resulting in the generation of FXa before TFPI is able to inhibit the process [612]. The duration of the initiation phase of secondary haemostasis is thus dependant to a large extent on the relative concentrations of TFPI and TF:FVIIa [600].

(iii) Protein C pathway and thrombomodulin

In addition to the central role of thrombin in initiating clot formation, it also plays an important role in downregulating the coagulation cascade by binding to thrombomodulin and activating protein C [613]. Thrombomodulin is a transmembrane protein synthesised by, and primarily found on, endothelial cells. When thrombin binds to thrombomodulin on the cell surface, thrombin undergoes a structural transformation such that it no longer activates platelets or coagulation factors but instead activates protein C [602]. Activated protein C (APC) then cleaves and inactivates FVIIIa and FVa [614, 615] in the presence of the cofactor protein S [616]. Important functions of the thrombin-thrombomodulin complex are to restrict clot development to the site of vascular injury and to maintain the anticoagulant properties of the endothelium under normal physiological conditions.

1.6.2 The fibrinolytic system

1.6.2.1 Overview of the fibrinolytic system

The fibrinolytic system comprises a number of proteins and inhibitors which break down fibrin deposits. The haemostatic and fibrinolytic systems are finely balanced for the

purposes of maintaining an intact and patent vascular system. The main effector enzyme of the fibrinolytic system is plasmin.

1.6.2.2 Generation of plasmin

Plasmin is generated by the proteolytic cleavage of the circulating zymogen plasminogen. Plasmin cleaves fibrin at specific lysine and arginine residues forming soluble fibrin degradation products [595]. When fibrin is cleaved, carboxy-terminal lysine residues are exposed which bind to lysine-binding sites present on both tPA and plasminogen, increasing fibrin binding, plasmin generation and lysis of the fibrin clot [617]. The main physiological activator of plasminogen is tissue-type plasminogen activator (tPA) which is released by endothelial cells in response to thrombin or injury [617]. Another activator, urokinase-type plasminogen activator (uPA), is also able to cleave plasmin from plasminogen and is synthesised and secreted as inactive pro-urokinase requiring activation by plasmin or one of the contact factors (FXII, kininogen and prekallikrein) [618]. Although both of these activators are synthesised by the endothelium, tPA is the primary plasminogen activator in the blood [619, 620] with uPA being more important in wound healing, vasculogenesis and proteolysis of the extracellular matrix [602], as well as playing a role in cell signalling [621].

1.6.2.3 Control of fibrinolysis

Despite an abundant supply of circulating plasminogen, very little plasmin is produced under normal circumstances because tPA is present in very small amounts and it is relatively inefficient at generating plasmin from non fibrin-bound plasminogen [622]. This is important in restricting fibrinolysis to sites of injury, where fibrin has been deposited, and avoiding widespread activation of plasminogen. In addition, a number of plasmin inhibitors and plasminogen activator inhibitors (PAIs) modulate the fibrinolytic process as outlined in Figure 9.

(i) Plasminogen activator inhibitors (PAI)

The activation of plasminogen is controlled by plasminogen activator inhibitors (PAIs) which circulate in plasma in great excess forming biologically inactive complexes with tPA and uPA preventing inappropriate plasmin generation [623]. Of the PAIs, PAI-1 is the most important biologically [619, 622] and it is synthesised by the vascular endothelium, adipose tissue and the liver [624-626] with large quantities being stored in platelets [627] rendering platelet-rich clots relatively resistant to fibrinolysis and protecting the developing clot from fibrinolysis [609]. Circulating PAI-1 is relatively unstable with a half-life of 1-2 hours [628],

however it binds to vitronectin, present in both the plasma and extracellular matrix [629, 630], which increases its half-life to 4-6 hours [628]. As the clot forms, the fibrin binding of tPA and plasmin/plasminogen within the thrombus limits the ability of PAI-1 to inhibit tPA, thereby allowing the generation of plasmin and fibrinolysis to occur [609]. Interestingly PAI-1 is also able to inhibit APC [631] and thrombin [632, 633] in the presence of vitronectin and/or heparin although the physiological significance of this remains unclear [609].

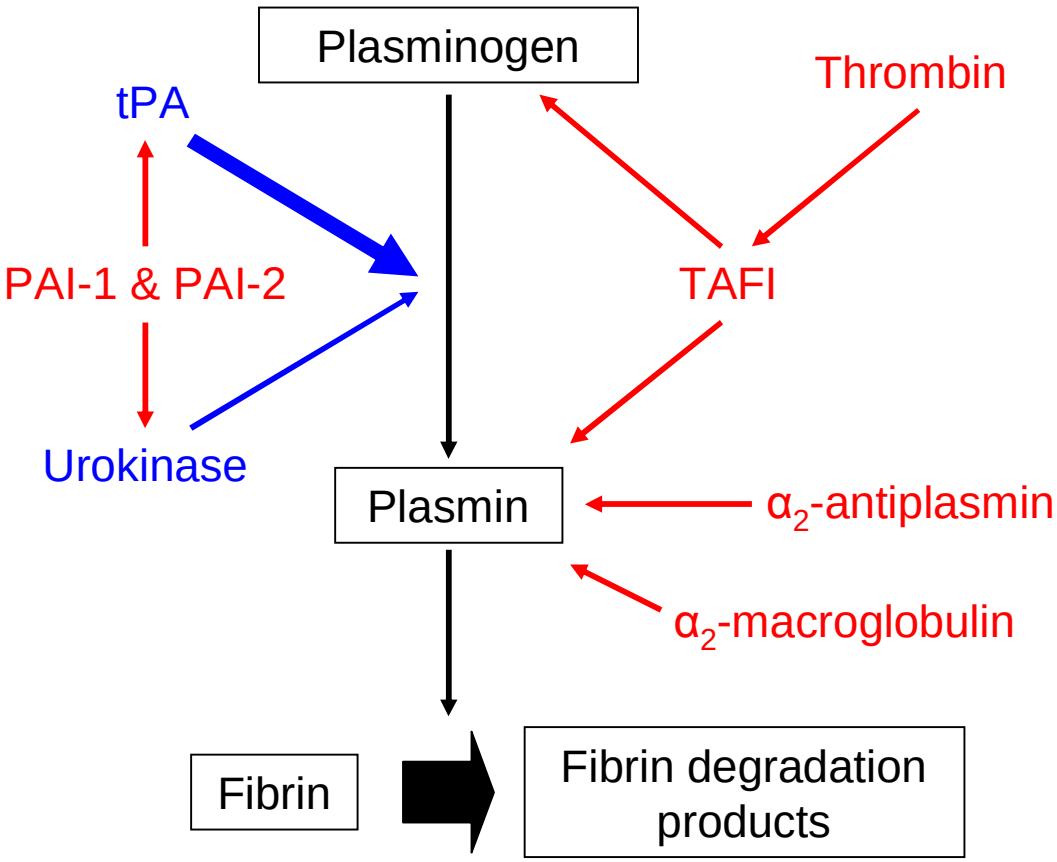


Figure 9 - The principal activators (blue) and inhibitors (red) of fibrinolysis. Plasmin is the main effector enzyme of the fibrinolytic system and it cleaves fibrin resulting in dissolution of the clot and the generation of soluble fibrin degradation products. Plasmin is formed by proteolytic cleavage of its zymogen, plasminogen, principally by the action of tissue plasminogen activator (tPA) but also, to a lesser degree, urokinase. Both tPA and urokinase are inactivated by complexing with plasminogen activator inhibitors (PAIs). Thrombin activated fibrinolysis inhibitor (TAFI) also inhibits plasmin and is formed when thrombin is present in high concentrations; other important physiological inhibitors of plasmin are α_2-antiplasmin and α_2-macroglobulin. (PAI: Plasminogen activator inhibitor, TAFI: Thrombin activatable fibrinolysis inhibitor, tPA: Tissue plasminogen activator).

(ii) Thrombin activatable fibrinolysis inhibitor (TAFI)

Thrombin activatable fibrinolysis inhibitor is a single chain plasma glycoprotein present in the circulation and stored in platelets [617]. It is activated by the presence of high

concentrations of thrombin but can be activated by much lower thrombin levels in the presence of thrombomodulin [634]. Activated TAFI (TAFIa) inhibits fibrinolysis by removing carboxy-terminal lysine and arginine residues from fibrin [635] preventing the binding of plasminogen and tPA.

(iii) α2-Antiplasmin

Of the plasmin inhibitors α_2-antiplasmin is the principal physiological inhibitor [602]. The C-terminal lysine residue of α_2-antiplasmin binds to the lysine binding site on plasmin, inactivating the enzyme and forming a complex which is subsequently cleared by the liver [617]. Plasmin binds to fibrin via the same lysine binding sites therefore α_2-antiplasmin is much more efficient at inactivating free rather than fibrin-bound plasmin [636]. Activated factor XIIIa also cross-links α_2-antiplasmin to fibrin enhancing the clot's resistance to fibrinolysis [637]. Other plasmin inhibitors include α_2-macroglobulin and the protease nexin [617, 623].

The foregoing sections on haemostasis and fibrinolysis detail the major mechanisms involved in the formation, and subsequent dissolution, of fibrin clots. The physical characteristics of fibrin clots, such as fibre thickness and pore size, can vary depending on the conditions under which they are formed. These different physical characteristics, in turn, are reflected in the properties of the clot such as clot permeability or susceptibility to fibrinolysis. An overview of the key determinants of fibrin clot structure and function is provided in the following section.

1.6.3 Determinants of fibrin clot structure and function

A number of genetic and environmental factors modify the fibrinogen molecule and its ability to interact with other molecules, such as FXIII and plasminogen, leading to alterations in the structure and characteristics of the formed clot. Studies of twins have shown the environmental factors to exert the greatest influence on the fibrin clot phenotype [638]. Some of the key environmental determinants of fibrin clot structure, in the context of this thesis, are discussed further here. Of prime importance in determining the structure of the fibrin clot, perhaps unsurprisingly given that it is the principal substrate, is the concentration of fibrinogen. *In vitro* studies have demonstrated that fibrin clot structure is greatly influenced by the kinetics of fibrin polymerisation [639]. Faster rates of polymerisation, as occur with higher fibrinogen or thrombin concentrations for example, result in the formation of clots composed of thinner fibres which are denser and less

permeable with smaller intrinsic pores in both purified fibrinogen [640-643] and plasma based [644-649] systems. A number of other environmental factors are also able to influence clot structure through different mechanisms; one such mechanism is through modifications to the fibrinogen molecule such as glycation. Fibrinogen glycation *in vitro* has been shown to decrease clot permeability and fibrinolysis susceptibility [650, 651]. Similarly fibrin clots derived from patients with diabetes appear to be less permeable and more resistant to fibrinolysis, when compared to patients without diabetes, with the clot characteristics being related to glyaemic control as measured by HbA1c levels [652-655]. A number of drugs can also alter clot structure. For example aspirin has been shown to increase clot permeability and fibre thickness [656-659], an effect likely mediated through acetylation of fibrinogen lysine residues [660]. Vitamin K is required for the γ-carboxylation of glutamate residues on the vitamin K dependant proteins, which include the coagulation factors II, VII, IX and X and the regulatory proteins C and S [661], in order that they can bind calcium and interact with phospholipids to carry out their physiological roles [662-668]. Therefore vitamin K antagonists, such as warfarin, can have a profound influence on fibrin clot assembly kinetics and the clot structure, for example increasing permeability [669].

Most relevant to the present thesis are the potential for oxidative stress and inflammation to alter the fibrin clot phenotype. Oxidative stress, inflammation and hyperfibrinogenaemia often tend to co-exist in patients making it hard to assess their individual contributions to the fibrin clot characteristics. The most likely mechanism through which oxidative stress may influence the fibrin clot phenotype is through oxidative modification of the fibrinogen molecule. This has been shown experimentally to increase the clotting time and reduce the clot density [670, 671], but not influence fibrinolysis susceptibility [671]. Fibrinogen oxidatively modified *in vitro* appears to polymerise at a slower rate [672-677] and this effect on the reaction kinetics may explain the mechanism of altered clot structure. Perhaps the most widely studied biomarker of oxidative stress in the setting of fibrin clot structure and function is prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) which is formed by the free radical-catalysed peroxidation of arachidonic acid [678]. Several observational studies have shown $PGF_{2\alpha}$ levels to be negatively correlated with clot permeability and positively correlated with fibrinolysis times [646, 648, 679, 680]. However, similar associations between these clot characteristics and the levels of CRP and fibrinogen were also reported in these studies which, combined with the observational nature of the study designs, makes it hard to differentiate the effects of oxidative stress from those of inflammation or fibrinogen levels.

As with oxidative stress markers, several studies have demonstrated associations between increased levels of inflammatory markers, such as IL-6, C3, CRP and orosomucoid, and fibrin clot parameters such as increased clot density, reduced permeability and fibrinolytic susceptibility [645, 646, 648, 649, 681-683]. Fibrinogen is an acute phase reactant [428], therefore trying to assess the effects of inflammation, independent of the effects on fibrinogen levels, from these data is not straightforward. Multiple linear regression statistical techniques have been used in an attempt to assess the individual contributions of predictor variables, e.g. inflammatory markers, on the various fibrin clot parameters while adjusting for other covariates, such as fibrinogen levels. Using this approach, several studies have reported independent associations between markers of inflammation and alterations in the fibrin clot properties [648, 679, 681]. Taken together, these would suggest that inflammation may lead to alterations in the fibrin clot by mechanisms other than simply influencing fibrinogen levels. One such mechanism may be the binding of inflammatory mediators to the fibrin molecule, such as CRP [684] or C3 [652, 682, 685, 686], thereby influencing fibrin network assembly. Complement may be particularly important in determining the fibrin clot phenotype. *In vitro* work has demonstrated that the fibrin clots formed in the presence of increasing concentrations of C3 are more resistant to fibrinolysis [686] and complement activation leads to reduced fibrin fibre thickness and increased tensile strength [687]. Human studies have shown that increased C3 levels are associated with prolongation of fibrinolysis times in healthy individuals and patients with diabetes [652, 688]. It therefore seems likely that complement component C3 is an important contributor to the fibrin clot structure and function. In addition to complement, the levels of PAI-1 may also be important in the setting of inflammation as they rise as part of the acute phase response [428] and increase fibrinolytic resistance. However, Sjoland *et al*. [645] reported a negative association between CRP levels and fibrinolysis times in experiments which included flufenamic acid, an inhibitor of PAI-1, suggesting increases in PAI-1 levels cannot fully explain the enhanced fibrinolytic resistance in the presence of inflammation. In practice, inflammation-induced hyperfibrinogenaemia is likely to be the principle driver behind the altered clot phenotype observed in the setting of inflammation, although changes in the levels of circulating factors, such as C3 and PAI-1, are also likely to be contributory.

From the data presented in this section, it can be seen that fibrin clot structure and function appear to be influenced by a number of environmental factors including oxidative stress, inflammation, hyperglycaemia and drug therapy. There is also considerable

evidence linking both fibrinogen levels and alterations in the fibrin clot phenotype with CV disease.

1.6.4 Haemostatic factors in cardiovascular disease

1.6.4.1 Elevated fibrinogen levels and cardiovascular disease

A large number of prospective epidemiological studies in non-renal populations, including two large meta-analyses [689, 690], have reported on the positive association between plasma fibrinogen levels and the risk of developing *de novo* CV disease. Furthermore, in patients with known CV disease, fibrinogen levels appear to be associated with the extent and severity of disease in a number of studies [691-694], although not all [695], with higher levels being predictive of future CV events and mortality [696-700]. Additionally in HD patients, in whom a number of the conventional CV risk factors such as hypertension and hypercholesterolaemia appear to behave differently as detailed in section 1.2.1, increased fibrinogen levels also appear to be associated with CV disease [45, 701]. However, fibrinogen levels are also known to be associated with many new and emerging risk factors for CV disease, such as smoking, elevated BMI, advancing age and inflammation [702-707]. This begs the question as to whether the association between hyperfibrinogenaemia and CV disease is an epiphenomenon, reflecting the contribution of these other risk factors, or represents a final common pathway through which these risk factors promote the development and progression of CV disease or, indeed, represents a different mechanistic pathway altogether. One potential mechanism through which fibrinogen levels may influence CV disease risk is through alterations to the fibrin clot structure.

1.6.4.2 Fibrin structure and function in relation to cardiovascular disease

In the 1990's, Fatah *et al*. demonstrated that male patients who had suffered a myocardial infarction before the age of 45 formed plasma derived fibrin gel networks that were tighter, more rigid, less permeable and were comprised of fibres with lower mass-length ratios than networks derived from healthy controls [644, 649]. This led to the concept of an "adverse clot phenotype", characterised by denser, less porous clots with increased fibrinolytic resistance, which a plethora of subsequent studies have consistently reported to be over represented in individuals either with, or at high risk of developing, CV disease. These include patients with premature CV disease [708] and their healthy first degree relatives [709], patients with acute coronary syndromes [648, 681], particularly with co-existent renal dysfunction [647] or coronary stent thrombosis [710], stroke patients [711,

712], patients with abdominal aortic aneurysms [713], peripheral arterial disease [714, 715] and their relatives [716], patients with idiopathic venous thromboembolism [717], chronic heart failure [718], diabetes [654, 655, 719, 720] and their relatives [688], patients with the metabolic syndrome [721] and smokers [679, 722, 723]. Similar alterations in the fibrin clot structure have also been described in patients on peritoneal dialysis [645] and HD [646].

In 2007, Sjoland *et al.* [645] compared the plasma derived fibrin clots from 22 peritoneal dialysis patients with 24 healthy controls. The clots formed from patient plasma were less permeable, less compactable and more resistant to fibrinolysis and were more architecturally complex when examined with electron microscopy. Additionally, a number of the clot characteristics, particularly fibre properties, permeability and fibrinolysis rate, were highly correlated with the levels of fibrinogen, IL-6 and CRP. However, the levels of these biomarkers were higher in the peritoneal dialysis patients compared to controls making it hard to discern the individual contributions of renal failure and dialysis to the clot phenotype [645]. Furthermore, there was little correlation between the clot parameters and markers of uraemia suggesting that the majority of the differences in clot structure may be attributable to the inflammatory milieu rather than the uraemic state. However routine indices of uraemia, such as serum urea and creatinine levels as reported by the authors, may be less reflective of retained toxins in a peritoneal dialysis population than in patients not on dialysis. The authors also reported no effect of PAI-1 levels on lysis rates which is somewhat counter-intuitive, although perhaps anticipated, as they added flufenamic acid to the fibrinolysis reaction mix which inhibits most fibrinolytic inhibitors including PAI-1 [724, 725]. The rates of fibrinolysis did, however, correlate with clot permeability and inversely with fibre diameter as had been reported by others [726] highlighting the link between fibrin clot structure and properties. Given the small patient numbers in the study and the differences in baseline characteristics, besides the presence of renal failure, it is hard to draw definitive conclusions about the influence of renal failure or peritoneal dialysis therapy on fibrin clot structure or function. It does, however, serve as pilot data suggesting that there may be differences in renal patients compared to non-renal controls.

Of greater relevance to the present thesis was the study by Undas *et al.* [646] which analysed fibrin clot parameters in a cohort of HD patients and examined how they were related to subsequent CV mortality. They undertook baseline fibrin clot analysis in 33 HD patients without evidence of active inflammation and 33 age and sex matched non-HD controls; patients were followed for 36 months for the occurrence of fatal CV events. To analyse the plasma derived fibrin clots they performed permeation studies and

spectrophotometric analyses of clot turbidity and lysis in addition to perfusion clot lysis analysis in which formed fibrin clots were perfused with buffer containing tPA and the concentration of D-dimer in the effluent measured every 20 minutes. The HD patients had higher levels of CRP and fibrinogen in addition to higher $PGF_{2\alpha}$ levels but similar levels of PAI-1. Again, the clots obtained from dialysis patients were less permeable, had higher maximum absorbencies using turbidimetric measurements and took longer to lyse [646]. In unadjusted analyses, the permeability coefficient was negatively correlated with $PGF_{2\alpha}$ and CRP levels and the lysis times positively correlated with the $PGF_{2\alpha}$ and fibrinogen levels; no significant associations were observed between any of the clot variables and the PAI-1 levels or duration of dialysis treatment. These suggest that the levels of inflammation and oxidative stress may be important in determining the fibrin clot phenotype in HD patients. The study also examined fibrin clot structure in relation to fatal CV events over 3 years. Baseline levels of fibrinogen and $PGF_{2\alpha}$, but not CRP, were significantly higher in the patients who experienced a fatal CV event during follow up and the fibrin clots from these patients were denser, less permeable and more resistant to fibrinolysis [646].

Data from the two studies examining fibrin clot structure in dialysis patients [645, 646] suggest that dialysis patients form fibrin clots that differ from healthy controls and exhibit the adverse clot phenotype seen in patients with, or at high risk of developing, CV disease i.e. compact, rigid, less permeable clots with reduced susceptibility to fibrinolysis. In the study of HD patients [646], alterations in the clot characteristics also appeared to be related to the subsequent risk of fatal CV events. It is hard to draw definitive conclusions, however, as other intergroup differences, such as the levels of inflammation, fibrinogen and oxidative stress, may well be responsible for these associations rather than any effects of uraemia or dialysis therapy.

1.6.4.3 Summary of haemostatic factors and cardiovascular disease

From the data presented in this section it appears that fibrinogen levels and alterations in the fibrin clot structure are associated with CV disease. As discussed in sections 1.4.3 and 1.5.3, increased inflammation and oxidative stress are also linked to increased rates of CV complications. It remains unclear whether the association between the haemostatic markers discussed and CV disease represents a mechanistic association or merely an epiphenomenon, reflecting the influence of other factors such as inflammation and oxidative stress. One way of addressing this uncertainty is to study the effect of interventions which reduce inflammation and / or oxidative stress on the haemostatic markers and also on CV outcomes. One such intervention, which has been extensively

studied in the setting of CV disease prevention, is Vitamin E (VE) which has both anti-oxidative and anti-inflammatory properties.

1.7 Vitamin E and cardiovascular disease

1.7.1 Overview of vitamin E

Vitamin E refers to a family of eight structurally related, water-insoluble compounds consisting of α-,β-,γ-, and δ-tocopherol and the corresponding tocotrienols [727]. Of these compounds, α-tocopherol is the most biologically active and γ-tocopherol is the most abundant form in the human diet [728, 729]. Vitamin E has been shown to have a number of pleiotropic actions, including anti-oxidant and anti-inflammatory activities.

1.7.1.1 Anti-oxidant activity of Vitamin E

Within cell membranes, α-tocopherol is the major defence against oxidative damage [730-732]. Vitamin E is able to function as an anti-oxidant by interrupting free radical propagated chain reactions leading to the formation of a tocopheroxyl radical [733]. This reaction involves the removal of a hydrogen atom from the hydroxyl group of the tocopherol by a peroxyl (oxidant) molecule forming a stable tocopheroxyl radical. Figure 10 outlines the reactions involved, along with the rate constants, in the α-tocopherol mediated interruption of lipid peroxidation. In the presence of an initiating radical (X•), a hydrogen radical may be extracted from unsaturated lipids (LH) forming a lipid radical (L•). The lipid radical then combines readily with oxygen to form a lipid peroxyl radical (LOO•) which propagates the radical chain if not scavenged. The reaction rate between LOO• and α-tocopherol (α-TOH) is much faster (k=10^5-10^6 $M^{-1}s^{-1}$) than the reaction rates between LOO• and LH (k=10^2 $M^{-1}s^{-1}$) which effectively means that α-tocopherol interrupts propagation at this stage by “neutralising” the LOO• radical to form an α-tocopheroxyl radical (α-TO•). Importantly, tocopherol can be recycled from the tocopheroxyl radical by a number of soluble anti-oxidants, such as vitamin C and ubiquitol [734], effectively increasing its antioxidant potential even at low concentrations.

1.7.1.2 Anti-inflammatory activity of Vitamin E

Vitamin E has been shown to influence several processes involved in inflammation and atherosclerosis. Monocytes isolated from healthy individuals [735] and patients with diabetes [736] have been shown to produce less superoxide, hydrogen peroxide, lipid peroxides and inflammatory mediators, such as IL-6 and IL-1β, following a period of α-tocopherol supplementation. Additionally, VE has been found to reduce the expression of

cell surface adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin [737, 738], and inhibit leukocyte adhesion *in vitro* [739, 740]. Studies in humans have also reported a reductions in circulating soluble adhesion molecules [741-743] and CRP levels [744, 745] following VE supplementation. A number of different mechanisms may explain the anti-inflammatory activity of VE including inhibition of the transcription factor NFκB [746], protein kinase C [747-749] and cyclo-oxygenase [750-752].

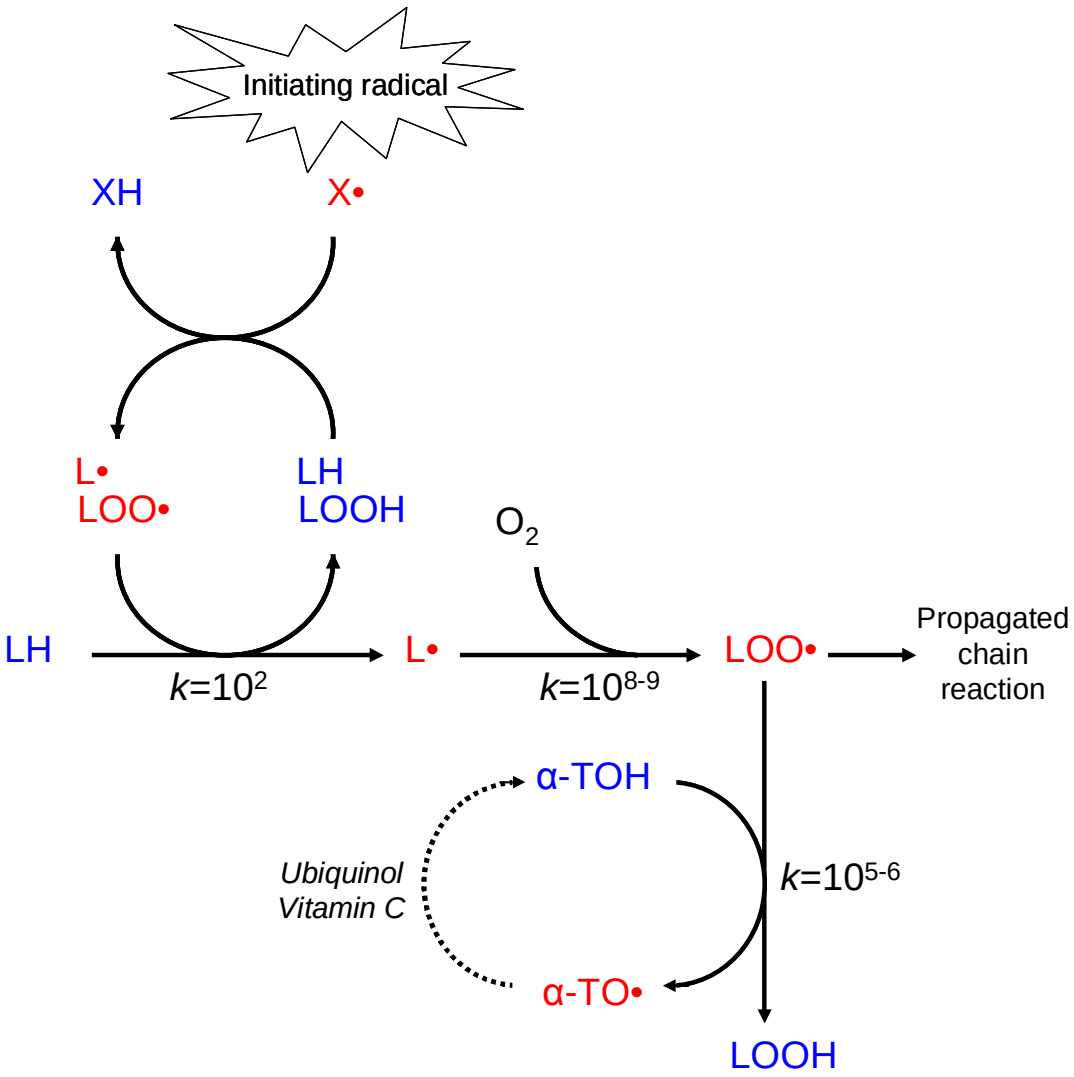


Figure 10 – Role of α-tocopherol in interrupting free radical propagated lipid damage. Free radicals (X•) react with unsaturated lipids (LH) to form a lipid radical (L•). This then rapidly combines with oxygen forming a lipid peroxyl radical (LOO•) which is capable of reacting with more LH, thereby propagating a chain reaction. The reaction rate constant (*k*) for the reaction between LOO• and α-tocopherol (α-TOH) ($k=10^{5-6}$) is much faster than that for LOO• with LH ($k=10^{2}$), therefore the presence of α-TOH effectively interrupts propagation at this stage. This results in the formation of the more stable α-tocopheroxyl radical (α-TO•) which can be recycled by antioxidants such as ubiquinol or vitamin C. (Adapted from Brigelius-Flohe [734]).

1.7.1.3 Haemostatic effects of vitamin E

A number of studies in different patient groups such as smokers [753] and patients with diabetes [754, 755] or coronary spastic angina [756], have demonstrated reductions in PAI-1 levels following VE supplementation. This may be important in terms of CV risk modification as elevated PAI-1 levels have been reported in patients with [757], or at high risk of developing [758], CV disease with PAI-1 deposition being observed in atheromatous plaques [759-761] and at sites of vascular injury [762]. Platelet activation is also important in atherogenesis [53, 763] and VE supplementation has been shown to reduce P-selectin levels [754, 764], a glycoprotein which mediates the adhesion of platelets to endothelial cells during inflammation and thrombosis, with circulating soluble P-selectin levels reflecting the degree of platelet activation [765, 766]. Vitamin E, particularly in high doses, appears to antagonise vitamin K [767, 768] which is required for the γ-carboxylation of glutamate residues on vitamin K-dependent proteins, which include the haemostatic factors II, VII, IX and X, proteins C and S [769]. Supplementation with VE in healthy individuals, however, appears not to influence laboratory clotting parameters, such as the bleeding [770] or prothrombin [768] times. This contrasts with data from patients taking coumarins or who are vitamin K deficient [771, 772] in whom VE supplementation exacerbates the coagulopathy. This latter point may be particularly relevant to HD patients who have been shown to have both low status and intake of vitamin K [773]. Vitamin E has also been shown to enhance endothelial function by increasing the release and amplifying the activity of nitric oxide [774], increasing the production of the vasodilating prostanoids PGE_2 and PGI_2 [775] and protecting the endothelium against damage from ROS, Ox-LDL and lipid peroxides [776, 777].

Given that VE has been shown to beneficially influence a number of processes implicated in the pathogenesis of CV disease, such as oxidative stress, inflammation and haemostasis, several studies have examined the potential utility of VE compounds to ameliorate CV risk.

1.7.2 Human studies of vitamin E

1.7.2.1 Vitamin E supplementation in non-dialysis patients

Data from observational studies, involving large numbers of patients, have suggested a reduction in CV disease endpoints for patients receiving VE supplements or those with a high dietary VE intake [778-782]. However, subsequent intervention studies in humans examining the role of oral VE supplementation in the primary [783-786] or secondary [787-

794] prevention of CV disease and subsequent meta-analyses [795, 796] have failed to demonstrate benefit in the general population. There are a number of potential reasons why the initial promise of VE in reducing CV disease, initially identified in observational studies, has not been subsequently observed in interventional trials. It is plausible that uncontrolled confounders may be responsible for the observed association. For example patients who take vitamin supplements may be more health conscious with regards to other aspects of their diet and lifestyle which may explain the association with improved CV outcomes rather than being a direct effect of the VE *per se*. The isoforms of VE found in the diet may have different biological activity [797] or bioavailability [798] to the synthetic VE compounds used in supplements to explain the apparent differential effects of VE in observation and interventional studies. However, despite the apparent absence of benefit from VE supplementation in the general population, given the heightened levels of oxidative stress and inflammation in HD patients, VE has also been extensively studied in dialysis patients.

1.7.2.2 Vitamin E supplementation in dialysis patients

Patients on HD do not appear to be deficient in VE. The levels of α-tocopherol have been reported to be similar, if not slightly higher, than non-dialysed controls [327, 389, 799] and γ-tocopherol levels have been variably reported as higher [389] or lower [800] in HD patients. Vitamin E compounds are lipid soluble and excreted in the bile [801, 802] thus dialytic losses of VE would not be anticipated. However the metabolites of VE, carboxyethylhydroxychromanols (CEHCs), are water soluble and are both excreted in the urine [797] and removed by HD [389]. The levels of α- and γ-CEHC, the respective metabolites of α- and γ-tocopherol, are significantly higher in HD patients compared to healthy controls, particularly following VE supplementation [389], suggesting dialytic clearance cannot not compensate for the loss of renal excretion. The biological activity of these metabolites is unclear but, for example, γ-CEHC has been shown to have anti-inflammatory properties *in vitro*, through the inhibition of cyclooxygenase-2 mediated prostaglandin synthesis [752].

Given that HD patients have heightened levels of inflammation, oxidative stress and CV disease, as well as altered VE metabolism leading to the accumulation of water soluble biologically active metabolites, there is a strong rationale for assessing the benefits of VE supplementation in HD patients despite the negative trials in non-HD populations. To this end a number of studies have investigated the effects of VE supplementation on markers of oxidative stress and inflammation as well as anaemia and CV outcomes as outlined in

Appendix A, Table 52. The results of these studies are heterogeneous although overall would tend to suggest improvements in markers of oxidative stress, such as MDA [368, 803-816], and anaemia parameters, such as haematocrit or ESA requirements [812, 817-821], and equivocal effects on markers of inflammation, such as CRP and IL-6 [389, 822-824], following supplementation with VE. The apparent contrasting results for some of these studies may reflect the different preparations of VE used, which differ in their bioavailability and relative concentrations of the various VE isoforms, and the non-standardised approaches for determining several of the oxidative stress endpoints reported such as MDA and TBARS levels. Furthermore, the studies consisted of small numbers of HD patients followed up over relatively short time periods and many of the studies selected sub-populations of patients, such as those with diabetes or low levels of VE. These factors limit the ability to generalise the results from the studies but do suggest that VE supplementation in HD patients may have a role in reducing some of the factors thought or known to contribute to CV disease.

The only published study to date examining VE supplementation and CV endpoints in HD patients was the SPACE trial [825]. In this study 196 patients with pre-existing CV disease were randomised to receive 800 IU/day of VE or placebo and were followed for a median of 519 days for the occurrence of CV events or death. There was a significant reduction in a primary composite CV endpoint, largely attributable to a reduction in the number of myocardial infarctions, but no mortality benefit for the VE treated patients, although it is worth noting that the study was not powered to detect a difference in mortality. Interestingly, Tepel *et al*. [826] conducted a placebo controlled trial with a different anti-oxidant, acetylcysteine, in 134 HD patients and again reported a reduction in a composite CV endpoint, but not mortality, after a median follow up of 14.5 months. Taken together, these would suggest that anti-oxidants may have a role in reducing CV morbidity, but not mortality, in HD patients.

It can therefore be seen that despite the attractive simplicity of administering oral VE supplements to ameliorate some of the factors known to contribute to CV disease in HD patients, no mortality benefit has been observed in the small number of HD patients studied to date. It may be that interventions aimed at lowering oxidative stress and / or inflammation may only have a minimal impact on lowering CV events or mortality rates, owing to the multiplicity of contributing factors in HD patients, or that the ability of VE to influence these factors is limited. With regards to the latter point, blood-membrane interactions are thought to contribute substantially to the oxidative and inflammatory

burden for HD patients and, as VE is lipid soluble, its ability to influence these reactions or neutralise the soluble ROS generated in the blood may be limited. In an attempt to address these issues, VE-bonded dialysis membranes have been developed. One small study has also demonstrated the superiority of membrane bound VE over oral supplementation in terms of its ability to reduce oxidative stress in HD patients [827]. Therefore, on the basis of these theoretical and practical considerations, a number of studies have evaluated VE bonded dialysis membranes in relation to inflammation and oxidative stress.

1.7.3 Vitamin E coated dialysis membranes

1.7.3.1 Dialysis membrane composition and biocompatibility

Before examining the role of VE bonded dialysis membranes in improving factors thought or known to be related to CV disease in HD patients, it is important to consider the membrane composition. As discussed in section 1.5.1, the process of passing blood over a dialysis membrane elicits an inflammatory response with the biocompatibility of a membrane relating to the magnitude of this response [828]. Membrane biocompatibility is a nebulous term lacking a consensus definition but can be evaluated by determining the ability of a membrane to generate complement activation products, such as C3a and C5a, upregulate pro-inflammatory cytokines, such as IL-1β, TNF-α or IL-6, and induce changes in the number and level of activation of circulating peripheral blood mononuclear cells during HD [829]. Dialysis membrane composition has changed over time, as have trends in usage, commensurate with improvements in dialysis and manufacturing technology and the costs involved. Cellulose membranes, such as Cuprophan, were the first to be manufactured and are generally deemed bioincompatible owing to the presence of large numbers of free hydroxyl groups on their surface capable of activating complement [479, 480]. Refinements to the original cellulose membranes, termed modified cellulose membranes, include substituted cellulose membranes in which acetate is chemically bonded to the surface reducing the number of free hydroxyl moieties, or cellulosynthetic in which a synthetic material, usually a tertiary amino compound, is added to liquefied cellulose during manufacture. The latest generation of membranes, termed synthetic membranes, are not based on cellulose but made from synthetic plastics and include polysulfone, polyacrylonitrile and polymethylmethacrylate. Synthetic membranes are the most biocompatible with regards to systemic complement activation owing to the absence of nucleophilic binding sites and, in the case of some membranes such as polyacrylonitrile, the ability to adsorb activated complement components onto their surface [830].

Despite the theoretical advantages, no large scale prospective randomised controlled trial to date has reported improved morbidity or mortality for biocompatible compared to less biocompatible membranes. Data from observational series [831-833] and a *post hoc* analysis of a multi-centre drug intervention study [834] however, have suggested higher rates of mortality and CV events for patients dialysing with unmodified cellulose compared to those using either modified cellulose or synthetic membranes. These observations may well be explained by the differing membrane compositions although other factors, such as improvements in patient care over time and reduced cellulose and increased modified cellulosic / synthetic membrane usage [835], are also likely to be important. A Cochrane review in 2005 [836] reported that there was no evidence of benefit for synthetic membranes over cellulose or modified cellulose membranes in terms of mortality, morbidity or dialysis-related adverse symptoms and that more research was needed. It is therefore not clear whether any clinically relevant difference in biocompatibility exists between modified cellulose and synthetic membranes. Grooteman *et al.* [837] compared several measures of biocompatibility in 31 patients undergoing chronic HD in a 3-week crossover study. Polysulfone use was associated with a slight reduction in dialysis-associated leucopenia when compared to modified cellulose but no difference in complement activation. Panichi *et al.* [838] reported greater reductions in CRP levels with polysulfone use compared to modified cellulose membranes although the polysulfone group contained a mixture of patients on conventional dialysis and haemodiafiltration, unlike the modified cellulose group who all received conventional dialysis. An observational series has reported lower mortality rates for patients dialysing with synthetic compared to modified cellulose membranes in centres where dialysers are reused [839], although dialyser reuse has declined rapidly in recent times with reductions in dialyser costs [840]. More recently Zhang *et al.* [841] undertook a 6 month prospective randomised controlled trial in 60 chronic HD patients to assess the biocompatibility of 4 different membranes. Patients were all dialysing on polysulfone membranes prior to study start and were then randomised to dialysis with either a polyethersulfone, cellulose triacetate or a polymethylmethacrylate membrane. No significant differences in markers of inflammation (CRP, IL-1β and IL-13) were observed but the levels of C5a rose more quickly during dialysis in patients dialysing with the cellulose triacetate membrane suggesting there may be differences in biocompatibility. A number of studies have compared markers of oxidative stress in patients using polysulfone dialysis membranes with those using modified cellulose membranes. Some studies [345, 355, 842-844], although not all [845-848], have reported improved levels of oxidative stress for patients dialysing with polysulfone compared to cellulose based membranes. For the purposes of this thesis, the

effects of VE coating modified cellulosic and synthetic membranes are considered separately since the membrane composition is known to impact a number of the factors which are also influenced by VE, such as inflammation and oxidative stress. It therefore seems prudent to examine the potential benefits of coating dialysis membranes with VE independent of the membrane composition.

1.7.3.2 Vitamin E coated modified cellulose membranes

Vitamin E modified cellulose membranes were the first to be developed [849] and a number of clinical studies have examined their anti-oxidant activity *in vivo* in addition to their effects on inflammation, erythrocyte lifespan, complement and leukocyte activation as detailed in Appendix A, Table 53. Taken together, these studies would appear to suggest that VE coating of modified cellulose membranes is associated with improvements in oxidative stress markers including TBARS and Ox-LDL [827, 844, 850-874], anaemia parameters including erythrocyte susceptibility to haemolysis, red blood cell lifespan and ESA requirements [856, 861, 868, 869, 875-879], markers of inflammation, such as IL-6 [854, 858, 880], and indices of complement activation, such as C3a and SC5b-9 levels [851, 857, 881-883]. A meta-analysis including many of these studies highlighted the anti-oxidant properties of these VE-bonded membranes [884]. It is important to note, however, that many of these studies should perhaps only be considered as pilot studies owing to the small number of patients evaluated, the relatively short duration of follow up and the lack of a control group in many of them. They do, however, serve as proof of concept regarding the potential utility of VE coated membranes to ameliorate some of the factors thought to contribute to CV disease in HD patients, such as anaemia, oxidative stress and inflammation. Perhaps more relevant in the modern treatment era is whether coating the latest generation biocompatible synthetic membranes with VE confers similar benefits.

1.7.3.3 Vitamin E coated polysulfone membranes

More recently VE coated polysulfone membranes have been developed [885] and continue to be refined [886, 887], with *in vitro* studies demonstrating their anti-oxidant activity and biocompatibility [885-888]. A number of clinical studies have reported on the effects of dialysis with a VE coated polysulfone membrane as detailed in Table 2. As with the VE bonded cellulose membrane, it can be seen that only relatively small numbers of patients have been studied to date and the results are somewhat equivocal with regards to anaemia, inflammation and oxidative stress [889-896]. Whether this is because there is no benefit of coating polysulfone membranes with VE, or that the effects are subtle

necessitating studies on larger groups of patients or specific patient subgroups in order to detect differences, is not clear.

Table 2 - Studies examining effects of vitamin E bonded polysulfone dialysis membranes on oxidative stress, anaemia and inflammation.

Study	*Duration*	*No. pts on VEM*	*Reported effects of VE membrane*
Andrulli *et al.*, 2010 [894]	8 months	9	→ERI, →CRP, →IL-6
Aoun *et al.*, 2010[1] [890]	4 weeks	7	↓Dialyser clotting, →Hb, →ESA
Calo *et al*, 2011 [891]	1 year	25	↓oxidative stress[2] (incl. ↓Ox-LDL), ↓PAI-1, →ESA, →Hb, ↓mononuclear cell activation, →Carotid intima-media thickness[3]
Mandolfo *et al*. 2011[4] [889]	6 months	16	→ESA, ↓ERI, →IL-6, →CRP, →TAC, →AGEs
Matsumura *et al*, 2010[5] [892]	10 months	8	↓Intra-dialytic hypotension, ↓Pre-dialysis BP, →Nitric oxide-related molecules, →Hb, →ESA dose
Morimoto *et al*, 2005 [895]	6 months	16	↓Ox-LDL, ↓ADMA, ↓MDA, ↓ESA
Paniichi *et al*, 2011 [893]	6 months	62	↑Hb, →ESA, ↓ERI, ↓CRP, ↓IL-6
Sanaka *et al*, 2013 [896]	12 months	151	→ERI, →CRP

Notes:
1. Paediatric study
2. Number of measures of improved oxidative stress reported: ↓Ox-LDL, ↓$p22^{phox}$, ↑heme oxygenase-1
3. Carotid intima-media thickness performed in a subset of 9 patients
4. All patients dialysing via CVC's
5. Patients with intra-dialytic hypotension selected for study inclusion

ADMA: Asymmetric dimethylarginine; AGEs: Advanced glycation end products; CVC: Central venous catheter; ESA: Erythropoiesis stimulating agent; ERI: ESA resistance index; MDA: Malondialdehyde; PAI-1: Plasminogen activator inhibitor-1; TAC: Total anti-oxidant capacity; VEM: Vitamin E-bonded membrane

1.8 Summary

Patients on dialysis have far higher rates of CV disease than members of the general population. Furthermore, the pathophysiology, risk factors and the clinical manifestations of CV disease differ markedly between these two groups. Anaemia, or more specifically ESAs for the treatment of anaemia, has been linked to the development of CV complications. Underlying both the high rates of CV disease and the association between anaemia and adverse CV outcomes may be the high levels of inflammation and oxidative stress encountered in HD patients. Additionally, there are a wealth of data linking haemostatic markers, such as fibrinogen levels and altered fibrin clot structure, with CV

disease in both dialysis and non-dialysis patients. Whether this represents a mechanistic association or an epiphenomenon is not clear. Vitamin E has both anti-inflammatory and anti-oxidative properties providing a strong rationale for examining its utility in ameliorating renal anaemia and improving CV outcomes in dialysis patients. Studies in non-renal patients, however, have largely been disappointing in this respect but studies in HD patients have shown some promise, perhaps reflecting the different nature of CV disease in these patients and the altered metabolism of VE. Blood-membrane interactions are thought to be a major contributor to both inflammation and oxidative stress in HD patients and VE is lipid soluble; hence supplementation may have a limited capacity to influence fluid phase reactions occurring at the blood-membrane interface. There is therefore a sound logic for investigating the potential of VE bonded membranes to improve inflammation, oxidative stress, renal anaemia and ultimately CV disease.

Chapter 2 : Aims

The main aims of this thesis were to examine the effects of switching prevalent HD patients to dialysis with VE bonded polysulfone dialysis membranes for a period of 12 months on:

- Renal anaemia (i.e. ESA resistance)
- Oxidative stress
- Inflammation
- Fibrin clot structure and function
- Cardiovascular events
- Mortality

These were achieved by undertaking a prospective randomised controlled trial in which 260 HD patients were randomised to dialysis with either VE bonded polysulfone dialysis membranes or non-VE bonded equivalent membranes. All of the factors under consideration, i.e. anaemia, oxidative stress, inflammation and fibrin clot structure, have been implicated in the development of CV disease and mortality in HD patients. This thesis will therefore provide an insight into the potential for VE-bonded membranes to ameliorate the high CV risk experienced by patients on HD.

Chapter 3 : Methods

3.1 Study design and intervention

The study forming the basis of this thesis was a prospective randomised controlled trial comparing a VE-bonded HD membrane (Vitabran-A, Asahi Kasei Medical Corporation, Japan) with a non-VE-bonded equivalent membrane (Rexeed-A, Asahi Kasei Medical Corporation, Japan). Both membranes were high-flux polysulfone membranes made by the same manufacturer with similar performance characteristics, differing only in the presence or absence of a VE coating on the dialysing surface. The Rexeed-A membrane was the standard membrane in use at Leeds Teaching Hospitals NHS Trust (LTHT) prior to this study. Participating patients were randomised to dialysis with either the Rexeed-A or Vitabran-A membrane for a period of 12 months. Both dialysis membranes were CE marked and were not classed as investigational medical products or devices (Medicines and Healthcare products Regulatory Agency (MHRA), personal communication January 2009, Ref: E/2008/1110).

3.2 Trial Approvals

The research protocol was approved by Leeds West Research Ethics Committee (reference: 08/H1307/144) and LTHT Research and Development (reference: RL 08/8779) prior to commencement. The study was also prospectively registered on the European Union Drug Regulating Authorities Clinical Trials (EudraCT) (reference: 2009-017505-11) and International Standard Randomised Controlled Trial Number (ISRCTN) (reference: 12650766) databases and was adopted onto the National Institute for Health Research (NIHR) portfolio (reference: 6789).

3.3 Funding

The study was jointly funded by Asahi Kasei Medical Corporation, Japan who manufactured and supplied the Vitabran-A and Rexeed-A membranes, by the LTHT Renal Charitable Trustees and the NIHR.

3.4 Study setting

The study was undertaken in HD units managed by LTHT which collectively provide dialysis services for a population of approximately 1.7 million people in West Yorkshire [897]. Study patients received dialysis at one of 9 dialysis units spread over 7 geographical

locations: (i) St James's University Hospital (wards 55 and 53), Leeds, (ii) Seacroft Hospital, Leeds (Frank Parson's dialysis unit and B-ward), (iii) Beeston Satellite Unit, Leeds, (iv) Calderdale Royal Infirmary, Halifax, (v) St. Luke's Hospital, Huddersfield, (vi) Clayton Hospital, Wakefield and (vii) Dewsbury District Hospital, Dewsbury. Midway through the study, the Clayton Hospital dialysis unit closed down and patients were transferred to a new satellite dialysis unit based at Pontefract General Hospital, Pontefract.

3.5 Research team

The research team was led by Dr Simon Lines and comprised the research nurses Rosalyn Wheatley (Principal Research Nurse for the study), Emma Giddings, Shyama Rughooputh, Stuart Turner, and a research assistant Frank Lee. The project supervisors for the study were Dr Mark Wright, Dr Emma Dunn (Consultant Nephrologists, LTHT) and Dr Angela Carter (Senior Lecturer in CV epidemiology, University of Leeds). The roles of the various members of the core research team are detailed in Appendix B.

3.6 Patients

3.6.1 Screening, recruitment and enrolment

All patients managed by the renal services at LTHT had information pertaining to their medical care stored on a locally configured computer-based information system (Proton, Clinical Computing Limited, UK), hereafter referred to as the Proton system. A list of all HD patients was obtained from the Proton system and screened by Dr Simon Lines. The inclusion criteria and exclusion criteria for study eligibility were as follows.

INCLUSION CRITERIA

- Established on HD for at least 3 months prior to entry into study
- Patients expected to remain on HD for at least 6 months
- Written consent and willingness to participate in the study
- Age ≥ 18 years at point of entry into study
- Patients on a 3 times a week dialysis schedule

EXCLUSION CRITERIA

- Unwillingness or inability to cooperate or give written informed consent
- Terminally ill patients (expected survival less than 6 months)

- Medical conditions requiring regular blood transfusions at the time of study enrolment
- Any serious medical, social or psychological condition that in the opinion of the investigator would disqualify a subject from participation
- Patients with a significant inflammatory illness within the last 3 months as defined by a CRP > 50 mg/L or 3 times the patient's baseline CRP.

Eligible patients were approached by a member of the research team when they attended for dialysis to introduce the study and to provide a copy of the patient information leaflet to take away and read. Patients were then approached again, no earlier than their next dialysis session, to answer any questions and obtain written informed consent for those wishing to participate. There was therefore a minimum time period of 48 hours between introducing the study and obtaining written informed consent and patients could request more time if they wished. If patients were non-English speakers, independent translators were used to explain the background to the study and to obtain written informed consent for those wishing to participate. The patient information sheets and the consent form were approved by the Leeds West Research Ethics Committee. Study patients who dialysed at another centre for more than two weeks during the study period discontinued the study after their last HD session at an LTHT unit prior to dialysing elsewhere; data collected up to that time point were analysed. This criterion was used as dialysis at a different centre often necessitated a change in dialysis membrane, non-protocolised ESA prescription (see section 3.8.1) and exposure to other factors, such as different dialysis water quality, which had the potential to influence the various study endpoints.

3.6.2 Randomisation

All patient records on the Proton system have a unique database number and this number was used to allocate patients into the control or intervention arm of the study on the basis of whether it was odd or even. An analysis of all LTHT HD patients was performed on this basis prior to starting the study and there was an even split in the number of patients and the two groups were comparable with regards to age, sex and length of time on RRT.

3.7 Data collection

3.7.1 Demographic, lifestyle and comorbidity data

The following baseline information was recorded for each of the study patients at baseline: age, sex, ethnicity, cause of ESRF, length of time on dialysis and smoking status coded as current smoker (i.e. smoked in the last 12 months), ex-smoker or never smoked. This information was obtained by reviewing the Proton system and from discussions between the study participant and a member of the research team. If patients did not speak English, translators independent of the study and research team were used. All data were entered and stored on a bespoke Microsoft Access 2003 (Microsoft Corporation, USA) database created by Dr Simon Lines which was password protected and stored in accordance with the Data Protection Act and the LTHT and University of Leeds data storage policies.

Comorbidity at baseline was determined for each patient using the method reported by Davies *et al.* [898]. This scoring system has been validated in patients with ESRF and shown to be predictive of survival [899, 900]. Information regarding the presence of comorbidity was obtained from discussions with the study participants and review of the clinical patient records. For the purposes of this scoring system, comorbidity was classified into seven domains as detailed below and, for each comorbid domain, evidence of disease not its severity was required. To be counted, comorbidities had to either be considered active, still present or controlled by on-going treatment. The comorbid score for each patient was determined by the number of domains affected giving a theoretical maximum of seven.

1. *Malignancy* - active, non-cutaneous disease e.g. myeloma, breast cancer
2. *Ischaemic heart disease (IHD)* - Previous myocardial infarction, angina pectoris, positive coronary angiography or other diagnostic procedure (e.g. exercise test, thallium or dobutamine stress test) or the presence of ischaemic changes on the resting electrocardiogram (as distinct from left ventricular hypertrophy).
3. *Peripheral vascular disease (PVD)* - Including distal aortic, renovascular, lower limb and cerebrovascular disease; either symptomatic disease in these vascular territories (e.g. cerebrovascular event, amputation, claudication) or significant stenoses (>50%) on vascular imaging or Doppler ultrasound.

4. *Left ventricular dysfunction* - Defined as clinical evidence of pulmonary oedema, not attributable to errors in fluid balance, or moderate to severe left ventricular dysfunction on echocardiography.
5. *Diabetes mellitus* - The presence of either type 1 or type 2 diabetes mellitus
6. *Systemic collagen vascular disease* - For example systemic vasculitis, rheumatoid arthritis and systemic sclerosis, either active or requiring treatment.
7. *Other significant pathology* - Defined as a condition severe enough to have an impact on survival in the general population such as severe chronic obstructive pulmonary disease, cirrhosis or psychiatric illness. Treatable conditions (e.g. peptic ulceration) or non-life threatening diseases such as severe osteoarthritis were not counted.

3.7.2 Medications

The current medications for all study participants were recorded at the time of the baseline, 6 and 12 months study visits. This information was obtained from the list of current medications stored in the Proton system following corroboration with the study participants. The data were stored in the study database as dummy variables by drug classes for those drugs which were deemed relevant to our study endpoints of anaemia, CV disease and fibrin clot properties. This list of drugs comprised: aspirin, warfarin, clopidogrel, dipyridamole, sulphonylureas, insulin, statins (3-hydroxy-3-methylglutaryl Coenzyme-A reductase inhibitors), renin-angiotensin medications (angiotensin converting enzyme inhibitors, angiotensin II receptor blockers or direct renin inhibitors) and β-adrenoceptor antagonists. The ESA and intravenous iron doses were recorded for all study patients. This information was obtained from the Proton system retrospectively at the end of the study by interrogating the database using a database query written by Dr Elizabeth Lindley (Clinical Scientist, Department of Renal Medicine, LTHT). As the Proton system was used for prescribing ESAs and intravenous iron for all LTHT managed HD patients [901] this method was robust.

3.7.3 Dialysis information

The monthly dialysis dose (urea reduction ratio), pre-dialysis blood pressure and post-dialysis weight were recorded for all study patients. For the blood pressure and weight, the median value of 3 readings performed over a week was recorded. Adherence to dialyser allocation was audited for all study participants by members of the research team. Following study commencement, all available dialysis worksheets were reviewed for the

first 6 weeks on a fortnightly basis. If there was >95% adherence to the allocated dialyser the monitoring schedule was reduced to a 2 week period every 6 weeks. If a unit failed to achieve 95% compliance they remained on fortnightly checks until adherence was >95%. Audit results were fed back to the senior sisters at each unit. The results of the routine water quality tests performed at all of the dialysis units during the study period were obtained retrospectively after study completion.

3.7.4 Blood sampling

Monthly pre- and post- dialysis blood samples were obtained from study subjects as part of routine clinical care. A summary of the blood testing schedule is shown in Table 3. The routine blood tests were taken by the clinical staff in line with LTHT standard operating procedures and the results collated from the Proton system using a database query written by Dr Elizabeth Lindley (Clinical Scientist, Department of Renal Medicine, LTHT).

Table 3 - Blood testing schedule. Details of the blood tests performed as part of routine clinical care the additional blood tests performed for the study.

Routine tests	Additional study tests
Monthly	*Baseline, 6 months and 12 months*
Pre- & post- dialysis urea and electrolytes	Lipid profile*
Full blood count	Clotting screen†
Ferritin	Clauss fibrinogen level
Calcium	C-reactive protein
Phosphate	Fibrin clot structure and function
Bicarbonate	Complement assays
HbA1c	Oxidative stress
3 monthly	
PTH	

*Cholesterol, high-density & low-density lipoprotein and triglyceride levels
†Clotting screen: Activated partial thromboplastin time and pro-thrombin time

Blood samples for the additional study tests were taken pre-dialysis by members of the research team via their dialysis access. For patients with AVFs and grafts, blood was aspirated via the dialysis needle after cannulation. For patients dialysing via a CVC, the CVC was prepared and connected to the dialysis machine as for routine dialysis. The blood pump was then started and once the column of blood had reached the bubble trap on the HD machine, approximately 30 seconds, blood was aspirated aseptically from the sampling port on the arterial limb of the dialysis circuit using a 19 Gauge butterfly. Approximately 35 mL of blood was collected and used to fill the sample bottles in the following order:

1. 9 mL of blood into 10 mL pre-cooled tube containing 1 mL of 0.109 M trisodium citrate
2. 6 mL of blood into pre-cooled ethylenediaminetetraacetic acid (EDTA) containing tube
3. 3.5 mL of blood into trisodium citrate tube
4. 4 mL of blood into each of 3 x gel tubes

Samples collected into pre-cooled bottles (1 and 2 above) were mixed by slow inversion, so as to minimise cellular activation, and immediately placed on ice. The trisodium citrate tube and 2 of the gel tubes were transported by hand to the LTHT Research and Development laboratories for analysis of lipids, CRP, Clauss fibrinogen, prothrombin time (PT) and activated partial thromboplastin time (APTT). The 10 mL trisodium citrate, 6 mL EDTA and the remaining 4 mL gel tube were transported to the LIGHT laboratories (the former two samples on ice) for centrifugation. The gel tubes were left for a minimum of 30 minutes to clot. All samples were then centrifuged at 3000g for 30 minutes in a centrifuge pre-cooled to 4^{o}C. Following centrifugation the supernatant was aliquoted into cryotubes and snap frozen in liquid nitrogen. The supernatant from the trisodium citrate samples were stored at -40^{o}C and the remainder stored at -80^{o}C prior to analysis.

3.7.5 Blood transfusions

Data on blood transfusions were initially obtained by asking patients at the time of blood sampling if they had received any blood transfusions in the preceding 6 months and by interrogating the Proton system; however it became apparent that only a few of the dialysis units were accurately recording this data. In an attempt to identify everyone who had received a blood transfusion a list was generated of all patients who had a >2 g/dL/month increase in their haemoglobin level during the study period. This list was then cross-referenced with the computerised records of the blood banks attached to units which were authorised to administer transfusions to determine which patients had received blood and the number of units they received.

3.7.6 Clinical events

Data on hospital admissions and deaths were collected weekly for study participants through a combination of interrogating the renal database, contacting the dialysis units by email and telephone, and encouraging study participants and clinical staff to notify the research team of clinical events such as hospital admissions. A spreadsheet was used to record the date and details of the clinical events which were coded into one or more of the

following categories: (i) cardiovascular, (ii) non-infective dialysis access event, (iii) infection requiring hospital admission, (iv) death or (v) other.

Dialysis access events were defined as any hospital admission or access procedure for non-infective dialysis access problems, e.g. thrombosed fistula, CVC blockage. Cardiovascular events were defined as death or hospital admission arising from CV disease including acute coronary syndromes, cerebrovascular events, peripheral vascular disease, acute arrhythmias and pulmonary oedema not attributable to errors in fluid balance.

3.8 Anaemia data

3.8.1 Anaemia management

The LTHT dialysis service has used a computer-based decision support system to inform ESA and iron dosing as part of routine clinical care for over 10 years [902]. By adopting a logic-based algorithmic approach anaemia practices are standardised for all patients, independent of supervising nephrologist and HD unit, permitting the objective evaluation of interventions with ESA-based outcomes as has been performed previously [903]. Recommendations from the decision support system are issued in the form of a report which is passed to either the supervising physician or independent nurse prescriber based at the respective dialysis unit to implement or reject any changes. In practice the recommendations are followed almost without exception providing an unbiased, stable platform for comparing ESA doses between the study groups.

The ESA-dosing algorithm used in this study was a predictive algorithm and the LTHT experience with this algorithm has been published [901]. All patients requiring an ESA were prescribed darbepoetin alfa (Amgen). In essence the predictive algorithm had a target haemoglobin level of 11.5 g/dL, the midpoint of the target haemoglobin range recommended by National Institute for Health and Clinical Excellence (NICE) at the time this study was conducted [904], and recommended ESA dose adjustments based on the haemoglobin level and its trajectory; full details of the algorithm are provided in Appendix D. In addition all patients received intravenous iron with dosing based on their haemoglobin, ferritin and CRP levels, mean red cell volume and the percentage of hypochromic red blood cells as detailed in Appendix D.

3.8.2 Erythropoiesis stimulating agent (ESA) resistance index

In line with other studies examining the effects of VE bonded membranes on ESA requirements [889, 893, 894], it was decided to use the ESA resistance index (ERI) as the outcome measure for ESA requirements. The ERI is a measure of how much ESA an individual patient requires to achieve a given haemoglobin concentration, adjusted for body weight, as defined below. For example, a lowering of the ERI in an individual patient would mean that they required a lower ESA dose to achieve a given haemoglobin level - i.e. they became less ESA resistant.

$$\text{ESA Resistance Index (ERI)} = \frac{\text{Weekly ESA dose (IU)*}}{\text{Weight (kg) x Haemoglobin (g/dL)}}$$

*Darbepoetin alfa (µg) doses multiplied by 200 to convert to IU units erythropoietin as per convention

3.9 Routine biochemistry and haematology assays

The clinical laboratories at LTHT performed the assays for the routine biochemistry and haematology tests detailed in Table 3, in addition to the lipid profile, clotting screen, Clauss fibrinogen and the highly sensitive CRP assays (see Appendix C for details). It became apparent after all of the sampes had been analysed that any samples with a Clauss fibrinogen level greater than 4.5 g/L were reported as >4.5 g/L rather than accurately quantified. This was because the autoanalysers were configured and optimised for clinical rather than research use. Although the raw data were still theoretically available, it was not possible to go back retrospectively and quantify the fibrinogen concentrations in the samples with high fibrinogen levels. Logistically it would have been difficult as it required taking one of the clinical analysers off-line for several hours to process even a few samples. Perhaps more importantly, the standard curve used to determine the fibrinogen concentration was optimised for analysing samples in the range 0-4.5 g/L. To retrospectively extrapolate data from outside the range of the standard curve would likely have introduced significant errors. In view of this, the fibrinogen dataset was analysed by dividing the samples into quartiles of fibrinogen concentration such that all the samples with fibrinogen concentrations >4.5 g/L were in the highest quartile.

3.10 Ox-LDL ELISA

The Ox-LDL assays were performed by Ms Helena Baker (Research Technician, LTHT Research and Development Laboratory) using commercial ELISA kits (Immundiagnostik AG, Bensheim, Germany) to analyse serum samples which were stored at -80 ^{o}C prior to analysis. To perform the assay, standards and quality control (QC) samples, buffers,

antibody, substrate and stop solutions included with the kit were reconstituted as per the manufacturer's recommendations. Serum samples were thawed prior to being diluted 1/10 with dilution buffer. The pre-coated 96-well microtitre plates were initially washed 5 times with 250 µL/well of washing buffer prior to loading 100 µL of standard, sample or QC into each well. The plates were sealed and placed on a plate shaker for 4 hours at RT. The contents of the wells were discarded and the wells washed 5 times with 250 µL of washing solution prior to loading with 100 µL/well of conjugated antibody solution. After a further 1 hour incubation at RT on a plate shaker, the contents of the wells were discarded and the wells washed 5 times with 250 µL of washing solution. To develop the plates, 100 µL of substrate was added to each well and the plates incubated for 15-25 minutes at RT in the dark; the reaction was stopped by the addition of 50 µL/well of stop solution. The absorption in each well was measured at 450 nm with a 620 nm reference filter. All samples were analysed in duplicate with an intra-sample coefficient of variation (CoV) of less than 10% deemed acceptable; samples with a CoV of greater than 10% were repeated. A linear best fit line was then plotted through the five standards and used to determine the concentration of Ox-LDL in the samples. The resultant data were provided by the R&D department as a spreadsheet (Excel®, Microsoft Corporation) containing sample identification numbers and Ox-LDL concentrations. With this approach 63% of the samples had a calculated concentration which was below the manufacturer stated lower limit of detection of the assay (4.3 ng/mL) and the inter-assay CoV for the low QC sample, which was nearest to the mean concentration of the study samples, was 26%.

The apparent low levels of Ox-LDL in the study cohort were an unexpected finding based on published studies using the same commercial assay both in renal [891] and non-renal [905] patients. It was therefore decided to obtain the raw optical density data from the R&D department for re-analysis (Dr Simon Lines). Visual inspection of these data suggested that fitting a straight line through the standards resulted in a poor estimation. Evaluation of the data using the curve fitting option on SPSS (SPSS Inc., IBM) revealed that a cubic curve fit was more appropriate. The equation for the best fitted cubic curve through the standards for each plate was determined using SPSS and the Ox-LDL concentration in each sample pair re-calculated using Microsoft Excel® (Dr Simon Lines). This revised dataset had a marginally improved inter-assay CoV of 19% for the low QC and 22% of the samples had an Ox-LDL concentration below the quoted lower limit of detection for the assay (4.3 ng/mL); these samples were assigned a concentration of 4.3 ng/mL for the purposes of data analysis. The inter-assay CoV of the high QC also marginally improved

on recalculation from 14% to 13%. The manufacturer quoted intra- and inter-assay CoVs were 3.9-5.0% and 9.0-11.0% respectively.

3.11 Thiobarbituric acid reactive species assay (TBARS)

3.11.1 Development and optimisation of TBARS assay

The assay used in this study was based on the method developed by Jentzsch *et al.* [906] and used by Eiselt *et al*. in a study of HD patients [866]. From the large body of literature on TBARS assays the key aspects appeared to be: (i) addition of an anti-oxidant to the reaction mix, most frequently butylated hydroxytoluene (BHT), to limit intra-assay oxidation [906]; (ii) precipitation of proteins and lipids, again to limit autoxidation of polyunsaturated fatty acids during the heating step and to reduce adsorption of the MDA:TBA adduct thereby theoretically reducing yield [907]; (iii) acidic reaction conditions and heating to render the carbonyl groups of MDA more susceptible to nucleophilic attack by TBA, thus decreasing reaction time and increasing yield [908] and (iv) butanol extraction to minimise the effects of reaction mixture turbidity [909, 910]. All of these considerations were addressed in the method published by Jentzsch *et al.* [906] which formed the basis of the assay used here. It was decided to use a 5mM concentration of BHT as previous work had suggested a threshold level of 3 mM for minimisation of intra-assay oxidation [906].

To generate the standard curve for the assay, a 5 mM MDA stock solution was prepared by acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) in 0.1 M hydrochloric acid at 37 ^{o}C. The MDA stock solution was serially diluted with ultrapure water to form standards of 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.6 µM, 0.8 µM and 0.4 µM; fresh standards were prepared for each assay. The assay was performed using fresh frozen serum aliquots, stored at -80 ^{o}C, which were thawed in cold water for 10-15 minutes, vortex mixed and centrifuged at 13,000 g for 1 minute immediately prior to analysis. A known volume of the serum, initially 150 µL, was combined with an equal volume of 0.2 M phosphoric acid (H_3PO_4) and 25 µL 70 mM BHT in an eppendorf tube. The contents were vortex mixed, 25 µL of 70 mM TBA added and the samples vortex mixed again. The eppendorf tubes were then transferred to a heat block pre-heated to 90 ^{o}C for 45 minutes. After the heating stage the eppendorf tubes were placed on ice for 5 minutes to stop the reaction prior to the addition of 350 µL butanol. The eppendorf tubes were then vortex mixed and centrifuged at 13,000g for 2 minutes. The supernatant was pipetted in aliquots of 150 µL into the wells of a 96-well microtitre plate and the absorbance measured at 540 nm with a 620 nm reference filter using an MRX plate reader (Dynex Technologies). A

cubic best fit curve was then calculated for the standards, plotted on a logarithmic abscissa, and used to interpolate the TBARS levels (i.e. MDA concentration) in the study samples. Several aspects of the published methodology [906] were identified for optimisation, these were the serum volume assayed and the incubation time.

To determine the optimal volume of serum to use in the assay, the total reaction volume was fixed at 450 µL and serum volumes of 100 µL, 150 µL and 200 µL were compared. In each case an equivalent volume of 0.2 M phosphoric acid was added, along with 25 µL of BHT and TBA; ultrapure water was used to make up the final reaction volume to 450 µL. The absorbance-concentration graphs for the standards from each of these experiments are shown in Figure 11. At low concentrations (i.e. below 3.1 µM MDA equivalents) the 100 µL volume performed worse, i.e. there was minimal change in absorbance for a change in concentration, but there was little difference between the 150 µL and 200 µL assays over the anticipated concentrations of TBARS in the study samples. In light of these considerations, and the volume of test serum available given that samples were to be analysed in duplicate and repeated if they yielded discordant results, it was decided to use a serum volume of 150 µL.

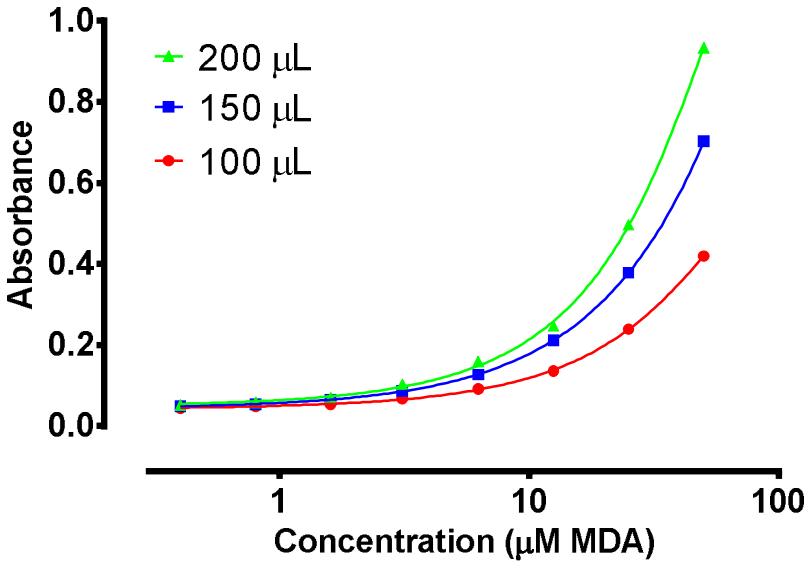


Figure 11 - TBARS assay standard curves from 3 experiments to determine the effects of altering the volume of serum or standard in the final reaction mix. At low TBARS concentrations the curves were relatively flat, particularly for the 100 µL assay, with little difference between the 150 µL and 200 µL assays.

The next optimisation step undertaken was to examine the effect of different incubation times. The vast majority of published TBARS methodologies heated the reaction mix to >90 ^{o}C although the incubation time varies from 15 minutes to three hours. The Jentsch *et*

al. [906] method involved heating for 45 minutes and it was decided to compare the performance characteristics of heating the reaction mix for different lengths of time. The standards were made up as for the previous experiments although the lowest 0.4 µM standard was omitted owing to the flatness of the curve in this region. Three samples of pooled serum were assayed as follows: (i) no spike, (ii) low concentration MDA spike (from MDA stock solution) and (iii) high concentration MDA spike. The effects of incubating the standards and test sera for 15, 30, 45, 60 and 75 minutes were compared and the results are displayed in Figure 12. On the basis of these experiments it was decided to heat the samples for 45 minutes.

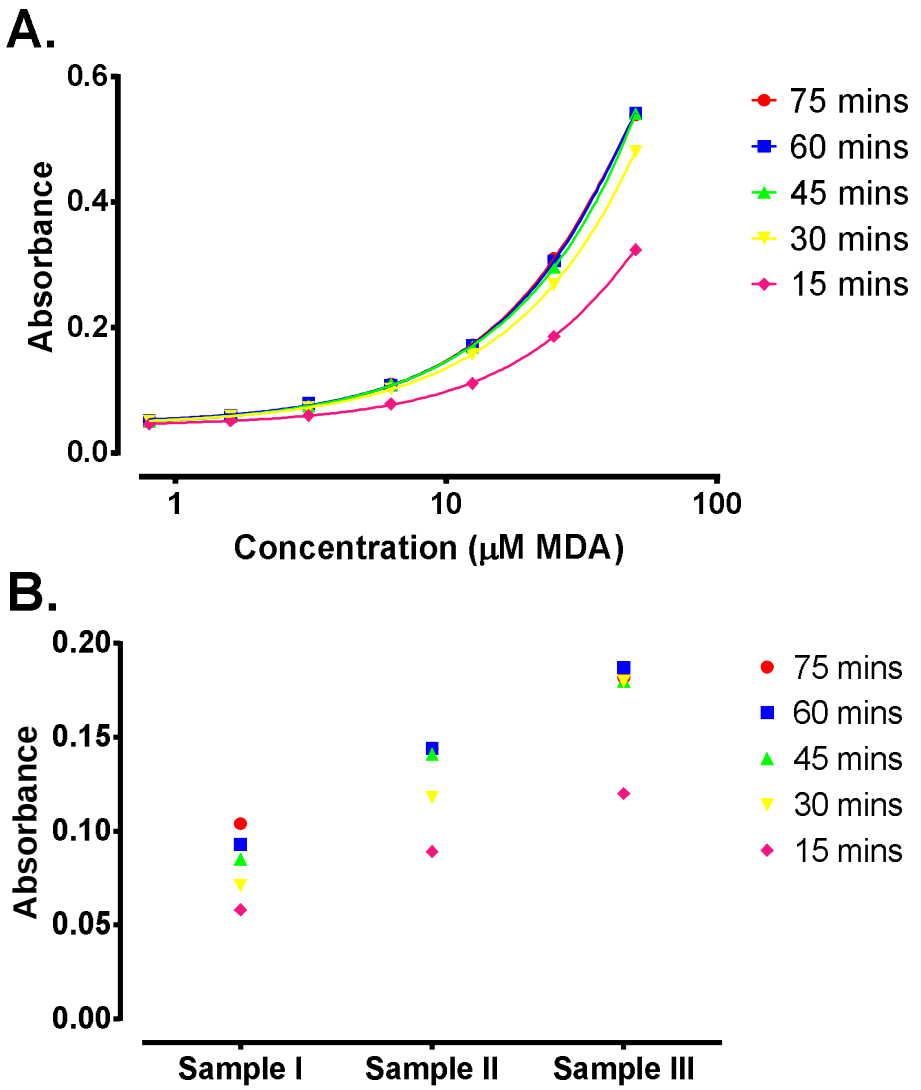


Figure 12 - Effects of different heating times in TBARS assay. (A) Standard curves and (B) Samples: (I) No-MDA spike; (II) Low concentration MDA spike and (III) High concentration MDA spike.

Spiking experiments were carried out to measure the recovery of MDA. Aliquots of pooled serum were spiked with 0.5 µM, 1 µM, 2 µM and 2.5 µM of MDA and the mean recovery rate was 84%; this was in keeping with the published experience of others [908]. Batches of high and low QC samples were generated in order that they could be run with each assay to determine the intra-assay CoV. The low QC consisted of a series of aliquots of fresh frozen serum and the high QC was generated by spiking a batch of low concentration QC with MDA standard prior to separation and storage.

3.11.2 Final TBARS assay protocol

Test serum samples, stored at -80 ^{o}C, were defrosted immediately prior to analysis by standing in water for 10 minutes at RT. All samples were vortex mixed then centrifuged at 13,000 g for 1 minute. Standards were prepared by serial dilution of the 5 mM stock MDA solution using ultrapure water to the following concentrations: 12.5 µM, 6.3 µM, 3.1 µM, 1.6 µM and 0.8 µM. One-hundred and fifty microlitres of standard or test serum were combined with 150 µL of 0.2 M H_3PO_4 and 25 µL of 70 mM BHT in eppendorf tubes; all standards and samples were analysed in duplicate. The contents were vortex mixed, 25 µL of 70 mM TBA added and the contents mixed again. The eppendorf tubes were transferred to a heat block pre-heated to 90 ^{o}C for 45 minutes. After this time they were placed on ice for 5 minutes to stop the reaction.

To extract the TBARS, 350 µL butanol was added to each eppendorf tube after cooling and the contents vortex mixed prior to centrifugation at 13,000 g for 2 minutes. One hundred and fifty microlitres of the supernatant were pipetted into the wells of a 96-well microtitre plate and the absorbance measured at 540 nm with a reference filter of 620 nm using an MRX plate reader (Dynex Technologies). The concentration of MDA equivalents in each sample was interpolated from a best fit cubic regression curve through the standards using the Revelation software package (Dynex Technologies). Any duplicates with concentrations below 5 µM and a CoV > 25% or concentrations above 5 µM and CoV's >10% were repeated. The intra-assay CoV's for the high and low QC were 2.1% and 18.5% respectively and the inter-assay CoVs 13% and 21% respectively. The average concentrations of the high and low QC samples were 9.4 and 2.1 µM MDA respectively.

3.12 C3 ELISA

A C3 ELISA developed previously in the Division of Cardiovascular and Diabetes Research (DCDR) by Mrs May Boothby and Mrs Jane Brown (Research Technicians, DCDR) and Dr Verena Schoeder (Visiting postdoctoral student, DCDR) was used to

determine C3 levels in citrated plasma which had been stored at -40 ^{o}C. A pooled citrated plasma sample was diluted in phosphate buffered saline with tween (PBS-T, see Appendix E) to dilutions of 1/25,000, 1/50,000, 1/100,000, 1/200,000 and 1/400,000 to generate the standards. As a new pool of plasma had been collected since the assay was previously run, the plasma pool used to generate the standards was calibrated against purified C3 (Quidel) to determine the concentration. To achieve this, a standard curve was generated using the purified C3 corresponding to concentrations of 4.8 mg/mL, 2.4 mg/mL, 1.2 mg/mL, 0.6 mg/mL and 0.3 mg/mL diluted 1/100,000. The normal pool was analysed at dilutions of 1/25,000, 1/50,000, 1/100,000, 1/200,000 and 1/400,000 and the calibration assay performed twice and used to calculate the concentration of C3 in the normal pool (0.86 mg/mL). The standards in the finalised assay therefore corresponded to sample concentrations of 3.44 mg/mL, 1.72 mg/mL, 0.86 mg/mL, 0.43 mg/mL and 0.22 mg/mL after adjusting for sample dilution.

On the day prior to performing the assays, each well of a 96-well microtitre plate (Nunc, Denmark) was coated with 100 µL rabbit anti-human C3 antibody (DAKO) diluted 1/10,000 with PBS. Plates were sealed and placed on a plate shaker at 400 rpm and incubated overnight at 4 ^{o}C. On the day of the assay the plate and all reagents were brought to RT. Test plasma samples were thawed in a water bath pre-warmed to 37 ^{o}C and serially diluted to 1/100,000 with PBS-T using a Microlab 500 diluter (Hamilton Company, USA).

Following overnight incubation the antibody coating solution was discarded and the wells washed 4 times with 200 µL of PBS-T. A blocking step was not included as it had been previously demonstrated not to influence the results (Dr Verena Schroeder). The plate was loaded with 100 µL/well of blank (PBS-T), standard, QC and test plasma samples in duplicate and incubated at RT on a plate shaker at 400 rpm for 2 hours. All samples pertaining to an individual patient were analysed on the same plate. The contents of the wells were discarded prior to washing 4 times with 200 µL PBS-T and the addition of 100 µL/well of detection antibody (goat anti-human C3, Quidel) at a dilution of 1/10,000 (in PBS-T). The plate was incubated at RT for 1 hour on a plate shaker at 400 rpm after which the contents of the wells were discarded prior to washing 4 times with 200 µL/well PBS-T. Subsequently 100 µL of horseradish peroxidise (HRP) conjugated rabbit anti-sheep antibody (DAKO), diluted 1/2000 in PBS-T, was added to each well and the plate incubated at RT on a plate shaker at 400 rpm for 1 hour. During this incubation, 4 tablets of OPD (14.08 mg of 1,2-phenylenediamine dihydrochloride per tablet, DAKO) were completely dissolved in 12 mL of deionised water before adding 5 µL 30% w/v hydrogen

peroxide. Following incubation, the contents of the wells were discarded and the plate washed 4 times with 200 µL/well of PBS-T. One hundred microlitres of OPD substrate solution was pipetted into each well by columns at 10 second intervals prior to placing the plate on a plate shaker at 400 rpm. Once the wells had turned dark yellow, after approximately 8 minutes, the reaction was stopped by the addition of 100 µL 1.5 M H_2SO_4 to each column at 10 second intervals. The plate was then sealed and placed on a plate shaker for 10 minutes prior to reading. The absorbance at 490 nm for each well was measured using an MRX microplate reader. The concentration of C3 in each sample was calculated using the Revelation software package by estimating the best fit quadratic regression curve through the standards. Each sample was analysed in duplicate and any sample with a duplicate CoV of greater than 10% was repeated. The intra- and inter-assay CoV's were 2.8% and 6.6% respectively. Of the 714 samples analysed, Dr Simon Lines carried out the ELISA for 238, Victoria Richardson (PhD student, LIGHT laboratories) 73 and Jia-Ying Lee (visiting PhD student, National Taiwan University) undertook 403.

In addition to C3 levels, it was decided to measure the levels of properdin, factor D and SC5b-9 in the study samples. Owing to the large number of study samples to be analysed, and the prohibitive costs of purchasing commercial ELISA kits, it was decided to develop and perform in-house ELISAs for these purposes. The properdin ELISA, as detailed in the following section, was based on an ELISA which had previously been developed in the department and which was re-optimised for this study. The ELISAs for quantifying factor D and SC5b-9 levels were developed *de novo* using commercially available antibody pairs as detailed in sections 3.14 and 3.15. During the development of the ELISAs, it was intended to perform the properdin, SC5b-9 and factor D ELISAs contemporaneously for the study samples so that all 3 assays could be performed using only 1 aliquot of fresh frozen plasma per patient sample. The coating, diluting and washing buffers and the diathenolamine solution used were the same for all three ELISAs and their compositions are detailed in Appendix E.

3.13 Properdin ELISA

3.13.1 Optimisation of properdin ELISA

A modified version of an in-house sandwich ELISA previously developed by Dr Riyaz Somani (PhD student, University of Leeds) was used for the measurement of properdin levels [911]. Briefly, the sequential steps involved in the ELISA were: (i) overnight coating of a 96-well microtitre plate with anti-human properdin monoclonal antibody (Thermo

Scientific), (ii) incubation with diluted plasma samples, (iii) incubation with a biotinylated anti-human properdin monoclonal antibody (Thermo Scientific), (iv) incubation with streptavidin and (v) development with p-nitrophenol phosphate. As the antibodies available were different lot numbers to those used previously, a matrix comparing different dilutions of the coating (anti-human properdin monoclonal antibody, Thermo Scientific) and conjugated (biotinylated anti-human properdin monoclonal antibody) antibody was performed by Jia-Ying Lee (visiting PhD student, National Taiwan University). This confirmed that the 1/5,000 dilutions, as had been used previously, were the most appropriate; these data is shown in Figure 13.

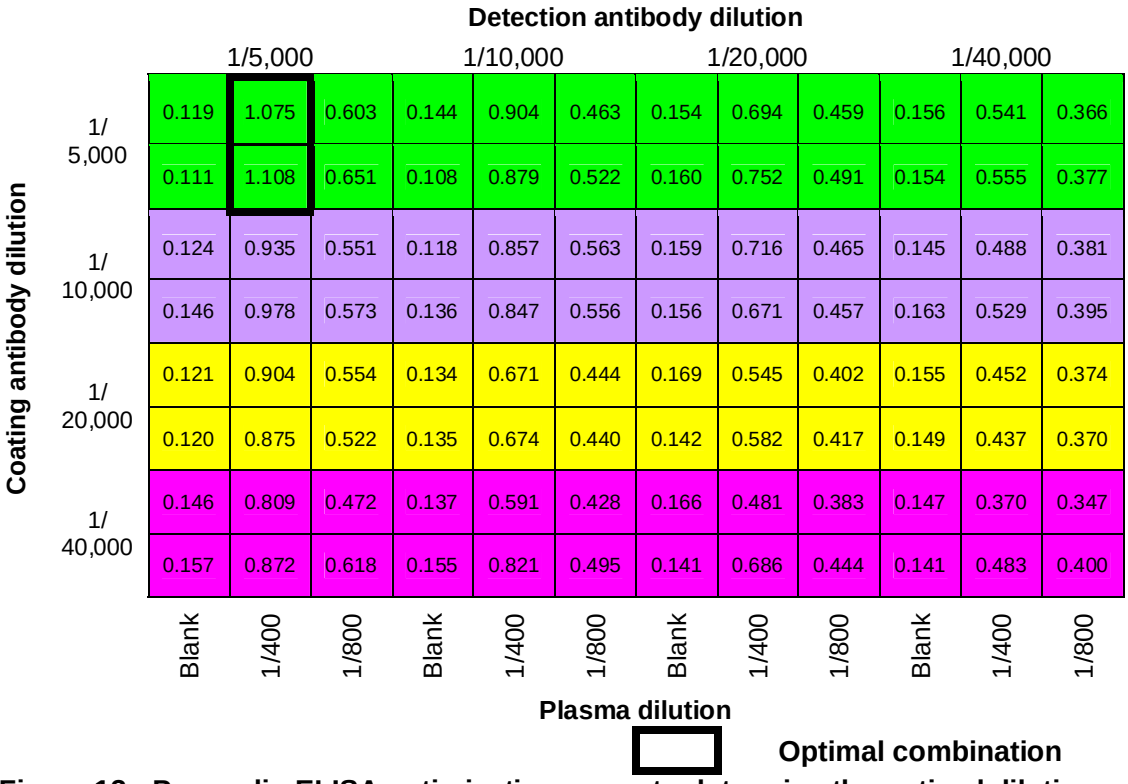


Figure 13 - Properdin ELISA optimisation assay to determine the optimal dilutions of coating and detection antibodies. Absorbancies measured at 405 nm with 540 nm reference filter for different dilutions of coating and detection antibody and plasma. The optimal combination was a 1/5000 dilution for both the coating and detection antibody and 1/400 for the plasma. Data kindly provided by Jia-Ying Lee (visiting PhD student, National Taiwan University).

The original assay [911] analysed samples at a dilution of 1/800 with a 5 point standard curve generated from serial dilutions of pooled plasma centred on this dilution. There was noted to be an increased signal using samples diluted 1/400 compared to 1/800 (see Figure 13) and an extended standard curve generated from serial dilutions of pooled plasma identified that the 1/400 dilution lay in the middle of the linear portion of the

standard curve (see Figure 14). The standard curve also flattened off at lower dilutions, i.e. higher properdin concentrations. It was therefore decided to analyse samples at a dilution of 1/400 and generate standards by diluting the pooled plasma samples: 1/100, 1/200, 1/400, 1/800, 1/1,600. After several plates were analysed, it was apparent that there was an edge effect which persisted even if all of the incubation steps were performed in the dark; the outer wells of the plates were therefore not used in subsequent assays.

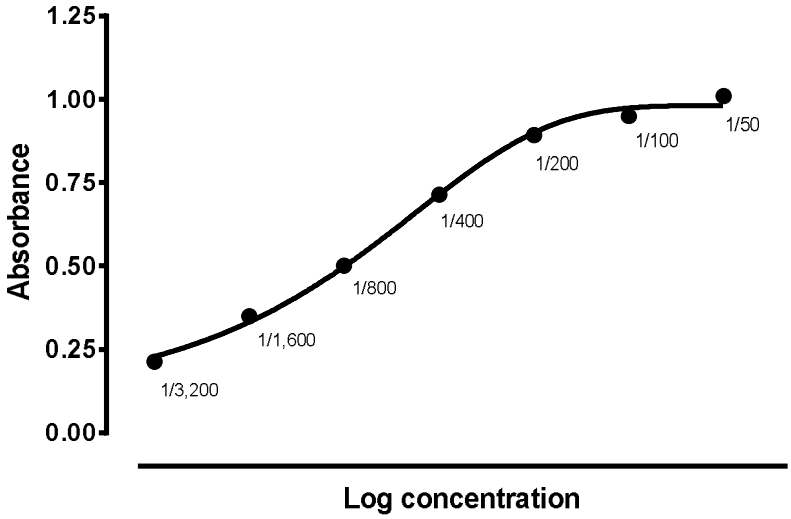


Figure 14 - Example standard curve for the properdin ELISA. Standards were generated by serial dilution of pooled plasma as labelled. The 1/400 dilution can be seen to lie on the linear portion of the standard curve. There was also a flattening off of the standard curve at lower dilutions / higher antigen concentrations.

To determine the concentration of properdin in the pooled plasma samples used to generate the standards, calibration assays were performed using purified properdin (Quidel). The purified properdin sample was diluted to form standards corresponding to sample concentrations of 110 µg/mL, 55 µg/mL, 27.5 µ/mL, 13.8 µg/mL and 6.9 µg/mL at a dilution of 1/400. Samples of the pooled plasma were analysed at dilutions of 1/100, 1/200, 1/400, 1/800 and 1/1,600. Four sets of dilutions were analysed on two separate plates to determine the concentration of properdin in the pooled plasma sample (70 µg/mL).

The next optimisation step was to investigate the effects of increasing the antibody-incubations steps from 1 to 2 hours to allow the properdin ELISA to be performed in parallel with the factor D and SC5b-9 ELISAs (see sections 3.14 and 3.15). Assays comparing 1 and 2 hour incubation steps with the antigen revealed greater well absorbance values and a lower CoV (8.4% vs 11.1%) in the 2 hour incubation assay. Similar experiments comparing 1 and 2 hour incubation periods with the conjugated antibody found no differences in the well absorbancies or the CoV. On the basis of these

results, and to facilitate performing the properdin ELISA in conjunction with factor D and SC5b-9 ELISAs (see sections 3.14 and 3.15), it was decided to increase the incubation periods with both the antigen and conjugated antibody to 2 hours.

The performance of the original properdin ELISA, on which the method detailed here was based, had previously been shown to be unaffected by the addition of a blocking step (Dr Riyaz Somani, Personal Communication). Assays performed using the optimised methodology here similarly demonstrated no effects of blocking with 1% BSA therefore no blocking step was included in the final protocol. Optimisation of the SC5b-9 ELISA, detailed in section 3.15.1, identified that the addition of 10 mM EDTA to the sample dilution buffer (see Appendix E) limited *in vitro* complement activation. The performance of the properdin ELISA was unaffected when assays using EDTA and non-EDTA containing sample dilution buffers were compared. It was therefore decided to use the EDTA containing buffer to facilitate the use of a single study sample dilution series to perform the properdin, SC5b-9 and factor D ELISAs. Finally, to assess the sensitivity of the ELISA at detecting different concentrations of properdin, spiking assays were performed using purified properdin (Quidel). Pooled plasma samples were combined with aliquots of purified properdin which equated to 30 µg/mL and 80 µg/mL after adjusting for the dilution factor of 1/400. Two sets of dilutions were performed in separate assays and the mean recovery of properdin was 127%.

3.13.2 Final properdin ELISA protocol

On the day prior to performing the assays each inner well of a 96-well microtitre plate (Nunc) was coated with 100 µL of anti-human properdin mouse monoclonal antibody (Thermo Scientific) diluted 1/5,000 in coating buffer (see Appendix E). Plates were sealed and placed on a plate shaker at 400 rpm overnight at 4 ^{o}C. On the day of the assay the plates and reagents were brought up to RT. Frozen EDTA plasma samples from study subjects and a QC sample, all stored at -80 ^{o}C prior to assay, were defrosted in a water bath pre-warmed to 37 ^{o}C and diluted to 1/400 in sample diluting buffer (see Appendix E) using a Microlab 500 diluter. An aliquot of pooled plasma, quantified for properdin as detailed above, was similarly thawed and diluted 1/100, 1/200, 1/400, 1/800 and 1/1,600 in sample diluting buffer to generate the standards which corresponded to sample properdin concentrations of 17.5 µg/mL, 35 µg/mL, 70 µg/mL, 140 µg/mL and 280 µ/mL at a dilution of 1/400.

Following overnight incubation the antibody coating solution was discarded and the wells washed 3 times with 200 µL washing buffer (see Appendix E). The inner wells of the plate were loaded with 100 µL/well of blank (sample diluting buffer), standard, QC and test plasma samples in duplicate and incubated at RT on a plate shaker at 400 rpm for 2 hours. All samples pertaining to an individual patient were analysed on the same plate. The contents of the wells were discarded prior to washing 3 times with 200 µL washing buffer. The wells were coated with 100 µL/well of biotinylated anti-human properdin monoclonal antibody (Thermo Scientific) diluted 1/5,000 with diluting buffer and incubated at RT for 2 hours on a plate shaker at 400 rpm. The contents of the wells were then discarded and the plates washed 3 times with 200 µL washing buffer prior to adding 100 µL/well streptavidin-alkaline phosphatase (Sigma), diluted 1/500 with diluting buffer, and the plate incubated at RT on a plate shaker at 400 rpm for 1 hour. During this incubation the substrate solution was made by dissolving 3 tablets of p-nitrophenol phosphate tablets (Sigma) in 15 mL of 1 M diethanolamine solution (see Appendix E). The contents of the wells were then discarded prior to washing 3 times with 200 µL washing buffer and 100 µL of substrate solution was added to each column at 10 second intervals prior to placing the pate on a plate shaker at 400 rpm. Once the wells had turned yellow, after approximately 30 minutes, the reaction was stopped by the addition of 100 µL 4 M NaOH to each well by columns at 10 second intervals. The plate was then sealed and placed on a plate shaker for 10 minutes prior to reading the absorbance on a MRX microplate reader at 405 nm with a reference filter of 540 nm. The concentration of properdin in each sample was interpolated using the Revelation software package by estimating the best fit cubic regression curve through the standards. Each sample was analysed in duplicate and any pair with a CoV of greater than 10% repeated. The intra- and inter- assay CoVs for the assay were 15.4% and 18.8% respectively which, although greater than the commonly accepted values of 5% and 10%, were similar to the manufacturer quoted CoVs for the commercial kits available at the time (Antibody Shop®, Denmark, March 2012).

3.14 Factor D ELISA

3.14.1 Development and optimisation of factor D ELISA

A search of the catalogues of the main antibody suppliers led to the identification of an antibody pair which were recommended for use in ELISAs for quantifying human factor D. These antibodies were an anti-human complement factor D monoclonal antibody (Santa Cruz Biotechnology Inc.) (coating antibody) and a biotinylated anti-complement factor D

monoclonal antibody (Thermo Scientific) (conjugated antibody). The initial step was to determine the optimal concentration of these two antibodies.

A 96-well microtitre plate was coated with 100 µL/well of coating antibody at concentrations of 1/250, 1/500, 1/1,000 and 1/2,000 and placed on a plate shaker at 4 ^{o}C overnight. The next day, after washing, the wells were incubated for 1 hour with blocking buffer (see Appendix E). The wells were then washed and the plate loaded with blanks (dilution buffer) and pooled plasma at a dilution of 1/100. After incubation for 1 hour and a subsequent plate wash, the plate was loaded with 100 µL/well of coating antibody at concentrations of 1/1,000, 1/2,000, 1/3,000 and 1/4,000. After a further 1 hour incubation the plate was washed and loaded with 100 µL/well of streptavidin-alkaline phosphatise (Sigma) at a dilution of 1/500 and incubated for a further 1 hour. After a final wash the plate was developed with p-nitrophenol phosphate substrate in 1 M diethanolamine (final concentration 1 mg/mL) and the reaction stopped by the addition of 4 M NaOH. Absorbance was then measured at 405 nm with a 540 nm reference filter using an MRX microplate reader - see Figure 15. On the basis of this assay, it was decided to use a coating antibody concentration of 1/500 and a detection antibody concentration of 1/4,000.

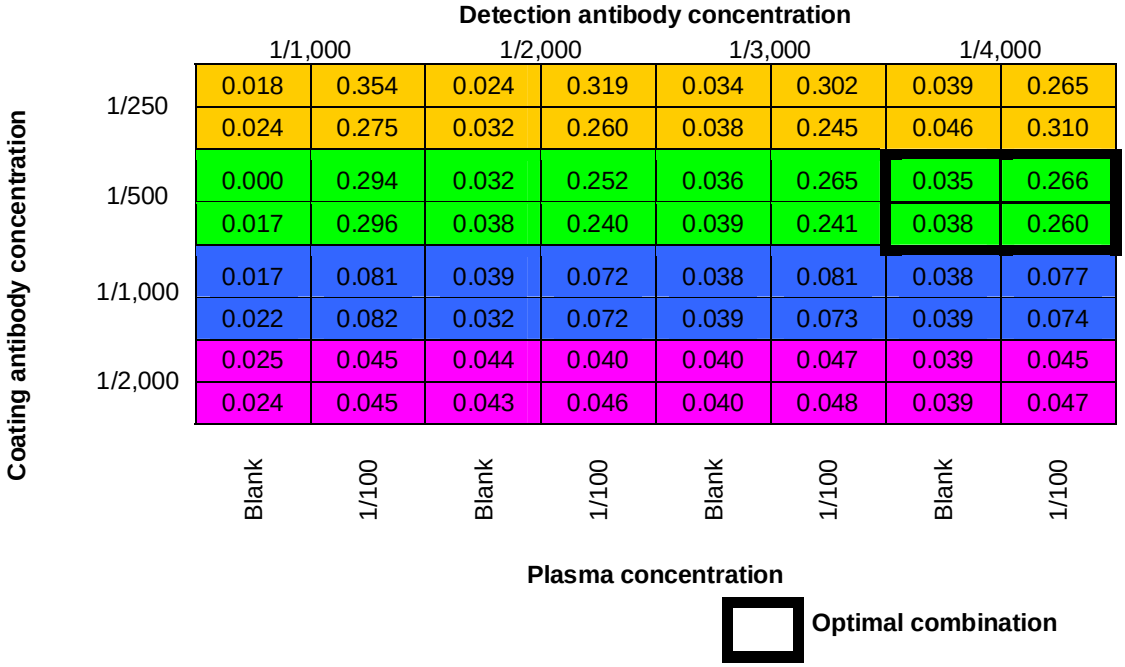


Figure 15 - Factor D optimisation assay to determine the optimal dilutions of coating and detection antibodies. From this assay, the optimal antibody concentrations were 1/500 for the coating antibody and 1/4,000 for the detection antibody.

To determine the optimal sample dilution an aliquot of the pooled plasma was diluted as follows: 1/5, 1/10, 1/20, 1/40, 1/80 and 1/160. A graph of absorbance against

concentration, plotted on a logarithmic abscissa, and a best fit curve through the data points is shown in Figure 16. It was decided to use a plasma dilution of 1/40 as, owing to the higher levels of factor D in HD patients [228], it was anticipated that the factor D levels in the majority of the study samples would fall on the linear portion of the standard curve.

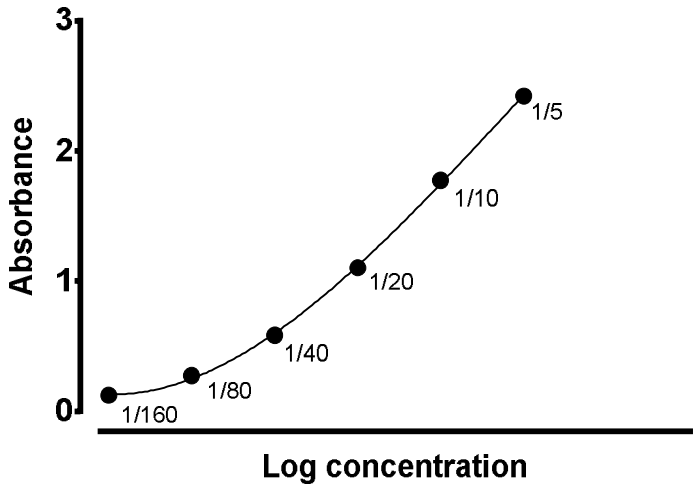


Figure 16 - Example standard curve for the factor D ELISA. Standards were generated by serial dilution of pooled plasma as labelled.

The next step was to determine the effects of increasing the incubation times with the antigen and conjugated antibody from 1 to 2 hours. For these experiments a pooled plasma sample was diluted: 1/10, 1/20, 1/40, 1/80 and 1/100. In addition, an aliquot of purified factor D was available (concentration unknown) and this was analysed undiluted alongside the diluted plasma samples. Experiments comparing 1 hour with 2 hour incubation times for the antigen and conjugated antibody were compared. Both assays were stopped after 20 minutes incubation with p-nitrophenol phosphate substrate by the addition of 100 µL/well of 4M NaOH and the results are shown in Figure 17. From these assays it can be seen that the antibodies were detecting factor D, as the wells incubated with the purified factor D returned a high signal, and lengthening the incubation times from 1 to 2 hours increased the well absorbancies. It was therefore decided to increase the incubation periods with both the antigen and conjugated antibody to 2 hours.

Next, experiments comparing the assay performance with and without the blocking step were undertaken in which half of the plate was incubated with blocking buffer and half of the plate was incubated with diluting buffer to act as control. The results of these experiments suggested that the blocking step had no significant impact on the final well absorbancies and it was therefore omitted. The SC5b-9 ELISA was found to be improved by analysing samples diluted in EDTA-containing buffer (see section 3.15.1) to limit *in vitro*

complement activation. The performance of the factor D ELISA was compared using EDTA and non-EDTA containing buffers and was found to be unaffected. It was decided to use the EDTA-containing buffer in order that the properdin, factor D and SC5b-9 ELISAs could be performed by serially diluting one aliquot of study plasma.

	Blank	1/10	1/20	1/40	1/80	1/100	Factor D
1 hour incubations	-0.024	1.179	0.672	0.380	0.141	0.113	0.826
	0.011	1.142	0.696	0.393	0.121	0.121	0.775
2 hour incubations	0.017	2.199	1.416	0.755	0.314	0.248	1.203
	0.020	2.049	1.344	0.737	0.305	0.242	1.103

Figure 17 - Factor D optimisation assay examining the effects of sample dilution and increased incubation times with the antigen and detection antibody

To determine the concentration of factor D in the pooled plasma sample used to generate the standards, calibration assays were performed using purified factor D (Quidel). Standards were generated using the purified factor D corresponding to factor D concentrations of 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL at a dilution of 1/40. The concentration of factor D in the pooled plasma, diluted 1/10, 1/20, 1/40, 1/80 and 1/160, was calculated by averaging data from two sets of dilutions in two separate assays (2.13 µg/mL). Spiking assays were performed in which the equivalent of 2 µg/mL, 4 µg/mL and 6 µg/mL of purfied factor D, at a dilution of 1/40, were added to pooled plasma. The assays were performed in duplicate and the average recovery of factor D was 88%.

3.14.2 Final factor D ELISA protocol

On the day prior to performing the assay, the wells of a 96-well microtitre plate were coated with 100 µL of anti-human complement factor D monoclonal antibody diluted 1/500 with coating buffer. The plates were sealed and placed on a plate shaker at 400 rpm and incubated at 4 ^{o}C overnight. On the day of the assay the plate and reagents were brought to RT. Frozen EDTA test plasma and QC samples, stored at -80 ^{o}C prior to assay, were defrosted in a water bath pre-warmed to 37 ^{o}C and diluted 1/40 in sample diluting buffer using a Microlab 500 diluter. The plate contents were discarded and the wells washed 3 times with 200 µL washing buffer. The wells were loaded with 100 µL of blanks (sample diluting buffer), standards, QC's and samples and the plate sealed and placed on a plate

shaker at 400 rpm and incubated at RT for 2 hours. All samples pertaining to an individual patient were analysed on the same plate. The contents of the wells were discarded and the wells washed 3 times with 200 µL washing buffer. Detection antibody (biotinylated anti-complement factor D) was diluted 1/4,000 in diluting buffer and 100 µL added to each well. The plates were sealed and placed on a plate shaker at 400 rpm and incubated at RT for 2 hours. The contents of the wells were then discarded and the wells washed 3 times with 200 µL washing buffer. The wells were loaded with 100 µL streptavidin-alkaline phosphatase, diluted 1/500 with diluting buffer, and the plate sealed and placed on a plate shaker at 400 rpm and incubated at RT for 1 hour.

During this incubation the substrate solution was made by dissolving 3 tablets of p-nitrophenol phosphate tablets in 15 mL 1 M diethanolamine solution. The contents of the wells were discarded prior to washing 3 times with 200 µL washing buffer. To develop the plate, 100 µL of substrate solution was added to each column at 10 second intervals prior to placing the plate on a plate shaker at 400 rpm. After 30 minutes the reaction was stopped by the addition of 100 µL 4 M NaOH to each well by columns at 10 second intervals. The plate was sealed and placed on a plate shaker for 10 minutes prior to reading the absorbance on a MRX microplate reader at 405 nm with a reference filter of 540 nm. The concentration of factor D in each sample was calculated using the Revelation software package by estimating the best fit cubic regression curve through the standards. Each sample was analysed in duplicate and any sample pair with a CoV of greater than 10% was repeated. For samples with factor D levels greater than the highest standard, a further assay was performed using a fresh serum aliquot analysed at a higher dilution. The intra- and inter- assay CoVs for the assay were 3.6% and 9.6% respectively.

3.15 SC5b-9 ELISA

3.15.1 Development and optimisation of SC5b-9 ELISA

The starting point of the SC5b-9 ELISA development was the identification of a suitable antibody pair. The capture antibody used was a monoclonal mouse antibody (aE11, Santa Cruz Biotechnology Inc.), originally developed by Mollnes *et al.* [912], and subsequently used in ELISAs for measuring SC5b-9 levels in human plasma [913]. This antibody binds to a neoantigen formed on the complement component C9 when it is incorporated into the SC5b-9 complex which is not present in uncomplexed C9. The conjugated antibody used for detection was a biotinylated anti-C6 monoclonal antibody (Quidel Corporation) as

recommended by Mollnes (personal communication, 2011). The specificity of using this approach to detect SC5b-9 has previously been demonstrated by Mollnes *et al*. [912].

To determine the optimal antibody dilutions a 96-well microtitre plate was coated with 100 µL/well of coating antibody at dilutions of 1/1,000, 1/2,000 and 1/3,000 and placed on a plate shaker at 400 rpm and incubated at 4 ^{o}C overnight. The next day the plate and all reagents were brought to RT. After discarding the contents of the plate and washing, the wells were incubated for 1 hour with blocking buffer (see Appendix E). The wells were then washed and loaded with 100 µL/well of blanks (dilution buffer) and pooled plasma, collected in iced citrate, diluted 1/10. After 1 hour incubation the plate was washed again and loaded with 100 µL/well of conjugated antibody at concentrations of 1/1,000, 1/2,000 and 1/3,000. After a further one hour incubation and subsequent plate wash, each well was loaded with 100 µL of streptavidin-alkaline phosphatase at a dilution of 1/500 and incubated for one hour. After a final wash the plate was developed with p-nitrophenol phosphate substrate in 1 M diethanolamine (final concentration 1 mg/mL) and the reaction stopped by the addition of 100 µL/well of 4 M NaOH after approximately 1 hour. Absorbance was measured at 405 nm with a 540 nm reference filter using an MRX microplate reader - see Figure 18.

Detection antibody concentration

Coating antibody concentration	1/1,000		1/2,000		1/3,000	
	Blank	1/10	Blank	1/10	Blank	1/10
1/1,000	-0.013	0.033	0.017	0.013	0.040	0.029
	-0.010	0.037	0.023	0.021	0.033	0.042
1/2,000	0.000	0.032	0.033	0.023	0.039	0.036
	0.009	0.034	0.033	0.023	0.040	0.039
1/3,000	0.010	0.037	0.037	0.034	0.043	0.043
	0.012	0.040	0.043	0.034	0.043	0.043

Plasma concentration

Figure 18 - SC5b-9 optimisation assay to determine optimal concentration of coating and detection antibody using 1 hour incubations. There was a low signal to noise ratio so the experiment was repeated using greater antibody concentrations.

The signal to noise ratio using these concentrations of plasma and antibodies was low, so a similar experiment was repeated using greater antibody concentrations of 1/250, 1/500 and 1/1,000 and plasma dilutions of 1/5 and 1/10. Furthermore the incubation times for the antigen and the conjugated antibody were both increased to 2 hours; the results of this assay are shown in Figure 19. From these two experiments it was decided to use a coating antibody concentration of 1/500 and a detection antibody concentration of 1/1,000, in conjunction with the longer incubation times, as this combination seemed to be the best compromise between greater signal to noise ratio and lowest antibody concentration and hence costs.

Detection antibody concentration

Coating antibody concentration	1/250			1/500			1/1,000		
	Blank	1/10	1/5	Blank	1/10	1/5	Blank	1/10	1/5
1/250	0.010	0.083	0.113	-0.009	0.081	0.125	0.038	0.087	0.122
	-0.004	0.086	0.123	0.015	0.089	0.128	0.041	0.088	0.147
1/500	0.007	0.135	0.199	0.027	0.143	0.215	0.047	0.137	0.195
	0.008	0.134	0.214	0.029	0.144	0.211	0.040	0.134	0.194
1/1,000	0.024	0.128	0.148	0.042	0.128	0.168	0.038	0.121	0.140
	0.032	0.132	0.149	0.040	0.143	0.164	0.041	0.107	0.133

Plasma concentration

Optimal combination

Figure 19 - SC5b-9 optimisation assay to determine optimal dilutions of coating and detection antibodies using 2 hour incubations. The optimal dilutions were 1/500 for the coating antibody and 1/1,000 for the detection antibody.

The next steps in the assay development were to determine the optimal sample dilution and to generate a standard curve which could be calibrated and used to determine the concentration of SC5b-9 in the study samples. Given that SC5b-9 is only present in low concentrations in serum, it was necessary to generate a sample standard with higher levels of SC5b-9 which, when diluted, could be used to generate a standard curve. To achieve this, a pool of fresh serum was obtained and incubated with 10 mg/mL of Zymosan A (Sigma), a glucan prepared from yeast cell walls which is a potent activator of complement [913], for 4 hours at 37 ^{o}C with frequent mixing. The specimen was then centrifuged and aliquots of the zymosan-activated serum (ZAS) supernatant were snap

frozen in liquid nitrogen and stored at -80 ^{o}C prior to use. Aliquots of the serum were also snap frozen in liquid nitrogen without ZAS activation to serve as QC samples.

Following overnight incubation with coating antibody at a concentration of 1/500, a microtitre plate was loaded with ZAS, serially diluted as follows: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1,024, 1/2,048, 1/4,096, 1/8,192 and 1/16,384, and with pooled serum samples diluted 1/2, 1/5 and 1/10. After a 2 hour incubation the plates were washed, conjugated antibody added at a dilution of 1/1,000, and the plates incubated for a further 2 hours; the remainder of the assay was carried out as detailed previously. The absorbancies in the wells containing the lower dilutions of ZAS, i.e. 1/1 to 1/32, exceed the maximum absorbance of the plate reader and were not quantified. A graph of the absorbance against concentration, plotted on a logarithmic abscissa, for the remaining diluted ZAS samples and a best fit curve through the data points is shown in Figure 20. From this graph it can be seen that the sample dilutions analysed provided broad spectrum coverage across the absorbance spectrum for the plate reader and that there was a flattening off of the curve at higher dilutions. In the same assay, pooled serum samples were analysed at dilutions of 1/2, 1/5 and 1/10 with mean absorbancies of 0.732 absorbance units (au), 0.553 au and 0.365 au respectively. Taking these findings together suggested that using ZAS diluted in the range 1/256 - 1/16,384 would provide adequate coverage of the SC5b-9 concentrations in the pooled serum sample diluted in the range 1/2 - 1/10.

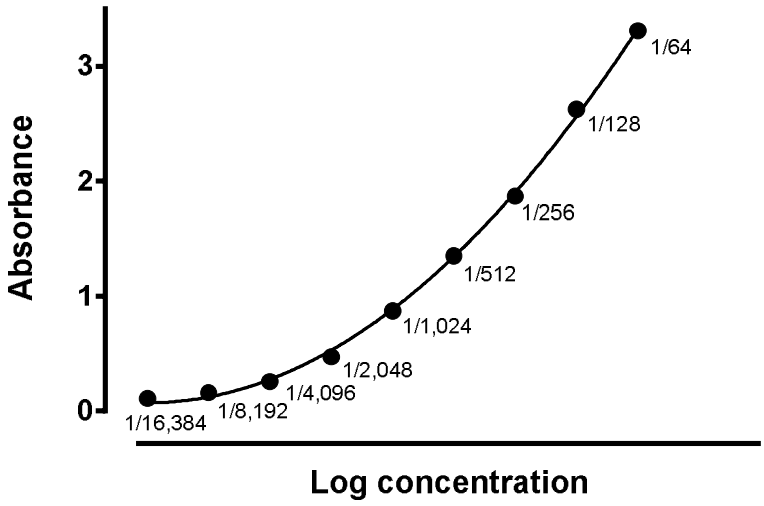


Figure 20 - Example standard curve using serially diluted zymogen-activated serum sample. At higher dilutions, the best fit line through the diluted zymogen-activated serum samples can be seen to flatten off.

The next optimisation assays performed were designed to establish the optimal time to read the plates after the addition of the the p-nitrophenol phosphate substrate. In these experiments, the concentration of SC5b-9 in the serum samples was assigned an arbitrary value and standards were made by diluting the ZAS as follows: 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400 and 1/12,800. The ELISA was carried out as detailed above and, following the addition of the p-nitrophenol phosphate substrate and prior to the addition of 4 M NaOH stopping solution, the plate was read after 30, 40, 50 and 60 minutes incubation. The results of these experiments, shown in Figure 21, revealed that the slope of the graph increased, particularly at higher concentrations of SC5b-9, with increasing incubation times thus improving the sensitivity of the assay to detect smaller differences in SC5b-9 concentrations. It was therefore decided to develop the plates for 60 minutes with the p-nitrophenol phosphate substrate prior to reading.

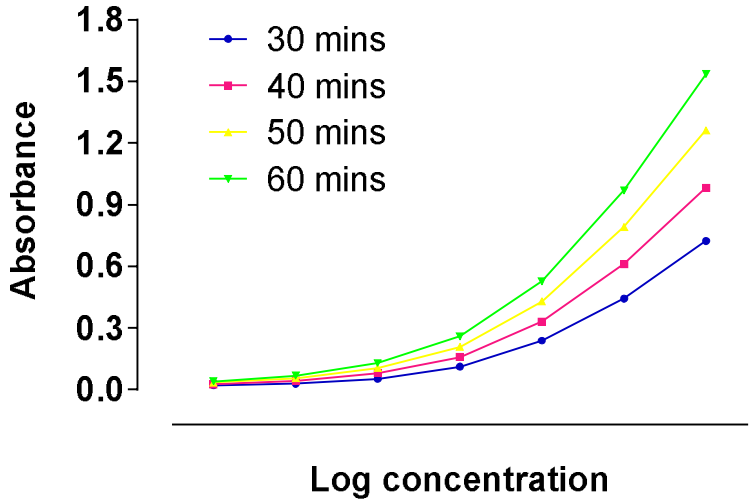


Figure 21 - Graph of absorbance against concentration for the zymosan activated serum standard curve following incubation with the substrate for 30, 40, 50 and 60 minutes. The slope of the graph, particularly at higher SC5b-9 concentrations, increased with the length of incubation time.

Experiments comparing the assay performance with and without the blocking step were undertaken. The results suggested the blocking step had no significant impact on the results and it was therefore omitted. Given the potential for ongoing *in vitro* complement activation following thawing of the sample aliquots prior to analysis, the effect of adding EDTA to the sample diluting buffer was investigated. (The presence of EDTA inhibits complement by chelating divalent metal ions, principally calcium and magnesium, which are required for complement activation [914]). This experiment was performed by loading half of an ELISA plate with a ZAS standard curve and normal pool serum (diluted 1/4) with EDTA containing buffer (10 mM) and the other half with the same samples diluted in a

similar buffer without EDTA. Again, after assigning an arbitrary concentration to the ZAS pool, the mean calculated SC5b-9 levels were 26.5% higher in the non-EDTA diluted samples suggesting ongoing *in vitro* complement activation. It was therefore decided to use an EDTA-containing buffer for sample dilution.

To determine the concentration of SC5b-9 in the ZAS, an arbitrary concentration was assigned to the ZAS pool and 36 samples, previously quantified for SC5b-9 using a commercial Quidel ELISA kit and spanning a wide concentration range (11.6 ng/mL to 525 ng/mL), were analysed on 2 separate assays. An average value for the concentration of SC5b-9 in the ZAS was then calculated (220 mg/mL). To examine the ability of the assay to detect differences in the SC5b-9 concentrations, in the absence of purified SC5b-9, two samples with differing levels of SC5b-9 were quantified for SC5b-9 and mixed in the ratios 25:75, 50:50, and 75:25. The SC5b-9 concentrations in each of the mixtures were measured and found to be 91.5%, 95.0% and 97.1% of the predicted concentrations respectively suggesting the ELISA was able to differentiate samples on the basis of SC5b-9 concentration. As the standard curve flattened off at low concentrations of SC5b-9, it was decided to assess the effects of analysing samples at lower dilutions i.e. 1/2. Data from these experiments suggested the changes in concentration levels at these lower dilutions were markedly non-linear therefore no samples were analysed at a dilution of less than 1/4.

3.15.2 Final SC5b-9 ELISA protocol

On the day prior to performing the assays the wells of a 96-well microtitre plate were coated with 100 µL of anti-human complement C9 monoclonal antibody (aE11) diluted 1/500 in coating buffer. The plates were then sealed and placed on a plate shaker at 400 rpm and incubated at 4 ^{o}C overnight. On the day of the assay the plate and reagents were brought to RT. Frozen EDTA-plasma samples, stored at -80 ^{o}C prior to assay, were defrosted in a water bath pre-warmed to 37 ^{o}C and diluted 1/4 in sample diluting buffer using a Microlab 500 diluter. An aliquot of ZAS was diluted with diluting buffer to make standards as follows: 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400, 1/12,800, 1/25,600. These corresponded to sample concentrations of SC5b-9 of 2200 ng/mL, 1100 ng/mL, 550 ng/mL, 275 ng/mL, 137.5 ng/mL, 68.8 ng/mL and 34.4 ng/mL respectively after adjusting for sample dilution. A fresh aliquot of pooled serum (high QC) and plasma (low QC) were diluted 1/4 and analysed on every plate.

On the day of the assay, the contents of the wells were discarded and the wells washed 3 times with 200 µL washing buffer. The wells were loaded with 100 µL of blanks (sample diluting buffer), standards, QC's and samples and the plate sealed and placed on a plate shaker at 400 rpm and incubated at RT for 2 hours. All samples pertaining to an individual patient were analysed on the same plate. The contents of the wells were discarded and the wells washed 3 times with 200 µL washing buffer. Conjugated antibody (biotinylated anti-human C6) was diluted 1/1,000 in diluting buffer and 100 µL added to each well. The plates were sealed and placed on a plate shaker at 400 rpm and incubated at RT for 2 hours. The contents of the wells were discarded and the wells washed 3 times with 200 µL washing buffer. The wells were loaded with 100 µL streptavidin-alkaline phosphatase (diluted 1/500 with diluting buffer) and the plate sealed and placed on a plate shaker at 400 rpm and incubated at RT for 1 hour.

During this incubation the substrate solution was made by dissolving 3 tablets of p-nitrophenol phosphate in 15 mL 1 M diethanolamine solution. The contents of the wells were discarded prior to washing 3 times with 200 µL washing buffer. To develop the plate 100 µL of substrate solution was added to each column at 10 second intervals prior to placing the pate on a plate shaker at 400 rpm. After 1 hour the reaction was stopped by the addition of 100 µL 4 M NaOH to each well by columns at 10 second intervals. The plate was placed on a plate shaker for 10 minutes prior to reading the absorbance on a MRX microplate reader at 405 nm with a reference filter of 540 nm. The concentration of SC5b-9 in each sample was calculated using the Revelation software package by estimating the best fit cubic regression curve through the standards. Each sample was analysed in duplicate and any pair with a CoV of greater that 10% repeated. Any sample with a SC5b-9 below the apparent lower limit of detection for the assay were assigned a concentration of 34.4 ng/mL - the lowest standard. For samples with SC5b-9 levels above the level of the standard curve, a fresh aliquot of serum was analysed at a greater dilution than 1/4. The intra- and inter- assay CoVs for the assay were 5.1% and 9.3% respectively.

3.16 Turbidimetric fibrin clot structure and function assays

Turbidimetric techniques were used to measure parameters related to the formation and breakdown of plasma-derived fibrin clots *in vitro* using assays developed for high throughput analyses as previously reported [721] and detailed below. All assays were performed using fresh frozen citrated plasma samples.

3.16.1 Turbidimetric clotting assay

In 96-well plates (Greiner), 25 µL plasma and 75 µL Tris-buffer were combined in each well and clot formation initiated by the addition of 50 µL of an activation mix comprising human thrombin (Calbiochem; final concentration 0.03 U/ml) and calcium chloride (final concentration 7.5 mmol/L) in Tris-buffer. The activation mix was added to columns of the 96-well plate on a plate shaker at intervals of 10 seconds. When the activation mix had been added to all of the wells, the plate was immediately transferred to an ELx-808 plate reader (BioTek Instruments) preheated to 32°C and the absorbance measured at 340 nm every 13 seconds for 60 minutes. The temperature of 32 °C was chosen as previous work demonstrated the formation of bubbles at 37 °C which interfered with assay interpretation (Dr Angela Carter, Personal communication). The time delay between adding the activation mix and transferring the plate to the plate reader was recorded and subsequent results adjusted accordingly. The resulting time and absorbance data were saved (KC4 software, BioTek Instruments) and imported into a spreadsheet (Microsoft Excel, Microsoft Corporation, USA). The raw data were analysed using bespoke software developed by Mr Simon Davy (School of Computing, University of Leeds) [721]. A diagram of the typical absorbance-time graph obtained from this software is illustrated in Figure 22, along with the calculated turbidimetric clotting assay parameters: lag time (Lag_C), maximum absorbance ($MaxAbs_C$) and clotting rate (CR_C).

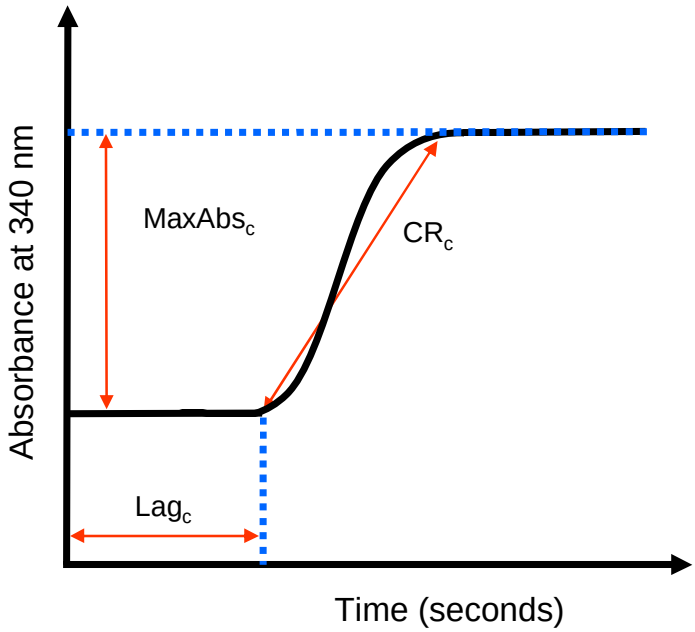


Figure 22 - Clotting assay parameters. Graphical representation of a typical absorbance / time graph obtained from the clotting assay illustrating the measured parameters: maximum absorbace ($MaxAbs_c$), lag time (Lag_c) and clot rate (CR_c).

The lag time was defined as the time between the addition of the activation mix and the time immediately prior to the exponential increase in absorbance. The maximum absorbance was representative of the final clot density and, for the purposes of these analyses, was defined as the absorbance value that remained stable for at least 3 readings (i.e. 39 seconds) after the lag time, corrected for the lag absorbance. A crude estimate of the clot rate was obtained by dividing the maximum absorbance by the time between the end of the lag phase and the point at which maximum absorbance was obtained. The intra- and inter-assay CoVs respectively for each of these variables were: lag time: 5.4% and 12.3%, maximum absorbance: 2.0% and 5.3% and clot rate: 4.9% and 16.8%.

3.16.2 Turbidimetric fibrinolysis assay

The method for the fibrinolysis assay was similar to the clotting assay with the addition of 12.5 ng tPA (Technoclone) per well in the Tris-buffer (final concentration 83 ng/mL). The tPA containing buffer was added to columns of a microtitre plate on a plate shaker, pre-loaded with 25 µL plasma, at 10 second intervals. After exactly 3 minutes, 50 µL of activation mix (see section 3.16.1) was added to each column of the plate at 10 second intervals. After the activation mix had been added to all of the wells the plate was transferred to an ELx-808 plate reader, pre-heated to 32 ^{o}C, and the absorbency measured at 340 nm every 13 seconds for 60 minutes. After one hour the frequency of the absorbency measurements was reduced to every 2 minutes for a further 9 hours. The time delay between adding the activation mix and the samples being read by the plate reader was recorded and subsequent results adjusted accordingly. Analyses of the raw data were undertaken using the bespoke software described in section 3.16.1. In addition to the parameters described in 3.16.1, several fibrinolysis parameters were also determined as shown in Figure 23.

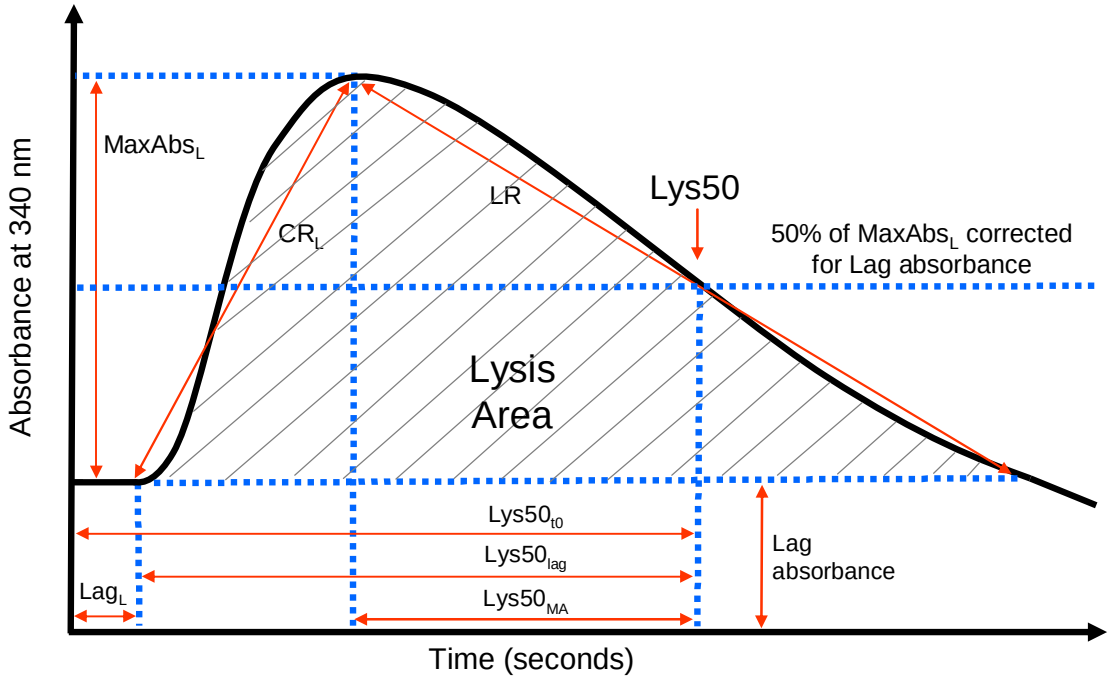


Figure 23 - Fibrinolysis assay parameters. Graphical representation of a typical absorbance / time graph obtained from the fibrinolysis assay illustrating the measured parameters: lag time (lag_L), maximum absorbance ($MaxAbs_L$), clot rate (CR_L), $Lys50_{t0}$, $Lys50_{lag}$ and $Lys50_{MA}$ times, lysis rate (LR) and lysis area.

The 50% lysis point (Lys50) was defined as the time at which the absorbance dropped to half the maximum absorbance after correcting for the lag absorbance. Three measures of clot fibrinolysis were determined as follows: $Lys50_{t0}$ - the time from initiation of clot formation to Lys50; $Lys50_{lag}$: the time from the end of the lag phase to Lys50 and $Lys50_{MA}$: the time between reaching maximum absorbance and Lys50. The lysis area was the area under the curve, corrected for lag absorbance, taken from the end of the lag phase to the point where the absorbance again dropped to the lag absorbance value following clot lysis. A crude estimate of the fibrinolysis rate was calculated by dividing the maximum absorbance by the time between the maximum absorbance being reached and Lys50. The intra- and inter-assay CoVs for the variables calculated in the fibrinolysis assay were as follows: lag time: 4.5% and 10.8%, maximum absorbance : 2% and 10.9%, clot rate: 6% and 21.5%, $Lys50_{t0}$: 3.4% and 6.6%, $Lys50_{lag}$: 5.4% and 9.7%, $Lys50_{MA}$: 9.5% and 15.9%, lysis rate: 16.0% and 32.9% and Lysis area: 16.0% and 27.8%.

For both assays a sample of pooled plasma was analysed on every plate for quality assurance and to determine the intra-assay CoV. All samples were analysed in duplicate and a mean value for each variable calculated. Prior to accepting the calculated values for a given sample, the absorbance-time graphs were inspected visually to ensure the computer algorithm was interpreting the data correctly. Results from sample pairs with

greater than 10% discrepancy in the maximum absorbance or $Lys50_{t0}$ variables were disregarded and the assay repeated using another fresh frozen citrate plasma sample. These variables were chosen for internal quality control as previous experience with the assay had revealed them to be the most reproducible and informative measurements.

3.17 Statistics

3.17.1 Overview of statistical methods used

All variables were assessed for conformity to an approximate normal distribution through a combination of visually inspecting frequency histograms and Q-Q plots, and calculating the sample skewness and kurtosis. For variables which approximated a normal distribution parametric tests were used otherwise non-parametric testing was employed. Normally distributed variables were reported as the mean [±standard error of the mean(SEM)] and non-normally distributed variables values reported as median [inter-quartile range(IQR)] unless otherwise stated. All statistical analyses were performed using either Stata version 12.1 (StataCorp, Texas) or SPSS version 16.0 (IBM Corporation, New York) and graphs were drawn using GraphPad Prism version 6.02 (GraphPad Software Inc., California). A $p<0.05$ was considered significant.

3.17.2 Power calculations

Based on the standard deviation of the log transformed ERI at baseline for the study population, a sample size of 118 patients in each group would detect a 25% reduction in the ESA dose with a power of 80% at a significance level 5%. Based on the standard deviation and differences observed in the measured maximum absorbance in a previous study using the same turbidimetric clotting assay in subjects with and without the metabolic syndrome [721], a sample size of 50 in each group would have an 80% power to detect a 10 percent difference at the 5% level.

3.17.3 Baseline between-group comparisons

To examine for differences in the continuous variables between the study groups, pairwise testing using either an unpaired t-test or a Mann-Whitney U test was performed depending on the underlying distribution. Categorical variables were compared using a Chi-squared test or Fisher's exact test as appropriate.

3.17.4 Analysis of baseline data

Initially bivariate analyses were performed in which the Spearman's rank correlation coefficients for the independent variables and the dependant variable of interest were calculated looking for significant monotonic relationships. For normally distributed dependent variables, the effect of categorical independent variables were tested using either an independent t-test (2 groups) or one way analysis of variance (ANOVA) (>2 groups) with Bonferroni *post hoc* testing if the omnibus ANOVA test was significant. For non-normally distributed dependent variables, the corresponding statistical tests used were a Mann-Whitney U test (2 groups) or a Kruskal-Wallis test (>2 groups) followed by *post hoc* Mann-Whitney U testing with Bonferroni correction for multiple comparisons if the omnibus Kruskal-Wallis test was significant.

Given the large number of predictor variables, multiple regression analysis was performed to identify the independent predictors of the ERI, oxidative stress, inflammation and the fibrin clot parameters in the dataset. All predictor variables associated with the dependent variable at a significance level of $p<0.2$ in unadjusted analyses, as a conservative cut-off suggested by Altman [915] and others [916], were included in the initial regression model. Again, given the large number of predictor variables and the exploratory nature of these analyses, a backwards stepwise variable selection procedure was used to construct a more parsimonious regression model and to identify which were the key independent determinants of each of the variables studied. All models were assessed for multicollinearity by reviewing correlation matrices of the variables included in the final model and inspection of the variance inflation factors (VIFs). As regression analysis can be sensitive to outliers, the analyses were initially performed on the whole dataset. A scatter plot of the residuals versus fitted values was reviewed for each model looking for a homoscedastic distribution of the errors and for outlying data points. If the distribution plot of errors was heteroscedastic, the dependent variable was transformed and the analyses repeated. If the dataset appeared to include influential outlying cases, the analysis was repeated after excluding these cases as specified in the text. In these situations, an objective method for identifying cases with outlying values for the dependent variable was adopted. A data point was considered to be an outlier if it was outside of 3 standard deviations from the mean for variables, raw or transformed, which approximated a normal distribution or if it was less than 1.5 times the interquartile range below the 25^{th} percentile or greater than 1.5 times the interquartile range above the 75^{th} percentile for non-normally distributed variables.

A number of the samples had analyte levels outside of the assay range (i.e. fibrinogen, SC5b-9 and Ox-LDL); in these cases the corresponding upper or lower limit of the assay was substituted for samples that were above or below the assay range respectively. For these variables, testing for significant associations with the predictor variables at baseline was performed by dividing the dataset into quartiles, such that all of the low or high substituted values were grouped together. The value of each of the continuous predictor variables were then compared across the quartiles by performing an ANOVA or Kruskall-Wallis analysis with *post hoc* testing as already described for normally or non-normally distributed continuous variables respectively. Categorical variables were compared between quartiles using either a Chi-squared of Fishers exact test as appropriate. To examine for the independent predictors of these variables, ordinal logistic regression was employed. The quartiles were modelled using a backwards stepwise variable selection procedure with all of the predictor variables associated with the independent variable at a significance of $p<0.2$ included in the initial model as already described. All models were tested to ensure that the Brant test of parallel regression assumption was met.

3.17.5 Analysis of longitudinal data

Between groups comparisons at a given time point were performed using either the independent sample t-test or the Mann-Whitney U test depending on the distribution of the variable. Baseline and 12 month values were compared within groups using either the paired sample t-test or the Wilcoxon-signed ranks test for normally or non-normally distributed variables respectively. To further assess the influence of time and study group allocation on the dependant variable of interest, repeated measures ANOVA were performed on raw or transformed data which approximated a normal distribution. For variables which were not normally distributed, the following transformations were performed and the data re-examined for normality: logarithmic, reciprocal, square-root and exponential. Logarithmic transformations were not attempted on datasets which contained 0 (e.g. ERI). For variables which did not approximate a normal distribution, either untransformed or following transformation, changes over time were assessed using the non-parametric Friedman's test. To graph the changes in the variables across study vistis, plots of the mean and 95% confidence interval were used. In the case of transformed variables, the confidence intervals were determined on the transformed dataset and back transformed to construct the graphs as advocated by Bland and Altman [917]. For variables which did not approximate a normal distribution, either before or after transformation, box-and-whisker plots were constructed with the whiskers representing the 5^{th} and 95^{th} centiles.

3.17.6 Analysis of clinical outcome data

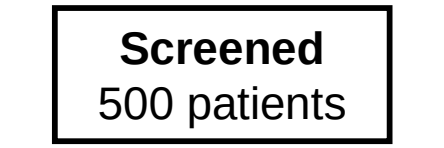
For each of the clinical outcome variables considered, i.e. mortality, CV events, infective episodes and dialysis access events, the associations with the categorical variables in the baseline dataset were explored by constructing Kaplan-Meier curves and calculating the logrank test statistic. The associations with the continuous variables were explored initially by comparing the event rates after dividing the study cohort into tertiles. This was undertaken to examine for non-linear associations which would invalidate Cox-regression analyses. Provided there was no evidence of a non-linear association, univariate Cox-regression analysis was then performed with each of the continuous variables to calculate the hazard ratio (HR) and 95% confidence interval. The effect of dialysis with the VE membrane was examined by constructing a Kaplan-Meier curve for the two study groups and calculating the logrank test statistic. All analyses were right-censored.

Chapter 4 : Patient characteristics and follow up

4.1 Patient recruitment, follow-up and baseline characteristics

A total of 500 patients were screened for study inclusion and 260 patients were enrolled as shown in Figure 24. The main reason for ineligibility was evidence of active inflammation at baseline and, of the eligible patients, the main reason for non-participation in the study was declined consent. Out of the 260 patients starting the study, 215 completed all 3 study visits (see Figure 25). The major reasons for study discontinuation were renal transplantation or death. Anaemia endpoint data were available for 220 patients (116 controls, 104 VE group) as 5 patients completed 12 months on the study but did not undergo the final 12-month blood sampling. Reasons for this were transplantation (n=2), death (n=1), withdrawal from dialysis (n=1) and protocol violation for more than two weeks dialysis at another centre (n=1).

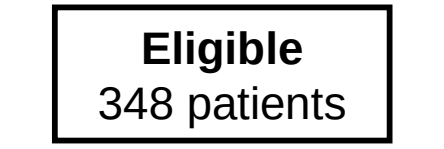
Following randomisation, a greater number of patients were allocated to the control arm of the study (137 vs 123). Patients were well matched in terms of demographics, dialysis parameters, medications and comorbidities with the exception of an excess of patients with diabetes (35% vs 23%, $p<0.05$) and a higher median post-dialysis weight (73.4 vs 69.7 kg, $p<0.05$) in the VE group (see Table 4). There were no significant differences in the biochemisty and lipid profiles between the study groups at baseline (see Table 5).



INELIGIBLE PATIENTS

Reason	*n*
High CRP / Inflammatory state	55
Large dialyser*	37
Not on x3/week dialysis schedule	27
On dialysis <3 months	12
Imminent switch of RRT modality planned	9
Frequent travel abroad	3
Unable to give consent	2
Short life expectancy	2
Poor compliance	2
Transfusion dependant	1
Intolerant of Rexeed dialysers	1
Lost to follow up	1
	152

*No vitamin E bonded equivalent
RRT: Renal replacement therapy



NOT ENTERED INTO STUDY

Reason	*n*
Declined consent	80
Died between consenting and starting study	6
Transplanted between screening and starting study	2
	88

Figure 24 - Patient recruitment and screening. Of the 500 patients screened, 260 were enrolled in the study.

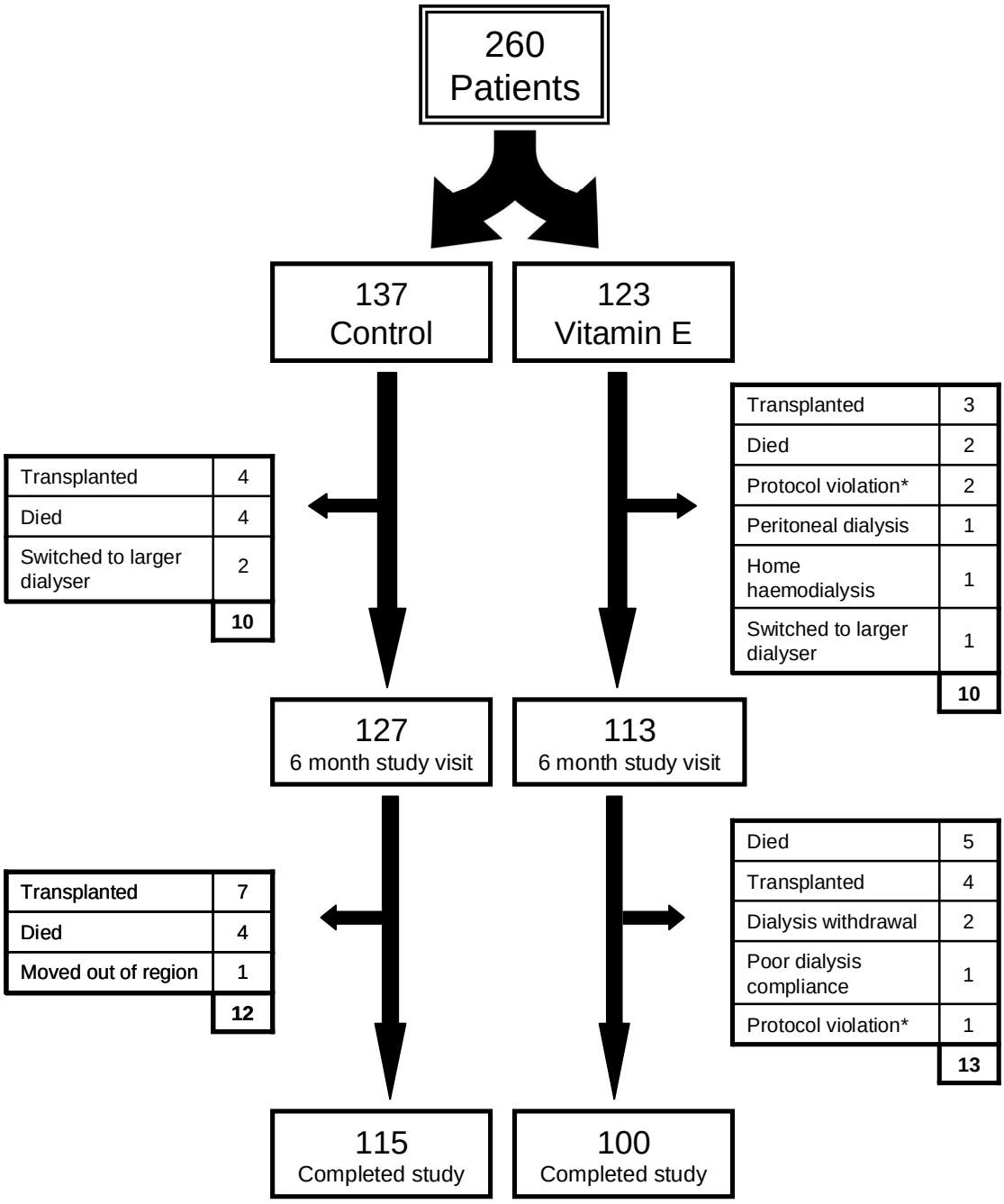


Figure 25 - Patients completing the study and reasons for discontinuation by treatment group. (*Patients dialysed at another centre for >2 weeks).

Table 4 - Comparison of baseline characteristics between study groups.

	Control	Vitamin E	p
N	137	123	
Sex			0.58
Male	80 (58%)	76 (62%)	
Female	57 (42%)	47 (38%)	
Age (yrs)	64.0 (±1.3)	62.6 (±1.5)	0.50
Ethnicity			0.96
White	106 (77%)	93 (76%)	
Asian	24 (18%)	22 (18%)	
Black	6 (4%)	7 (6%)	
Other	1 (1%)	1 (1%)	
Smoking history			0.21
Never smoked	56 (41%)	62 (50%)	
Current smoker	35 (26%)	22 (18%)	
Ex-Smoker	46 (34%)	39 (32%)	
Time on renal replacement therapy (yrs)	3.9 [1.8 – 7.6]	3.2 [1.2 – 6.6]	0.18
Dialysis access at baseline n (%)			0.13
Fistula	109 [80%]	109 [89%]	
Dialysis Catheter	25 [18%]	13 [11%]	
Graft	3 [2%]	1 [1%]	
Pre-dialysis systolic blood pressure (mmHg)	135 [±2.1]	139 [±2.2]	0.18
Pre-dialysis diastolic blood pressure (mmHg)	71 [±1.2]	72 [±1.2]	0.75
Post dialysis weight (kg)	69.7 [56.8 – 78.9]	73.4 [61.2 – 87.0]	0.03
Dialysis dose (urea reduction ratio)	0.76 [±0.01]	0.75 [±0.01]	0.30
Cause of ESRF n (%)			0.54
Diabetes	27 (19.7%)	30 (24.4%)	
Autosomal dominant polycystic kidney disease	9 (6.6%)	9 (7.3%)	
Chronic pyelonephritis	10 (7.3%)	8 (6.5%)	
Glomerulonephritis	30 (21.9%)	16 (13%)	
Hypertension	14 (10.2%)	10 (8.1%)	
Renal vascular disease	12 (8.8%)	8 (6.5%)	
Other	16 (11.7%)	19 (15.4%)	
Unknown	19 (13.9%)	23 (18.7%)	
Co-morbidity n (%)			
Diabetes	31 (22.6%)	43 (35.0%)	0.03
Ischaemic heart disease	43 (31.4%)	31 (25.2%)	0.27
Peripheral vascular disease	37 (27.0%)	34 (27.6%)	0.91
Left ventricular dysfunction	11 (8%)	13 (10.6%)	0.48
Malignancy	9 (6.6%)	8 (6.5%)	0.98
Systemic collagen disease	9 (6.6%)	3 (2.4%)	0.11
Other significant pathology	32 (23.4%)	24 (19.5%)	0.45
Overall Score	1 (0 – 2)	1 (0 – 2)	0.94
Drugs at baseline n (%)			
ACEi / A2RB	41 (29.9%)	42 (34.1%)	0.47
B-blockers	26 (19%)	29 (23.6%)	0.37
Statins	79 (57.7%)	68 (55.3%)	0.70
Aspirin	56 (40.9%)	61 (49.6%)	0.16
Clopidogrel	9 (6.6%)	11 (8.9%)	0.47
Dipyridamole	0	3 (2.4%)	0.07
Warfarin	12 (8.8%)	4 (3.3%)	0.07
Sulphonylureas	5 (3.6%)	8 (6.5%)	0.29
Insulin	26 (19%)	28 (22.8%)	0.45

Data presented as mean [±SEM] or median [IQR] unless stated. A2RB: Angiotensin II receptor blocker; ACEi: Angiotensin converting enzyme inhibitors.

Table 5 - Baseline biochemistry and lipid profiles.

	Control	Vitamin E	p
Biochemistry			
C-reactive protein (mg/L)	7.1 [2.9 - 14.4]	5.6 [1.6 - 17.9]	0.29
Albumin (g/L)	38 [±0.4]	38 [±0.4]	0.97
Calcium (mmol/L)	2.38 [±0.02]	2.39 [±0.02]	0.63
Phosphate (mmol/L)	1.49 [±0.04]	1.51 [±0.05]	0.75
Parathyroid hormone (pmol/L)	21 [12 - 39]	23 [9 - 44]	0.62
Bicarbonate (mmol/L)	21.9 [±0.2]	22.0 [±0.2]	0.81
HbA1C (%)	7.7 [±0.3]	7.4 [±0.2]	0.42
Lipid profile			
Cholesterol (mmol/L)	3.9 [±0.1]	4.0 [±0.1]	0.23
Low-density lipoprotein (mmol/L)	2.0 [±0.1]	2.2 [±0.1]	0.14
High-density lipoprotein (mmol/L)	1.2 [±0.0]	1.1 [±0.0]	0.56
Triglycerides (mmol/L)	1.4 [1.1 - 2.0]	1.4 [1 - 2.2]	0.94

Data presented as mean [±SEM] or median [IQR]

4.2 Dialyser adherence

Dialyser adherence was monitored throughout the study as detailed in section 3.7.3 and a summary of the dialyser monitoring checks for each dialysis unit by study arm is provided in Table 6. None of the units fell below the 95% adherence standard at any of the audit visits and the overall adherence in both treatment groups was 98.8%.

Table 6 - Results of dialyser adherence monitoring.

Dialysis unit	*%correct* (Number of sessions audited)		
	Control	***Vitamin E***	***TOTAL***
Frank Parsons Unit, Seacroft Hospital	*99.9%* (1411)	*97.4%* (2010)	*98.5%* (3421)
Ward 55, St James's Hospital	*99.9%* (1226)	*96.2%* (1129)	*98.0%* (2355)
Beeston Dialysis Unit	*100%* (1207)	*99.6%* (427)	*99.9%* (1634)
Dewsbury Dialysis Unit	*99.6%* (734)	*97.6%* (1137)	*98.3%* (1871)
Wakefield Dialysis Unit	*99.9%* (929)	*99.8%* (580)	*99.9%* (1509)
Huddersfield Dialysis Unit	*99.7%* (867)	*96.2%* (541)	*98.4%* (1408)
Halifax Dialysis Unit	*100%* (623)	*99.1%* (736)	*99.5%* (1359)
B-ward, Seacroft Hospital	*100%* (731)	*97.5%* (427)	*99.1%* (1158)
Ward 53, St James's Hospital	*100%* (60)	*96.3%* (116)	*97.8%* (176)
TOTAL	***99.9%* (7788)**	***97.6%* (7103)**	***98.8%* (14891)**

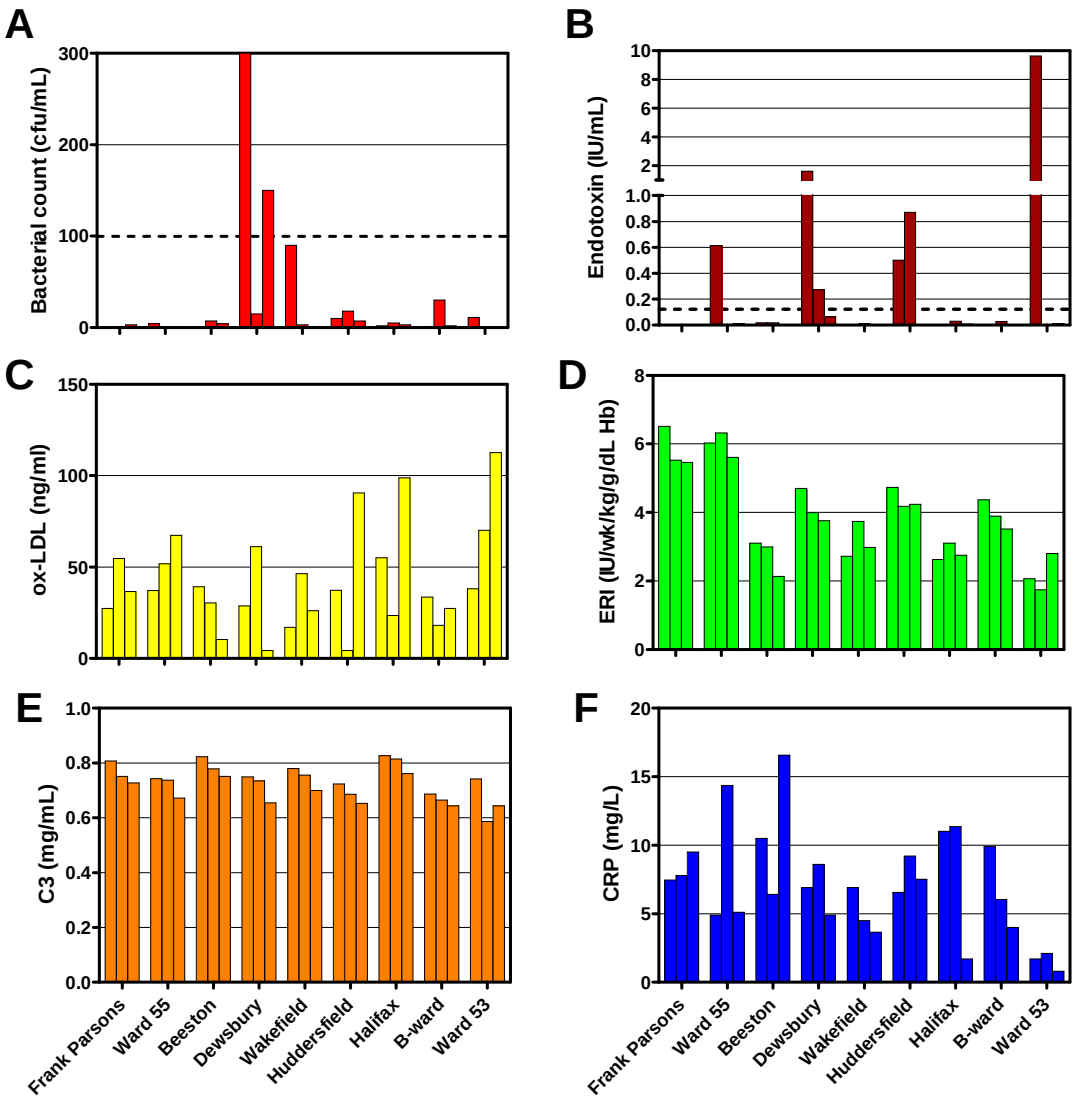
4.3 Water quality data

4.3.1 Microbiology data

As part of routine clinical care, the quality of the water used to generate the dialysate was monitored regularly at each dialysis unit; the results of these tests were obtained retrospectively after study completion. Figure 26 shows the bacterial growth and endotoxin count for each of the dialysis units at the time the study samples were taken, along with the median ERI, CRP and Ox-LDL levels and the mean C3 levels. It can be seen that there was significant variation between the units in terms of the water quality but this did not appear to be reflected in the ERI, CRP, Ox-LDL levels or C3 levels. Additionally, the bacterial count at Dewsbury at the time of the baseline and 12 month study visit, the endotoxin levels on wards 55 and 53 at baseline, and at Dewsbury and Huddersfield for both the baseline and 6-month study visits exceed the Quality of Dialysis Fluid for Haemodialysis and Related Therapies (BS ISO 11663_2009) recommended limits. (These limits are a bacterial count of <100 colony forming units per mL and an endotoxin concentration of <0.25 endotoxin units per mL). No water quality data were available from wards 55 and 53 at the time of the 6 month study visit as the main water treatment plant was being replaced and all patients were dialysed using individual reverse osmosis units fed from the main hospital water supply.

4.3.2 Chlorine levels

Figure 27 shows the results of the chlorine levels from the routine weekly water tests for the various units along with the ERI, haemoglobin and the bilirubin levels as a crude measure of haemolysis, the principal complication of excess chlorine in the dialysis water supply [918]. No data on chlorine levels were available for the 6-month sampling period at ward 55 or ward 53 as the dialysis unit was undergoing replacement of the water plant and all patients were dialysing via individual reverse osmosis units; data were also unavailable for the 12-month sampling period for the Wakefield dialysis patients. The Quality of Dialysis Fluid for Haemodialysis and Related Therapies (BS ISO 11663_2009) recommends a maximum concentration of chlorine in water used for dialysis is <0.1 mg/L. On 2 occasions the levels of chlorine in the dialysis water breached this standard: during the baseline sampling at Huddersfield and the 6-month sampling at B-ward. However, the levels were only marginally above the standard and not associated with any evidence of haemolysis on the basis of the parameters examined.



-- BS ISO 11663_2009 recommended limits: bacterial count <100 cfu/mL; endotoxin count <0.125 IU/mL

Figure 26 - Bacterial and endotoxin counts, Ox-LDL levels, ESA resistance index (ERI), C3 and CRP levels grouped by dialysis unit at the time of the baseline, 6- and 12-month study visits. The bacterial count at Dewsbury at the time of the baseline and 12 month study visit, the endotoxin levels on wards 55 and 53 at baseline and at Dewsbury and Huddersfield for both the baseline and 6-month study visit exceed the BS ISO 11663_2009 recommended limits.

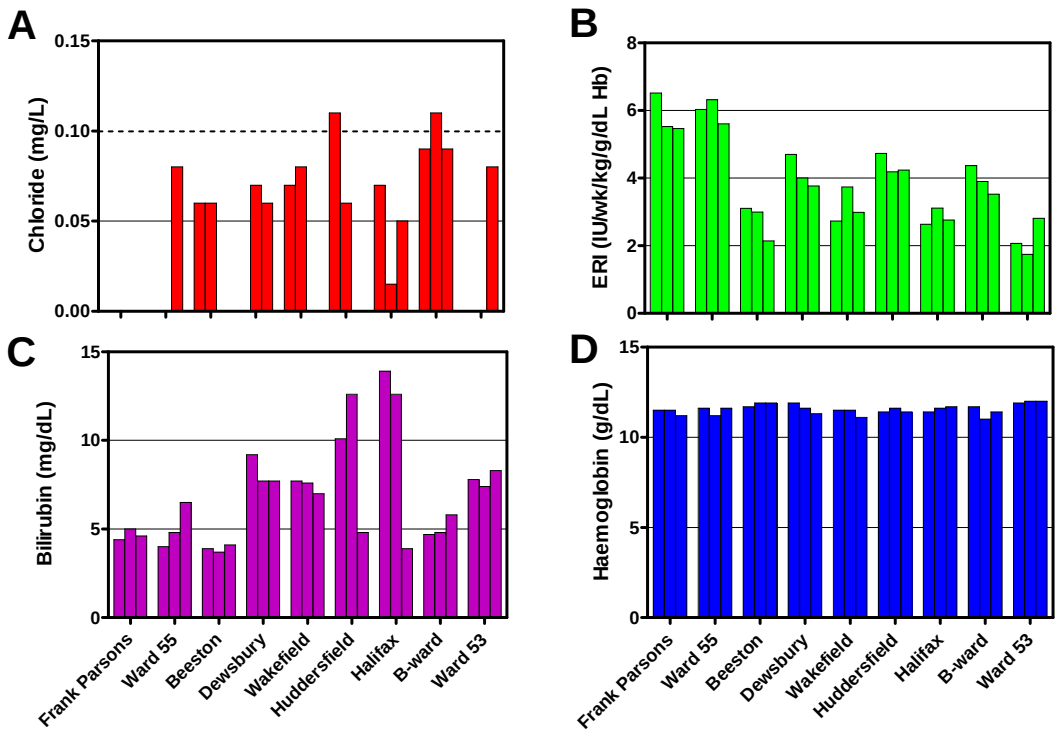


Figure 27 - Chloride levels, ESA resistance index (ERI), bilirubin and haemoglobin levels grouped by dialysis unit at the time of the baseline, 6- and 12-month study visits. At the time of the baseline sampling at Huddersfield and the 6-month sampling at B-ward the chlorine levels exceed the BS ISO 11663_2009 recommended standard.

4.4 Discussion

A total of 260 patients were recruited and 215 completed 12-months in the study and all 3 study visits. In terms of patient characteristics, both groups were well matched with the exception of an excess of patients with diabetes and a higher median post dialysis weight in the VE group. There were no significant inter-group differences in the baseline biochemical and lipid parameters measured. One of the main exclusion criteria was the presence of active inflammation at baseline, defined as a CRP > 50 mg/L as others have done [919]. This approach was taken as the levels of inflammation in patients with significant inflammation at baseline would be anticipated to reduce. It would therefore be difficult to discern in these patients if any reductions in inflammation were attributable to the use of the VE-bonded membrane or the results of changes to the factors which were contributing to the inflammation. Given that all of the endpoints in this study, namely ESA requirements, oxidative stress, fibrin clot structure and function and inflammation itself, would be influenced by the presence of active inflammation this approach was adopted.

The principal reasons for study discontinuation were patient death or renal transplantation and a detailed analysis of the patient outcomes are presented in Chapter 9.

The results of the dialyser adherence audits suggested a high level of overall compliance with the study protocol, thus permitting a meaningful evaluation of the VE membrane. The adherence was higher in the control group, as may have been anticipated, because the dialysers used in the control arm were the standard dialysers in use by the units, both prior to and during the study, hence there was a greater chance of a study patient being dialysed with a control membrane rather than vice versa. The quality of the water used for dialysis at the various units was broadly in keeping with the BS ISO 11663_2009 (Quality of dialysis fluid for haemodialysis and related therapies) recommended guidelines with the notable exceptions of high endotoxin levels in 3 of the units at various points during the study. However all LTHT dialysis machines were fitted with endotoxin filters and no temporal relationships between the excursions in endotoxin levels and either inflammatory markers or ERI were observed, suggesting no clinically relevant sequelae.

It can be seen that in the present study a large number of patients were recruited and followed for 12-months. Consequently this study allowed meaningful investigation into the determinants of ESA-resistance, oxidative stress, inflammation, fibrin clot structure and function and clinical outcomes in a prevalent, non-inflamed UK HD population in addition to examining the effects of HD with VE-bonded dialysis membranes on these parameters.

Chapter 5 : Anaemia

As discussed in section 1.3, anaemia is frequently encountered in HD patients and several studies have reported an association between greater ESA requirements and mortality [50-52]. It still remains unclear, however, whether this association is a consequence of the higher ESA doses themselves or the factors which render patients ESA resistant and hence requiring of higher ESA doses. Many of the factors which are known to increase ESA requirements, such as inflammation and oxidative stress, have also been implicated in the development of CV disease. Therefore interventions aimed at improving these factors, such as dialysis with a VE-bonded membrane in the present study, may both lower the ESA requirements, with their potential for harm and significant attendant healthcare costs, and improve patient outcomes. In this chapter, the significant determinants of ESA resistance in the study cohort at baseline were examined prior to evaluating the effects of 12 months HD with VE-coated polysulfone membranes.

5.1 Determinants of baseline ESA resistance index

Table 7 and Table 8 detail the results of the bivariate analyses examining for the significant determinants of ERI at baseline. The ERI was negatively correlated with length of time on renal replacement therapy and bicarbonate levels, and positively correlated with systolic blood pressure and CRP (see Table 7). The ERI was also higher in females, Blacks compared to Caucasians and in patients dialysing with CVCs (see Table 8). The ERI did not differ significantly when patients were compared under the broad categories of diabetes, ischaemic heart disease, malignancy, peripheral vascular disease or left ventricular dysfunction; similarly comorbidity score, as defined in section 3.7.1, was not correlated with ERI ($r=0.04$; $p=0.52$).

Table 7 – Determinants of ESA resistance index at baseline: continuous variables.

	n	*r*	*p*
Patient factors			
Age	260	0.02	0.80
Pre-dialysis systolic blood pressure	260	0.12	0.046
Pre-dialysis diastolic blood pressure	260	0.03	0.64
Dialysis factors			
Time on renal replacement therapy	260	-0.19	0.002
Urea reduction ratio	260	-0.11	0.08
Laboratory parameters			
Ferritin	260	0.08	0.22
CRP	260	0.15	0.02
Albumin	260	0.04	0.50
Cholesterol	259	0.03	0.69
High-density lipoprotein	259	-0.07	0.29
Low-density lipoprotein	254	0.00	1.0
Triglycerides	259	0.02	0.75
Bicarbonate	260	-0.14	0.02
Calcium	260	0.00	0.99
Phosphate	260	0.07	0.25
Parathyroid hormone	248	0.01	0.92
HbA1c	72	-0.03	0.79

r: Correlation coefficient

Multivariate regression analysis modelling the baseline ERI using a backwards stepwise variable selection procedure and the variables listed in Table 7 and Table 8 with a significance level of $p<0.2$, as described in section 3.17.4, was carried out. This indicated that an increased ERI was independently associated with higher CRP levels, female sex, lower dialysis dose and was higher in Blacks compared to Asians or Caucasians. The final model had an adjusted-R^2 of 0.12 indicating that the model explained 12% of the variance in the baseline ERI. Review of the residual versus fitted regression diagnostic plot, however, highlighted a number of cases with extreme values. A total of 9 cases fulfilled the *a priori* criteria for having an outlying high ERI value (see section 3.17.4); no cases fulfilled the criteria for having outlying low values. Repeating the modelling procedure after excluding these 9 cases indicated an independent positive association between the ERI and CRP levels, lower dialysis dose and female sex, but did not retain the variable coding for ethnicity. The adjusted-R^2 for this model was 0.06 indicating that this final model explained 6% of the variance in the baseline ERI for this subset of patients.

Table 8 - Determinants of ESA resistance index at baseline: categorical variables.

			n	ERI (IU/wk/kg/g/dL Hb) Median [IQR]	p
Patient factors:	Sex	Male	156	3.92 [2.02 - 7.50]	<0.05
		Female	104	5.19 [2.74 - 8.49]	
	Ethnicity	Caucasian	199	3.94 [2.11 - 7.36]	0.02†
		Asian	46	5.25 [2.73 - 7.67]	
		Black	13	7.82 [4.33 - 17.1]	
		Other*	2	8.42 [4.36 - 12.5]	
	Smoking history	Never	118	4.90 [2.70 - 7.86]	0.18
		Current	57	3.42 [2.03 - 6.27]	
		Ex-smoker	85	4.90 [2.14 - 7.86]	
	Dialysis access	Fistula	218	4.10 [2.12 - 7.37]	0.02
		Catheter	38	6.71 [3.42 - 10.7]	
		Graft*	4	4.35 [0.60 - 7.40]	
Co-morbidities:	Diabetes	Yes	74	5.16 [2.48 - 7.54]	0.40
		No	186	4.14 [2.17 - 7.78]	
	Ischaemic heart disease	Yes	74	4.69 [2.03 - 7.54]	0.87
		No	186	4.30 [2.30 - 7.81]	
	Malignancy	Yes	17	5.29 [3.73 - 10.9]	0.15
		No	243	4.36 [2.17 - 7.61]	
	Peripheral vascular disease	Yes	71	4.62 [1.92 - 7.81]	0.89
		No	189	4.36 [2.50 - 7.62]	
	Left ventricular dysfunction	Yes	24	4.09 [1.13 - 8.01]	0.35
		No	236	4.56 [2.38 - 7.62]	
Drugs:	ACEi / A2RBs / DRI	Yes	83	4.83 [2.42 - 7.82]	0.55
		No	177	4.54 [2.10 - 7.65]	
	β-blockers	Yes	55	5.20 [3.41 - 7.82]	0.07
		No	205	4.12 [2.09 - 7.65]	
	Statins	Yes	147	4.36 [2.02 - 7.31]	0.14
		No	113	5.04 [2.76 - 7.96]	
	Aspirin	Yes	117	4.83 [2.35 - 8.02]	0.69
		No	143	4.43 [2.15 - 7.36]	
	Clopidogrel	Yes	20	5.25 [3.55 - 6.79]	0.55
		No	240	4.39 [2.17 - 7.80]	
	Dipyridamole	Yes	3	6.33 [2.00-11.12]	0.61
		No	257	4.54 [2.28 - 7.65]	
	Warfarin	Yes	16	5.98 [1.89 - 9.38]	0.48
		No	244	4.49 [2.27 - 7.62]	
	Sulphonylureas	Yes	13	493 [1.23 - 7.02]	0.64
		No	247	4.54 [2.29 - 7.76]	
	Insulin	Yes	54	5.48 [2.97 - 8.25]	0.16
		No	206	4.16 [2.17 - 7.61]	

* Excluded from statistical analysis owing to small group size; †Caucasians vs Blacks on *post hoc* testing. ACEi: Angiotensin converting enzyme inhibitors; A2RBs: Angiotensin II receptor blockers; DRI: Direct renin inhibitors

5.2 Temporal changes in anaemia parameters and the effects of vitamin E

The baseline anaemia parameters are shown for both groups in Table 9. The unadjusted ESA dose was significantly higher for patients in the VE group. However patients in the VE group had a higher median post dialysis weight, and the ESA dose and weight were significantly correlated ($r=0.12$, $p<0.01$). Therefore the weight adjusted ESA doses were compared between study groups and found not to differ. There were no significant differences between the groups in the markers of iron status, ferritin and red cell hypochromasia, nor in the doses of intravenous iron administered.

Table 9 - Comparison of the baseline anaemia parameters between study groups.

	Control	Vitamin E	p
n	137	123	
Haemoglobin (g/dL)	11.7 [±0.1]	11.4 [±0.1]	0.09
Mean corpuscular volume (fl)	97 [±0.6]	96 [±0.5]	0.32
Red cell hypochromasia (%)	7.2 [±0.6]	6.5 [±0.6]	0.47
Packed cell volume (%)	37 [±0.4]	36 [±0.4]	0.26
Darbepoetin alfa dose (µg/wk)	20 [7.5 - 30]	20 [10 - 40]	0.049
Weight (kg)	69.7 [56.8 - 78.9]	73.4 [61.2 - 87.0]	0.03
Weight adjusted Darbepoetin alfa dose (µg/kg/wk)	0.25 [0.13 - 0.40]	0.28 [0.14 - 0.45]	0.16
ESA resistance index (ERI) (IU/wk/kg/g/dL Hb)	3.96 [3.45 - 4.96]	5.06 [2.33 - 8.09]	0.13
Patients not requiring ESA at baseline	13 [9.5%]	8 [6.5%]	0.38
Ferritin (µg/L)	490 [±20]	460 [±19]	0.26
Iron sucrose dose (mg/week)	25 [25 - 50]	25 [25 - 50]	0.24

Data presented as mean [±SEM] or median [IQR]; ESA: Erythropoiesis stimulating agent; Hb: Haemoglobin; wk: week.

5.2.1 12-month changes in haemoglobin and ferritin levels

Figure 28 shows the mean monthly haemoglobin concentrations for study patients. Throughout the study period, the mean haemoglobin levels in both groups were centred on the mid-point of the haemoglobin target range: 11.5 g/dL (see section 3.8.1). There were no significant differences in the mean haemoglobin levels between the groups at baseline ($p=0.09$) or 12 months ($p=0.98$). Pairwise comparisons of the mean baseline and 12 month haemoglobin levels revealed no significant differences in either the control group ($p=0.12$)

or the vitamin E group (p=0.64). Additionally, a repeated measures analysis found no significant effect of time (p=0.44), study group (p=0.33) or significant interaction between time and study group (p=0.38) on the haemoglobin concentration indicating the haemoglobin levels did not change significantly over 12 months and there was no effect of dialysis with the VE membranes.

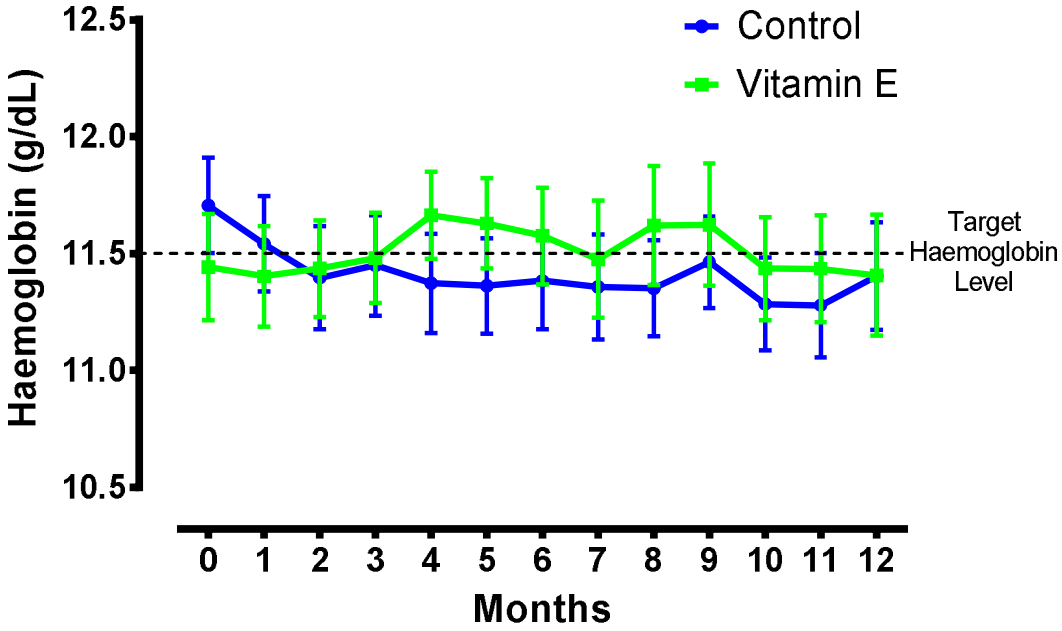


Figure 28 - Monthly mean haemoglobin concentrations (±95% CI) for the two study groups. There were no significant differences between the two groups with the haemoglobin levels centred on the midpoint of the target range: 11.5 g/dL.

There were no significant differences in the mean ferritin levels at baseline (p=0.26) or 12-months (p=0.50), nor when the 12-month changes in ferritin levels were compared between study groups (p=0.66). The mean ferritin levels increased during the study period (p for trend <0.001) but there was no effect of study group (p=0.88) nor significant interaction between study group and time (p=0.57) as shown in Figure 29. The LTHT assay used for measuring ferritin levels was changed shortly after the study was begun and the new assay reported approximately 30% higher ferritin levels (Dr Mike Bosomworth, Head of Blood Sciences, LTHT, personal communication 2010) to likely explain the significant increasing trend in the ferritin levels observed; the wide inter-assay variability of ferritin assays has been reported previously [920]. Changing the ferritin assay resulted in the gradual increase in the mean ferritin among study patients, as shown in Figure 29, rather than an abrupt change. This is a consequence of the staggered study commencement times for patients, spanning an approximate 5 month period, and the fact

that it was not introduced for all patients at the same time. Thus the length of time that patients had been on the study at the time the new assay was introduced varied and the proportion of patients undergoing ferritin determinations with the new assay increased as the study progressed.

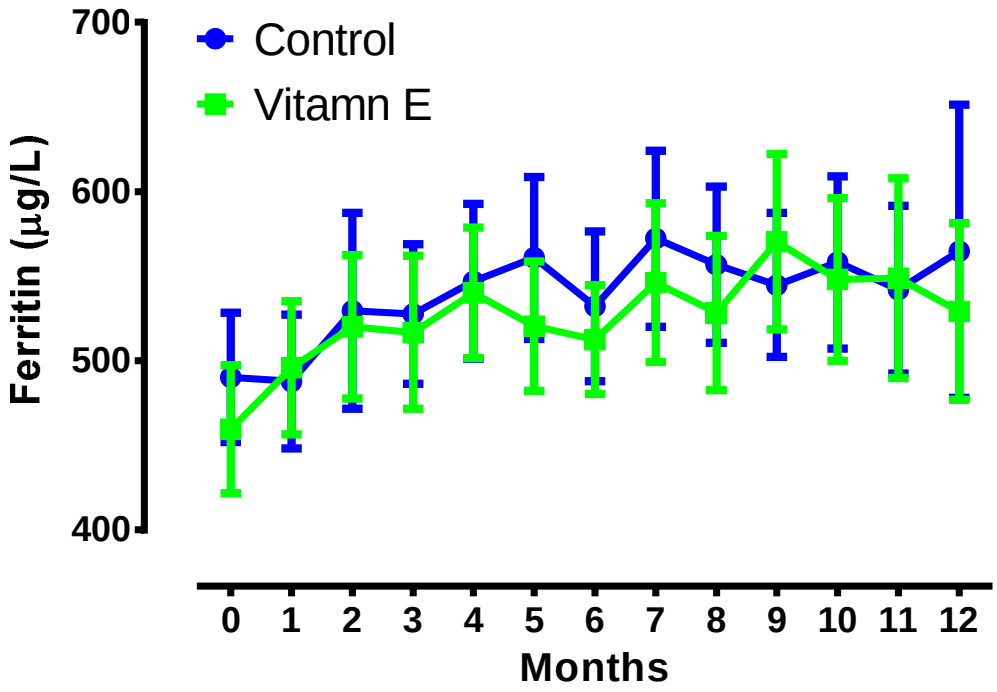


Figure 29 - Mean monthly ferritin concentrations (±95% CI) for the two study groups. The ferritin levels increased significantly over time, likely as a consequence of a change in the ferritin assay. There was no differential effect of dialysis with the VE-bonded membrane.

A comparison of the number of units of blood transfused into patients during the study revealed no statistical differences between groups, either in the number of patients receiving a transfusion (VE: 6 vs Control: 7 patients, p=0.93) or the total number of units of blood transfused (VE: 35 vs Control: 23 units of blood, p=0.43). In the VE group, one patient received 11 units and a second patient received 10 units which explained the non-statistically significant discrepancy in the total number of units transfused despite a similar proportion of patients receiving a transfusion.

5.2.2 12-month changes in ESA resistance index

Figure 30 displays the monthly ERI for the two study groups. There were no significant differences in the median ERI between the groups at baseline (p=0.13) or at 12-months (p=0.20), nor any significant differences between the median baseline and 12 month ERI in either the control (p=0.30) or VE (p=0.60) groups. Similarly a comparison of the change

in ERI at 12-months from baseline found no statistically significant difference between the control and VE groups (p=0.08) as shown in Figure 31.

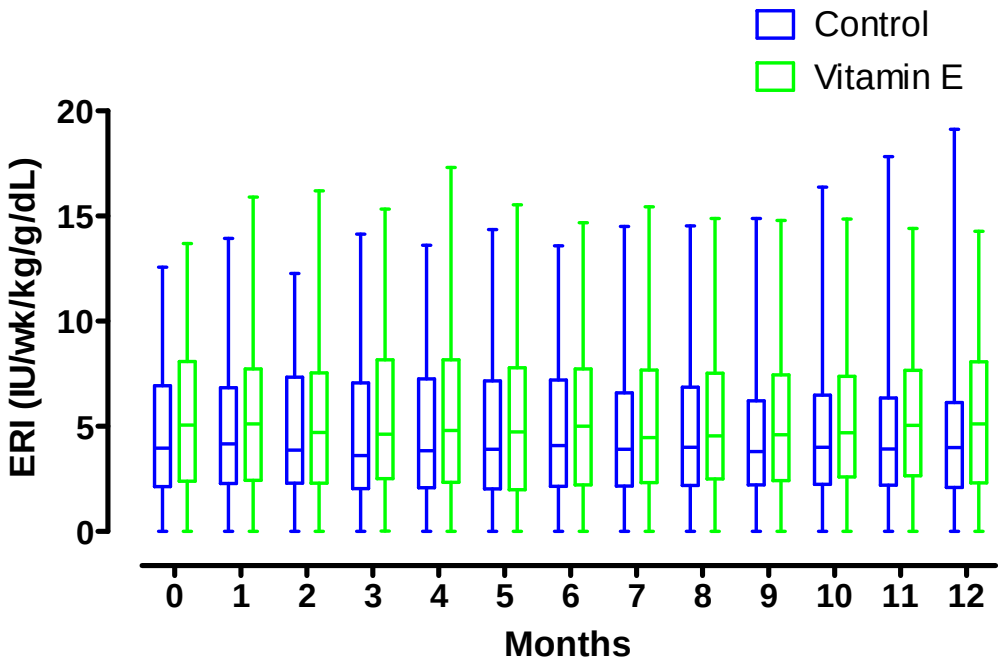


Figure 30 - Box and whisker plot of the monthly ERI by study group. There were no significant differences between the groups nor any significant changes over time within either group.

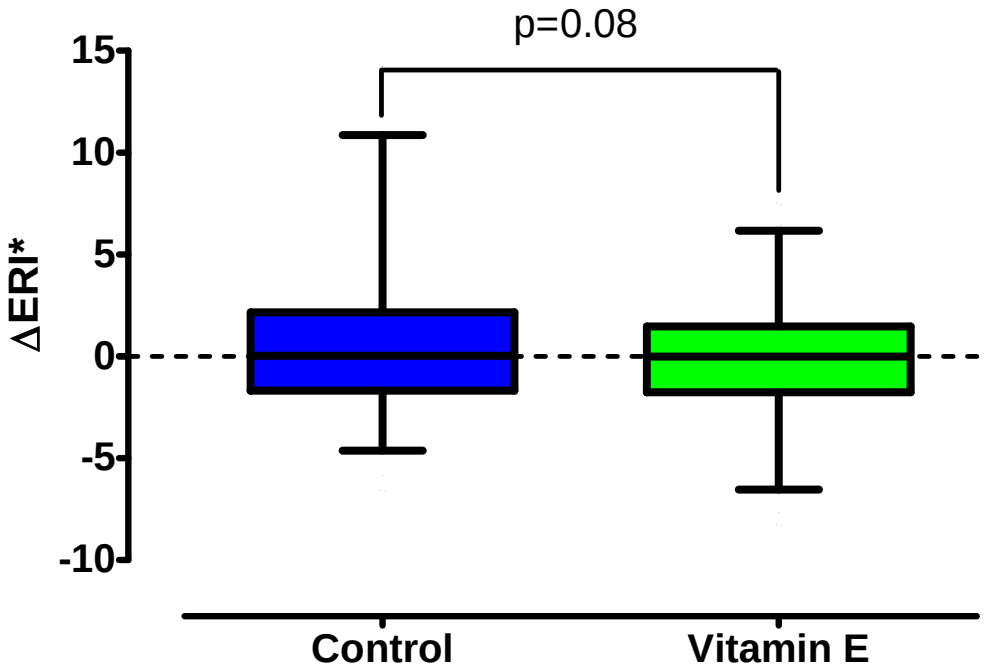


Figure 31 - Comparison of the change in ERI over 12 months between groups showing no significant difference. (*ΔERI = $ERI_{12months}$ - $ERI_{baseline}$)

Data from the two UK Renal Registry Reports, which collate data from the majority of renal units in the UK, conducted immediately prior to [921] and during [4] the study period, indicated that both the mean and median ESA doses for LTHT dialysis patients were the lowest in the country, despite haemoglobin levels close to the national median; thus suggesting low levels of ESA resistance in LTHT dialysis patients. It was therefore decided to perform *post hoc* subgroup analysis after stratifying patients into tertiles of ERI at baseline to determine if there was a differential effect of VE for those patients with a higher ERI at the start of the study. Table 10 shows the baseline and 12-month ERI data stratified by tertiles of ERI at baseline and study group. A chi-squared analysis of the number of patients from the two study groups within each tertile of ERI at baseline was not statistically significant (p=0.18) suggesting an even distribution.

Table 10 - Baseline and 12 month ESA resistance indices for study patients, stratified by ERI at baseline and study group.

		BASELINE		12 MONTHS		
		n	**ERI (IU/wk/kg/g/dl Hb)**	**n**	**ERI (IU/wk/kg/g/dl Hb)**	***p****
Highest tertile	**Control**	39	9.45 [7.62 - 12.3]	31	8.14 [4.44 - 15.6]	*0.41*
	Vitamin E	48	9.28 [7.70 - 12.5]	41	7.70 [5.34 - 12.7]	*0.01*
	***p**†*		*0.72*		*0.60*	
Middle tertile	**Control**	50	4.40 [3.53 - 5.45]	45	4.04 [2.60 - 6.02]	*0.87*
	Vitamin E	36	4.70 [3.83 - 5.36]	31	5.18 [3.04 - 6.56]	*0.49*
	***p**†*		*0.50*		*0.60*	
Lowest tertile	**Control**	48	1.66 [0.000 - 2.23]	40	1.91 [0.351 - 3.50]	*0.02*
	Vitamin E	39	1.53 [0.603 - 2.30]	32	2.13 [1.28 - 3.55]	*0.03*
	***p**†*		*0.84*		*0.52*	

*p-value for baseline vs 12 months; †p-value for between group comparisons

From the data shown in Table 10 it can be seen that there was an increase in the ERI after 12 months for patients in the lowest ERI tertile at baseline irrespective of study group, no significant changes for patients in the middle tertile, and a reduction in ERI for patients in the highest tertile dialysing with the VE-bonded, but not the control, membranes. Interestingly the between group comparisons of ERI at the baseline and 12-month time points were not statistically significant. Performing a Friedman's test for each group within each tertile, assessing for significant changes over time across the 12 month study period using the monthly ERI data, revealed the change in ERI was only statistically significant for

the VE group in the highest tertile (p<0.001); the significance persisted at the 5% level after applying a Bonferroni correction for multiple comparisons. To further explore if there was a differential effect of the VE-bonded membrane depending on the ERI at baseline, the 12-month change in ERI was compared between study groups after stratifying into tertiles of ERI at baseline as shown in Figure 32. It can be seen that there was an apparent beneficial effect of dialysing with the VE-bonded membrane for patients in the highest tertile in terms of a reduction in the ERI.

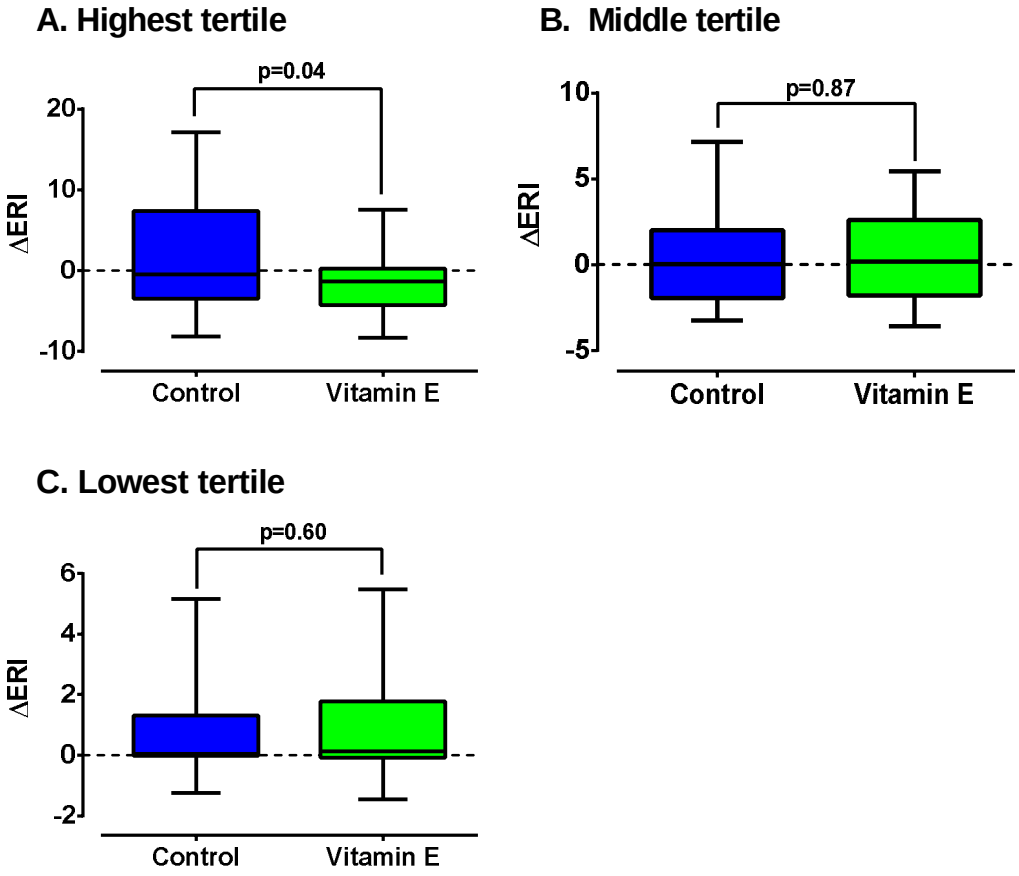


Figure 32 - Comparison of change in ERI between groups stratified by tertiles of ERI at baseline. Among patients in the highest tertile of ERI at baseline, there was a significant reduction in ERI after 12 months for those patients dialysing with the VE membrane. ($\Delta ERI = ERI_{12months} = ERI_{baseline}$).

As there appeared to be a differential effect of the VE membrane depending on the baseline ERI, a regression model for the 12-month change in ERI was constructed comprising the baseline ERI and study group in addition to an interaction term for these two variables. The regression co-efficients for the study group and baseline ERI variables were not significant (p=0.17 and 0.28 respectively) but the regression coefficient for the interaction term was statistically significant (p<0.01), indicating an effect of the VE-bonded

membrane on the 12-month change in ERI conditional on the starting ERI. This is depicted graphically in Figure 33 which is a scatter plot of the change in ERI against the baseline ERI and best-fit linear regression lines through the data points for the two study groups. The slope of the regression line through the control group data points did not differ significantly from zero (p=0.30) indicating no effect of the baseline ERI on the 12-month change in ERI observed. The slope of the regression line through the VE group data points was negative and differed significantly from zero and the other regression line (p<0.01 in both cases). This suggests a differential effect of the VE-bonded membrane with greater reductions in ERI for patients with a higher ERI at baseline.

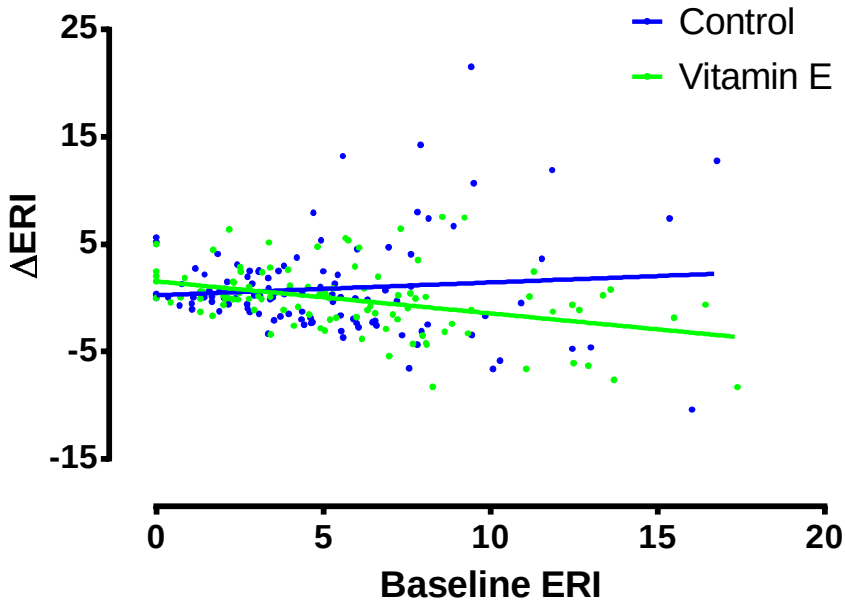


Figure 33 - Scatter plot for the change in ERI against baseline ERI with best fit regression lines through the data points for each group. The regression line for the control group did not differ significantly from zero (p=0.30), whereas the regression line for the Vitamin E group had a negative slope (β -0.30 [±0.07]) which differed significantly from zero and from the control regression line (p<0.01 in both cases). ($\Delta ERI = ERI_{12months} - ERI_{baseline}$).

Given that there was a reduction in ERI for those patients in the highest tertile dialysing with the VE membrane, further analyses were undertaken to determine the characteristics of this group. To this end all of the variables which were associated with ERI at baseline at a significance level of p<0.2, the selection criterion for inclusion in the multivariate analyses, were compared between tertiles; the results of these analyses are shown in Table 11. From these results it can be seen that selecting patients on the basis of a higher ERI was selecting patients with higher levels of inflammation, increased CVC usage, lower dialysis dose, lower pre-dialysis bicarbonate levels and shorter length of time on RRT.

Table 11 - Factors significantly related to baseline ERI compared between the tertiles of ERI at baseline.

	Baseline ERI			
	Lowest tertile	**Middle tertile**	**Highest tertile**	**p†**
n	87	86	87	
ERI (IU/wk/kg/g/dl Hb)	<3.05	3.05 - 6.33	>6.33	
Sex				0.18
Males	68%	57%	55%	
Females	32%	43%	45%	
Ethnicity				0.14
Caucasian	84%	76%	70%	
Asian	14%	18%	20%	
Black	2%	3%	9%	
Other*	0%	1%	1%	
Smoking				0.14
Never smoked	39%	49%	48%	
Current smoker	24%	27%	15%	
Ex-smoker	37%	24%	37%	
Dialysis access				0.01[1]
Fistula	91%	85%	76%	
Central venous catheter	7%	14%	23%	
Graft*	2%	1%	1%	
Time on RRT (yrs)	4.5 [2.7 - 8.6]	3.8 [1.3 - 8.4]	2.7 [1.0 - 5.5]	0.001[1]
Urea reduction ratio	0.76 [±0.01]	0.76 [±0.01]	0.74 [±0.01]	0.046[2]
C-reactive protein (mg/mL)	5.7 [2.2 - 14.5]	5.6 [1.7 - 10.5]	9.8 [4.2 - 22]	0.002[1,3]
Bicarbonate (mmol/L)	22.3 [±0.2]	22.2 [±0.3]	21.3 [±0.2]	0.01[1]
Systolic blood pressure	132 [±2.6]	140 [±2.6]	138 [±2.7]	0.10
Malignancy	3%	8%	8%	0.38
β-blockers	13%	29%	22%	0.03[4]
Insulin	15%	22%	25%	0.23
Statins	64%	58%	47%	0.07

Data presented as range, mean [±SEM] or median [IQR]. ERI: ESA resistance index; RRT: renal replacement therapy. *Excluded from statistical testing due to small group size; †Significance test for trend

Post-hoc pairwise testing between tertiles: [1]Highest vs lowest tertile; [2]No significant differences on pairwise testing; [3]Highest vs middle; [4]Lowest vs middle tertile

To determine if a change in any of these factors might explain the observed reduction in ERI for patients in the high ERI tertile dialysing with the VE-bonded membrane, the 12-month changes in these parameters were compared between study groups. From Table 12 it can be seen that the changes in these variables did not differ significantly between study groups. This suggests that the reduction in ERI may be attributable to the VE-bonded membranes and not to the changes in these variables.

Table 12 - Between group comparisons of change in each variable over 12 months for patients in the high ERI tertile. Variables listed are those which differed significantly when patients compared on the basis of ERI tertile. (In each case change was calculated by subtracting the baseline from the 12-month value.)

	Change in variable over 12 months		
Variable	***Control***	***VE***	***p***
C-reactive protein (mg/L)	0 [-7.5 - 8.2]	-0.3 [-10.3 - 3.2]	0.42
Urea reduction ratio	0.02 [-0.03 - 0.06]	-0.01 [-0.06 - 0.04]	0.34
Bicarbonate (mmol/L)	1 [-2 - 2]	0 [-3 - 2]	0.33
Central venous catheter usage (n)	-2	-1	1.0

Data presented as median [IQR] unless stated

5.3 Discussion

One of the principal aims of this study was to determine if switching prevalent HD patients to dialysis with a VE bonded dialysis membrane for 12 months had any effect on ESA requirements; the metric used to assess ESA requirements was the ERI. There are a number of advantages to analysing the ERI in preference to unadjusted ESA doses. The weekly ESA dose is not a continuous variable, as it is restricted to the available pre-filled darbepoetin alfa syringe sizes available (10 µg, 15 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 80 µg, 100 µg, 130 µg, 150 µg, 300 µg and 500 µg), therefore analyses of the unadjusted ESA doses were unlikely to be sensitive to small changes in ESA responsiveness. The use of an endpoint such as ERI was particularly pertinent in the present study given the non-blinded study design. If the endpoint was simply ESA dose, there would be the potential for investigators to modify their ESA-prescribing practices, either consciously or subconsciously, thereby possibly influencing the outcome of the study. This is negated by adjusting the ESA dose by the haemoglobin concentration in the calculation of the ERI. The ability of prescribers to influence the outcome in the present study is further minimised by the use of a computer based predictive ESA-dosing algorithm. Additionally, other studies evaluating the potential of VE-bonded polysulfone membranes to improve anaemia [889, 893, 894] have reported effects on the ERI allowing the results from the present study to be placed in context.

5.3.1 Baseline determinants of ESA resistance index

One of the main purposes of analysing the baseline dataset was to gain an understanding of the determinants of ERI in the study population prior to examining the influence of VE-bonded dialysis membranes. At baseline, the unadjusted median ESA dose and post-dialysis weight were significantly higher in the VE group. However, after adjusting for weight, the ESA requirements and ERI were found not to differ between groups. With

regards to the determinants of ERI, the principle findings were that the baseline ERI was negatively correlated with dialysis dose and length of time on RRT, positively correlated with CRP and was higher in women, Blacks compared to Caucasians and in patients dialysing via CVCs. All of these findings are consistent with the published literature concerning ESA responsiveness as discussed further here.

The association between markers of inflammation, such as CRP as measured in the present study, and ESA resistance is well documented in HD patients [922-926]. Inflammation increases ESA requirements through a number of mechanisms. Inflammatory cytokines, such as IL-1, TNF-α and IFN-γ, have been shown to have a direct suppressive effect on early erythroid progenitor cell growth *in vitro* [158, 159]. Erythrocyte life span is also reduced in the presence of inflammation due to accelerated destruction of immunoglobulin or immune complex coated red blood cells by activated reticulo-endothelial macrophages [927] and enhanced complement mediated lysis in HD patients [227]. Importantly, inflammation also exacerbates functional iron deficiency through hepcidin induction [152, 928], the uptake of lactoferrin by activated macrophages [156, 157] and impairment of intestinal iron absorption [147]. The influence of functional iron deficiency on anaemia in the present study, however, was mitigated to a large extent by the protocolised administration of supplemental intravenous iron as detailed in section 3.8.1 and Appendix D.

The finding in the present study regarding the association between greater dialysis dose and reduced ERI has been similarly reported in other studies [177-180]. The most likely explanation for this is the removal of as yet unidentified “uraemic inhibitors of erythropoiesis” by the dialysis process. Evidence for the existence of these uraemic inhibitors comes from early *in vitro* work in which uraemic serum was found to inhibit erythropoiesis in animal cell lines [181-183] and improvements in anaemia observed following the initiation of dialysis in uraemic individuals [174-176] or an increase in dialysis dose [177-180]. The exact nature of these inhibitors remains elusive.

It is interesting that CVC usage, inflammation and lower dialysis dose were associated with increased ERI on bivariate analyses but the association with CVC usage did not persist in the multivariate analysis; an association between CVC usage and increased ERI has been reported previously [162, 929, 930]. The association between CVC usage and ERI in the present study did not appear to be solely due to heightened inflammation, which is generally enhanced in patients dialysing with CVCs [453], as there was no statistically

significant difference in the CRP levels at baseline when patients dialysing with CVCs and fistulas were compared (9.0 [2.1-19.7] vs 6.3 [2.0-14.2] mg/L respectively; p=0.28). Patients dialysing via CVCs received, on average, a lower dialysis dose compared to patients dialysing with fistulas (URR: 0.73 [±0.01] vs 0.76 [±0.00] respectively, p=0.03) which is likely a consequence of lower achievable blood flow rates for patients dialysing via CVCs, although no data were available to corroborate this. Thus it is possible that the bivariate association between catheter usage and ERI became non-significant in a model which adjusted for inflammation and dialysis dose because the association was mediated through these factors.

On bivariate analysis it was evident that patients who had been on RRT for longer had a lower ERI. One explanation for this may be that time on RRT was a proxy for general health, with healthier patients surviving, and hence dialysing, for longer. The converse may also be true with the healthiest patients receiving a renal transplant leaving those patients deemed medically unsuitable for a transplant to remain on dialysis. Thus the people who have been on dialysis for longer periods may, in fact, represent an intermediate group in terms of disease burden suggesting comorbidity may not be the explanation for the association between dialysis vintage and ERI. Comorbidity, as defined by the Charlson Index [931], was found to be related to ESA requirements in a large observational Spanish study [162] but not in the present study where comorbid disease burden was quantified using a different method developed by Davies *et al*. [898] (r=0.04, p=0.52). Furthermore, the exclusion from the present study of patients with evidence of active inflammation, approximately 10% of the screened population (see Figure 24), may have diluted any association between disease burden and ERI in contrast to the observational study by Lopez-Gomez *et al*. [162] which had no such exclusion criteria. Thus the apparent contrasting findings between these two studies may be a result of different approaches to defining comorbidity and the different populations studied. Length of time on dialysis was also strongly positively correlated with dialysis dose (r=0.28, p<0.0001) thus the association between ERI and time on dialysis may simply be a confounding effect rather than a causal relationship. This is further supported by the exclusion of the variable coding for length of time on dialysis in favour of dialysis dose in the stepwise multivariate regression analysis.

The multivariate regression model identified that the ERI was greater in women, independent of the other variables considered, which is consistent with the findings of others [162, 932]. In health, women have a lower haemoglobin level than men [933]. The

reasons for this are not completely understood although possible explanations include the influence of sex hormones on the release of iron from the reticuloendothelial cells during erythropoiesis [934], the positive effects of androgens on erythropoiesis [934, 935] and menstrual blood loss. The latter finding, however, is likely to be of minor relevance in HD patients as premenopausal women are frequently amenorrhoeic [936].

The initial regression model suggested that the ERI was higher in Blacks, although this association did not persist after excluding the 9 cases with outlying high values for the ERI; 4 of these 9 patients were of Black ethnicity. This suggests that it was a small number of patients with high ERIs driving the association between ethnicity and ERI in the initial analysis. The finding of higher ESA resistance in Blacks compared to Caucasians has been widely reported in dialysis series from the United States [179, 937-939], although the racial mix is likely to differ from the patients included here. Some factors which can influence anaemia are known to differ among races, such as haemoglobin variants and socioeconomic factors [940], and this may explain some of the differences, although a large observational series from America of non-dialysis patients found the higher incidence of anaemia among Black individuals to persist even after controlling for a number of these factors [941]. Thus, despite being widely acknowledged, a comprehensive explanation for the racial differences in anaemia and ERI remains enigmatic. Furthermore, it is hard to draw definitive conclusions about racial differences in ERI from the present study given the vastly discrepant group sizes (13 Blacks vs 199 Caucasians). From the analyses of the baseline dataset, it appears that the study patients were representative of a typical dialysis population thus providing a suitable platform to test the effects of a VE-bonded dialysis membrane on anaemia outcomes.

5.3.2 The effects of vitamin E on anaemia

A number of studies have compared VE-bonded modified cellulose membranes with non VE-bonded membranes with respect to anaemia parameters, such as haemoglobin levels, ESA requirements and red blood cell lifespan, and generally reported improvements with VE [856, 861, 868-870, 875-879, 942, 943]. However many of these studies were non-randomised, did not have a parallel group study design and examined anaemia parameters as a non-primary outcome measure. More recently VE-bonded versions of the latest generation biocompatible synthetic membranes have become available such as the Vitabran-A membrane tested here. The primary finding in the present study with regards to anaemia was that 12 months dialysis with a VE-coated polysulfone membrane offered no improvements in ESA resistance, when compared to an equivalent non-VE-coated

polysulfone membrane. *Post hoc* subgroup analysis, however, did reveal a reduction in ERI for patients with a higher ERI at baseline dialysing with the VE membranes, but not the control membranes, and a regression analysis examining the interaction between baseline ERI and study group found a significant effect of the VE-membrane on the 12-month change in ERI conditional on the ERI at baseline. Further analysis of the subgroup of patients with the highest ERI at baseline revealed a higher proportion of these patients were dialysing via CVCs, that they had higher levels of CRP and lower pre-dialysis bicarbonate levels and were receiving a lower dose of dialysis compared to the remaining patients; each of these factors were also significantly associated with ERI at baseline. To try and determine if the reduction in ERI for those patients in the highest tertile of ERI at baseline dialysing with the VE membrane was an effect of the VE-membrane, or due to changes in any of these parameters, the change in each of these variables over 12 months were compared between study arms and found to be non-significant. Thus, on the basis of these analyses, it may be that the observed improvement in ERI for this subset of patients was attributable to dialysis with the VE-membrane, although an effect of an unmeasured factor or factors cannot be discounted. It is worth noting, however, that although the distribution of patients across the tertiles analysed using a chi-squared test did not differ from the expected values, suggesting an even distribution of the two study groups, more patients in the highest tertile were dialysing with VE membrane (48 vs 39). Patients with the highest ERI at baseline were, on subsequent determinations, more likely to have a reduction in ERI rather than further increases; the phenomenon of regression to the mean. Thus the effect of regression to the mean, rather than a true effect of the VE membrane, cannot be entirely discounted as a reason for the observed reduction in ERI for the patients in the highest ERI tertile dialysing with VE. It is important, however, to consider the data from the present study in the context of other studies examining the utility of VE-bonded membranes in improving renal anaemia.

Several studies have investigated the effects of VE-coated polysulfone membranes on anaemia parameters. A number of these studies [891, 892, 894, 896], including a paediatric study [890], failed to demonstrate a convincing reduction in ESA requirements with the VE membranes. One of the first studies to suggest a benefit in terms of renal anaemia with the VE membranes was published by Morimoto *et al*. [895] who followed 31 patients for 6 months, 16 of whom dialysed with the VE-membrane. Although anaemia was not the primary focus of the study, they reported significantly lower ESA doses in the VE group despite similar haemoglobin levels. Several subsequent studies, all of which were published after the present study was commenced, have specifically addressed the ability

of VE bonded polysulfone membranes to reduce ESA requirements. In a pilot study Andrulli *et al.* [894] followed 19 patients for 8 months, 10 of whom were randomised to dialysis with a VE bonded polysulfone membrane. The ERI decreased significantly in all patients over the study period with no apparent benefit observed with the VE membrane. After adjusting for the levels of intact PTH, α- and γ-tocopherol, all of which were significantly correlated with ESA dose at baseline, in a *post hoc* multivariate regression analysis the reduction in ERI in the VE group compared to the control group reached statistical significance (p=0.04). This study, as stated by the authors, was a pilot study but does point to a possible ESA-sparing effect of the VE-membranes. Mandolfo *et al*. [889] followed 16 patients, all of whom were dialysing with CVCs, in a 12-month cross-over study comprising 6 months dialysis with a VE bonded polysulfone membrane and 6 months with a non-VE bonded polysulfone membrane; no details on the performance characteristics of the comparator membrane were provided. During the 6 month period dialysing with the VE membrane they reported lower ESA doses and a lower ERI, with no significant differences in haemoglobin levels, again suggesting that the VE membrane improved ESA responsiveness. This second study differs markedly from the present study in that only patients with CVCs were included, a factor which was found to be associated with ESA resistance in the present dataset, making direct comparisons impossible.

In perhaps the most comprehensive study published to date examining anaemia outcomes, Panichi *et al.* [893] enrolled 62 patients in a 13-month multicentre randomised controlled cross-over design study comparing a low-flux VE-bonded polysulfone membrane with a low-flux non VE-bonded polysulfone membrane. After 6-months dialysis with the VE membrane they reported stable ESA requirements and an increase in haemoglobin levels which translated into a statistically significant improvement in the ERI; no significant changes in the haemoglobin level, ESA dose or ERI were observed following 6-months dialysis with the non VE membrane. Thus there was an apparent benefit of the VE membrane in terms of reducing ERI. The Panichi *et al.* [893] study differs from the present study in that low-flux membranes were used in contrast to the high-flux membranes used in the present study. However, published data have shown that membrane flux has little impact on anaemia [919, 944-946] suggesting that the use of membranes with different permeability characteristics may not explain the discrepant findings of the two studies.

More recently, Sanaka *et al*. [896] published the results of a 12-months prospective, multi-centre, randomised controlled trial evaluating the effects of a VE-bonded polysulfone HD

membrane on the relative change in ERI. A total of 305 patients from 48 haemodialysis facilities in Japan were divided into two groups on the basis of their haemoglobin levels, 10-10.9 g/dL and 11-11.9 g/dL, and then randomised to 12-months dialysis with either a high-flux VE-bonded membrane or a non-VE bonded equivalent membrane; data on 213 patients completing the study were analysed. The primary outcome was relative ERI, defined by dividing the monthly ERI by the ERI at baseline. Overall there was no effect of the VE-membrane on the 12-month relative change in ERI; although no data were provided to assess equivalence of ERI at baseline. Further analyses, defined *a priori*, identified a reduction in the relative ERI for patients in the higher haemoglobin group dialysing with the VE-membrane who were receiving darbepoetin alfa, but not rHuEPO, at 12 months. Analysing all of the patients in the higher haemoglobin group together, regardless of ESA used, revealed a significantly higher 12-month relative ERI for patients dialysing with the non VE-bonded membrane, compared to the VE-bonded membrane, although in both groups the 12-month relative ERIs did not differ significantly from baseline.

There are a number of methodological concerns with this study including the 2x2x2 study design (i.e. haemoglobin group x dialyser membrane x ESA used) which significantly reduced the power of the study to detect a significant effect of the membrane; no reference was made in the manuscript regarding the statistical power of the study. Furthermore, no statistical adjustments were made for the multiple pairwise comparisons of the relative ERI at each of the monthly time points and the associated increase in familywise error rate. Therefore the aforementioned apparent significant differences between the 12-month relative ERIs for patients in the high haemoglobin group dialysing with the different membranes may represent a type I statistical error. It would, perhaps, have been more informative to stratify patients on the basis of ERI at study start, rather than the haemoglobin level, given that change in ERI was the primary outcome measure. No data were provided comparing ESA requirements between the patients in the two “haemoglobin groups” although the median ESA doses appeared to be smaller in the higher haemoglobin group, suggesting that these patients may have been less ESA resistant. If, indeed, this was the case it is possible that the VE-bonded membrane may have had a role in limiting increases in ERI over time, as were observed for patients dialysing with the control membrane, for less ESA-resistant patients. This is the converse of the present study in which the more ESA-resistant patients appeared to benefit from dialysis with the VE-bonded membrane. This interpretation of the Sanaka *et al*. [896] data, however, remains conjecture as these points were not specifically addressed in the manuscript. It

may, however, provide further circumstantial evidence that subgroups of patients, identified on the basis of ESA-resistance, may benefit from dialysis with a VE-bonded membrane rather than advocating wholesale switching of all prevalent HD patients to dialysis with VE-bonded membranes.

It is important to note that the patients in the present study differed from those in the previously cited studies reporting a beneficial effect of VE-bonded membranes on anaemia. The patients in the present study, for example, had significantly lower ERI's at baseline than the other studies evaluating VE-bonded polysulfone membranes in this setting (see Table 13). Interestingly, the baseline ERIs for those patients in the highest tertile of ERI in the present study, who appeared to benefit from dialysis with the VE-membrane, were closer to the published studies which demonstrated an ESA-sparing effect of the VE-membranes [889, 893]. This suggests that the VE-bonded membranes may be beneficial in ESA-resistant patients although such a supposition requires testing in an appropriately designed study.

Table 13 - Comparison of studies evaluating vitamin E bonded polysulfone membranes with a primary anaemia endpoint.

Study	*No. patients*	*Study duration*	*Baseline ERI* (IU/wk/kg/g/dl Hb)*	*Overall effect of VE membrane*
THIS STUDY	260	12 months	4.6 [2.3 - 7.7]	ERI →
Andrulli *et al.* [894]	20	8 months	7.7 [4.8 - 11.6]	ERI →
Panichi *et al.* [893]	62	6 months	10.7 [9.7 - 11.8]	ERI ↓
Mandolfo *et al.* [889]†	16	6 months	12.2 [± 5.6]	ERI ↓
Sanaka *et al*., [896]	305	12 months	*Not stated*	(Relative) ERI →

*Data presented as median [IQR] or mean [±standard deviation] as reported by the Authors; †Study included only patients dialysing through central venous catheters. ERI: Erythropoiesis stimulating agent resistance index

The apparent low ERI for patients in the present study is further highlighted by the LTHT patients having the lowest mean and median ESA doses in the country, despite haemoglobin levels close to the national median, from data published in the UK Renal Registry reports [4, 921]. The reasons for the apparent low ERI in the study cohort are not immediately obvious. Patients with significant inflammation, which is associated with ESA resistance as already discussed, were excluded from study and this is likely to be an important factor although a number of the other published studies [893, 894] similarly excluded patients with active inflammation yet reported higher patient ERIs. This fact would also not explain the UK Renal Registry data [4, 921] as information on all HD

patients is collated. Perhaps the most striking difference between the present study and the published studies and data from the other UK dialysis units included in the UK Renal Registry is the use of a predictive ESA-dosing algorithm. The published experience with this approach [901] suggests it leads to efficient ESA usage and thus may explain, at least in part, the apparently low doses of ESA used by LTHT HD patients.

Another factor potentially complicating the comparison of the results from the present study with those already published is the use of the conversion ratio of 1:200 for converting rHuEPO doses (µg) to equivalent darbepoetin doses (IU) in order to calculate the ERI. This conversion factor is recommended by the manufacturers of rHuEPO as the ratio at which products are cost-neutral under European licensing [903], and is adopted by the UK Renal Registry [4, 921]. There have been concerns for some time that this conversion ratio may not represent clinical equivalence [947]. A meta-analysis examining the relative doses of ESAs in HD patients identified that after switching patients from rHuEPO to Darbepoetin alfa at a conversion rate of 1:200, further dose reductions averaging 30% could be made while maintaining haemoglobin levels [948]. Using this conversion rate, therefore, will likely overestimate the ERI for patients receiving darbepoetin alfa. In the present study, where all patients used the same ESA, this is maybe of less relevance although in other studies, such as the Panichi *et al*. study [893], where a mixture of rHuEPO and darbepoetin alfa were used, applying a potentially erroneous correction factor to a subset of patients may give misleading results. In the Panichi *et al*. study, however, the authors stated that the same ESA was used by each patient for the duration of the study and this, combined with the cross over study design, means it is unlikely to have materially affected their overall results. It is therefore likely that ERIs calculated in patient groups using different ESAs may not be directly comparable and this may form part of the explanation for the apparent discrepant findings of the studies evaluating VE-bonded polysulfone membranes detailed in Table 13. The use of different ESAs was specifically addressed by Sanaka *et al*. [896] who reported a benefit of the VE-membrane for patients with higher haemoglobin levels at baseline receiving darbepoetin alfa, but not those who received rHuEPO, suggesting the possibility of a differential effect of the VE-bonded membrane depending on the ESA used. The mechanisms through which such a situation may occur are not obvious. The effect of different dialysis membranes on the biological activity of different ESAs has not been specifically studied to date. If, for example, the various ESA preparations had differing susceptibilities to oxidative modification, which in turn resulted in attenuation of their biological activity, it

would be conceivable that the use of the putatatively anti-oxidant VE coating on the dialysis membrane may exert differing effects depending on the ESA used.

It is important to note that in the present study only prevalent HD patients were included, i.e. established on dialysis for greater than 3 months, and patients with active inflammation at baseline were excluded which together ruled 13% of the screened LTHT HD population ineligible for this study. These factors, in addition to the other parameters shown to be important determinants of ERI in this study population, such as race, CVC usage, dialysis dose and the prevailing levels of inflammation and ESA-resistance, need to be considered when generalising the results from the present study to other dialysis populations. It is also important to consider the statistical power of the present study. The power calculation indicated a sample size of 118 patients in each arm would have an 80% power to detect a 25% difference in ESA doses (see section 3.17.2). The number of patients with 12-months of ESA data (VE group: 116 and control group: 104) was less than this thus the failure to show any significant benefit with the VE membrane may represent a type 2 statistical error. However, this study enrolled more patients that the other VE studies which did report reductions in ERI with Panichi *et al.* [893] reporting an 11% reduction in ERI and Mandolfo *et al.* [889] a 28% reduction. The baseline and 12 month ERI for patients in the VE group in the present study were similar (5.1 [2.3-8.1] IU/wk/kg/g/dl Hb in both cases, p=0.68 for pairwise comparison) suggesting an effect of the VE membrane on ERI at the study population level was unlikely, despite the recruitment of fewer patients identified in the power calculation. The apparent significant difference between the groups in terms of the reduction in ERI for the highest tertile patients dialysing with the VE membrane also needs to be interpreted in context, given that it could represent a false positive finding in light of the inflated type 1 error rate associated with multiple pairwise statistical testing. However, the regression coefficient for the interaction term of study group and baseline ERI in a multiple regression model of the 12-month change in ERI did suggest a significant effect of the VE membrane conditional on the baseline ERI, strengthening the case that the VE membrane may be beneficial for patients with increased ESA-resistance.

The positive finding with respect to a significant reduction in ERI for patients in the highest tertile of ERI at baseline dialysing with the VE-membrane needs placing in clinical context. The median reduction in ERI for this group of patients was 1.29 IU/wk/kg/g/dL Hb which, using the median weight and average haemoglobin level for this subgroup of patients, equates to a reduction of 5.5 µg/week. The median ESA dose for patients in the highest ERI tertile at baseline was 40 µg/week therefore this represents a greater than 10%

reduction in the ESA dose. It is difficult to calculate what this dose reduction would translate to in terms of a cost saving given the variation in ESA pricing between different units and the use of pre-filled syringe sizes. However changes of this magnitude may well translate into significant cost savings in terms of ESA expenditure. An important question, howeverm would be whether the increased costs of the VE-membrane could be wholly or partially offset by the reductions in ESA requirements.

From the information presented in this chapter, it is perhaps not surprising that in a unit which already has efficient ESA prescribing [901], low levels of ESA-resistance [4, 921] and which uses the latest generation of biocompatible membranes no additional benefits of the VE membrane on overall ESA resistance were observed; akin to the law of diminishing returns. Given that the comparator membrane was a latest generation biocompatible membrane, further attempts to improve the biocompatibility of an already biocompatible membrane by coating with VE are likely to result in more modest improvements than those observed with coating the less biocompatible modified cellulose membranes.

In conclusion, no ESA-sparing effect was observed in the present study when prevalent HD patients were dialysed with a VE-bonded membrane for a period of 12 months. For patients with higher levels of ESA resistance, VE-bonded membranes may offer some benefit although trials specifically designed to answer this question are needed. Further studies are required to determine which, if any, patients or subsets of patients may benefit from dialysis with a VE-bonded membrane. Such considerations would also need to factor in the increased costs associated with the VE-membranes and the potential to offset these with reductions in ESA expenditure or improved clinical outcomes.

Chapter 6 : Oxidative stress

Levels of oxidative stress, measured using a variety of different biomarkers, have been shown to be increased in HD patients [326-337] due to the increased generation of ROS and reduced anti-oxidant defences as outlined in section 1.4.2. Furthermore, enhanced oxidative stress is thought to underlie a number of complications encountered by HD patients such as ESA-resistance (see section 1.3.2) and CV disease (see section 1.4.3). Two biomarkers of oxidative stress were measured in the present study, Ox-LDL and TBARS, and analyses of these data are presented in this chapter. Initially the determinants of each biomarker in the study population were evaluated prior to examining the effects of 12 months dialysis with a VE-bonded polysulfone membrane.

6.1 Determinants of baseline oxidative stress

6.1.1 Determinants of baseline Ox-LDL levels

Out of 260 baseline samples 257 were analysed owing to missing labels on three samples. Of the 257 samples, 57 (22%) were below the lower limit of detection for the assay and were assigned a value of 4.3 ng/mL as described in the methods section 3.10; for this reason the frequency distribution of the Ox-LDL dataset was heavily positively skewed and had a long tail owing to high Ox-LDL levels in a small number of samples as shown in Figure 34. Correlation analysis to look for significant associations with continuous variables in the dataset, given the distribution of the data and the large number of samples with Ox-LDL levels below the limit of detection, would be unlikely to yield meaningful results. The data were therefore divided into quartiles as described in the methods (section 3.15), thereby ensuring that all of the 22% samples with Ox-LDL levels below the lower limit of detection were grouped together in the lowest quartile. The results of the analyses examining for significant determinants of Ox-LDL at baseline are shown in Table 14 and Table 15.

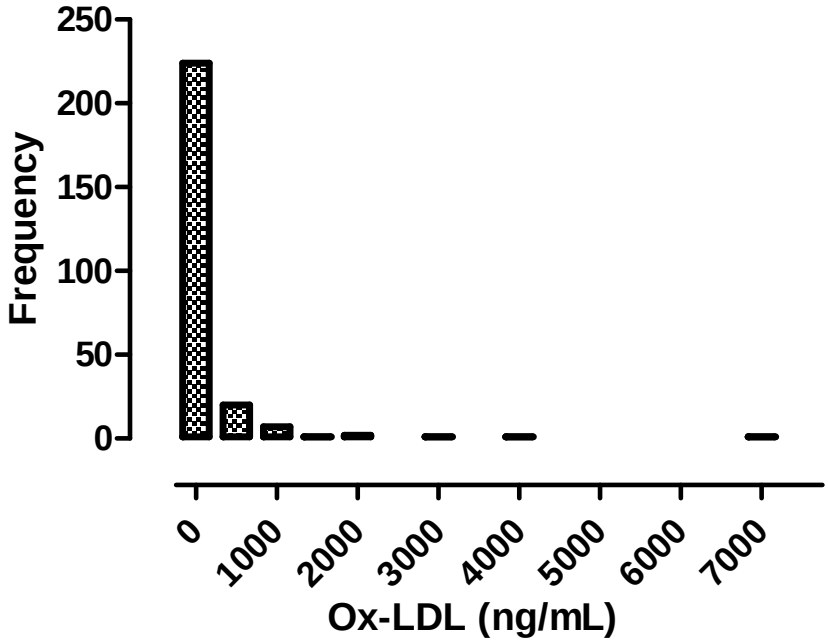


Figure 34 - Frequency distribution of Ox-LDL levels at baseline. The distribution of data was heavily positively skewed. Of these samples 22% were below the lower limit of detection for the assay and high levels of Ox-LDL were present in a small number of samples.

From the analyses in Table 14 and Table 15 it can be seen that the Ox-LDL levels were significantly associated with PTH concentration and were higher in patients with ischaemic heart disease, peripheral vascular disease and left ventricular dysfunction. To examine for independent predictors of Ox-LDL in the dataset, stepwise ordinal logistic regression modelling the quartiles of Ox-LDL levels was performed as detailed in section 3.17.4. The independent variables initially entered into the model were those variables associated with Ox-LDL at a significance of $p<0.2$ in the bivariate analyses. The resultant model identified that a history of ischaemic heart disease, left ventricular dysfunction, higher phosphate and lower PTH levels were independently associated with higher quartiles of Ox-LDL concentration. The model satisfied the Brant test of parallel regression assumption and had an overall adjusted-R^2 of 0.03.

Table 14 - Determinants of Ox-LDL levels at baseline: analysis of continuous variables between quartiles of Ox-LDL.

	Quartiles of Ox-LDL				
	Q1	***Q2***	***Q3***	***Q4***	***p****
n	65	64	64	64	
Ox-LDL (ng/mL)	<8.9	8.9 - 34.0	36.2 - 96.8	>96.8	
Patient factors					
Age (yrs)	65.2 [±1.9]	63.2 [±1.9]	59.9 [±2.0]	65.5 [±2.2]	0.18
Weight (kg)	73.6 [59.4-84.1]	73.3 [62.1-85.5]	69.9 [56.7-81.2]	68.2 [57.0-78.6]	0.28
Pre-dialysis systolic BP (mmHg)	140.8 [±3.1]	132.7 [±2.7]	136.6 [±3.6]	136.5 [±2.8]	0.32
Pre-dialysis diastolic BP (mmHg)	71.2 [±1.7]	71.6 [±1.6]	72.5 [±1.9]	70.2 [±1.5]	0.79
Dialysis factors					
Time on renal replacement therapy (yrs)	3.7 [1.3-5.9]	3.4 [2.0-7.4]	3.4 [1.3-7.7]	3.9 [1.3-8.6]	0.77
Urea reduction ratio	0.75 [±0.01]	0.76 [±0.01]	0.74 [±0.01]	0.76 [±0.01]	0.39
Laboratory parameters					
Haemoglobin (g/dL)	11.6 [±0.2]	11.4 [±0.1]	11.7 [±0.2]	11.7 [±0.2]	0.76
Ferritin (µg/L)	454 [±32]	490 [±21]	464 [±31]	491 [±27]	0.73
C-reactive protein (mg/L)	6.9 [2.9-14.9]	6.5 [2.8-19.9]	6.8 [1.8-13.9]	6.5 [1.7-14.3]	0.80
Albumin (g/L)	37.8 [±0.6]	38.2 [±0.5]	37.9 [±0.5]	38.0 [±0.6]	0.97
Cholesterol (mmol/L)	3.8 [±0.1]	4.1 [±0.1]	3.9 [±0.1]	4.0 [±0.1]	0.45
High-density lipoprotein (mmol/L)	1.1 [±0.0]	1.1 [±0.1]	1.1 [±0.0]	1.2 [±0.1]	0.50
Low-density lipoprotein (mmol/L)	2.0 [±0.1]	2.2 [±0.1]	2.1 [±0.1]	2.1 [±0.1]	0.73
Triglycerides (mmol/L)	1.4 [0.9-2.0]	1.6 [1.2-2.2]	1.5 [1.1-2.0]	1.4 [1.0-2.1]	0.56
Bicarbonate (mmol/L)	21.9 [±0.3]	22.5 [±0.2]	21.6 [±2.3]	21.8 [±2.8]	0.22
Calcium (mmol/L)	2.39 [±0.03]	2.40 [±0.02]	2.4 [±0.02]	2.39 [±0.02]	0.88
Phosphate (mmol/L)	1.41 [±0.06]	1.49 [±0.06]	1.61 [±0.06]	1.50 [±0.07]	0.15
Parathyroid hormone (pmol/L)	23.5 [9.2-47.3]	24.3 [15.0-49.4]	22.9 [11.9-40.9]	15.6 [7.9-27.8]	0.04[†]
HbA1c (%)	8.0 [6.65-9.7]	7.1 [6.6-7.7]	6.8 [6.0-8.3]	6.9 [6.2-8.4]	0.22

Data presented as range, mean [±SEM] or median [IQR]. *The p-value was obtained by comparing the values between quartiles. If this omnibus test statistic was significant, pairwise comparisons between quartiles were performed looking for significant differences at the 5% level (after applying a Bonferroni correction to the α-level for multiple comparisons) with significant findings indicated on the table**.** ([†]Q4 vs Q2). BP: Blood pressure.

Table 15 - Determinants of Ox-LDL levels at baseline: categorical variables.

			n	Ox-LDL (ng/mL) Median [IQR]	p
Patient factors:	Sex	Male	154	38.3 [9.0 - 103.1]	0.29
		Female	103	32.1 [4.3-87.9]	
	Ethnicity	Caucasian	198	39.5 [11.5-101.7]	0.30
		Asian	44	31.8[7.4-72.7]	
		Black	13	4.3[4.3-174.8]	
		Other*	2	26.4 [25.1 - 27.6]	
	Smoking history	Never smoked	116	30.1 [4.3-60.7]	0.17
		Current smoker	57	44.3 [13.7-121.6]	
		Ex-smoker	84	40.0 [18.1-107.7]	
	Dialysis access	Fistula	215	31.3 [6.8-81.5]	0.18
		Catheter	38	51.5 [9.8-120.8]	
		Graft*	4	60.0 [25.7-324.1]	
Co-morbidities:	Diabetes	Yes	73	40.1 [4.3-109.6]	0.87
		No	184	33.1 [11.9-86.3]	
	Ischaemic heart disease	Yes	73	42.8 [20.6-174]	0.01
		No	184	30.2 [5.7-69.7]	
	Malignancy	Yes	17	31.3 [10.2-341.1]	0.60
		No	240	35.1 [7.9-96.5]	
	Peripheral vascular disease	Yes	69	48.9 [16.2-205.3]	0.02
		No	188	32.0 [6.7-68.2]	
	Left ventricular dysfunction	Yes	24	57.4 [16.1-363.9]	0.047
		No	233	33.2 [6.2-80.4]	
Drugs:	ACEi / A2RBs / DRI	Yes	82	32.1 [4.3-59.6]	0.15
		No	175	38.1 [13.4-105.5]	
	β-blockers	Yes	54	38.3 [20.0-80.1]	0.58
		No	203	33.3 [6.6-101.4]	
	Statins	Yes	144	32.3 [6.3-100.8]	0.65
		No	113	40.2 [8.8-92.3]	
	Aspirin	Yes	116	32.7 [8.8-100.6]	0.59
		No	141	38.4 [7.2-97.6]	
	Clopidogrel	Yes	19	54.5 [11.6-224.8]	0.29
		No	238	33.6 [7.4-96.0]	
	Dipyridamole	Yes	3	15.0 [4.3-58.1]	0.40
		No	254	35.1 [8.5-99.1]	
	Warfarin	Yes	16	31.0 [9.7-282.9]	0.82
		No	241	36.2 [8.2-97.6]	
	Sulphonylureas	Yes	13	42.8 [14.2-73.9]	0.72
		No	244	33.5 [7.9-100.7]	
	Insulin	Yes	53	39.1 [4.3-114.1]	0.51
		No	204	33.6 [13.0-86.3]	

*Excluded from statistical analysis owing to small group size. ACEi: Angiotensin converting enzyme inhibitors; A2RBs: Angiotensin II receptor blockers; DRI: Direct renin inhibitors

6.1.2 Determinants of baseline TBARS levels

In addition to the Ox-LDL levels, a second marker of oxidative stress, TBARS, was also measured in the study participants. To examine the relationship between TBARS and Ox-LDL levels, TBARS levels were compared across quartiles of Ox-LDL (see Figure 35) and were found not to differ significantly (p=0.13).

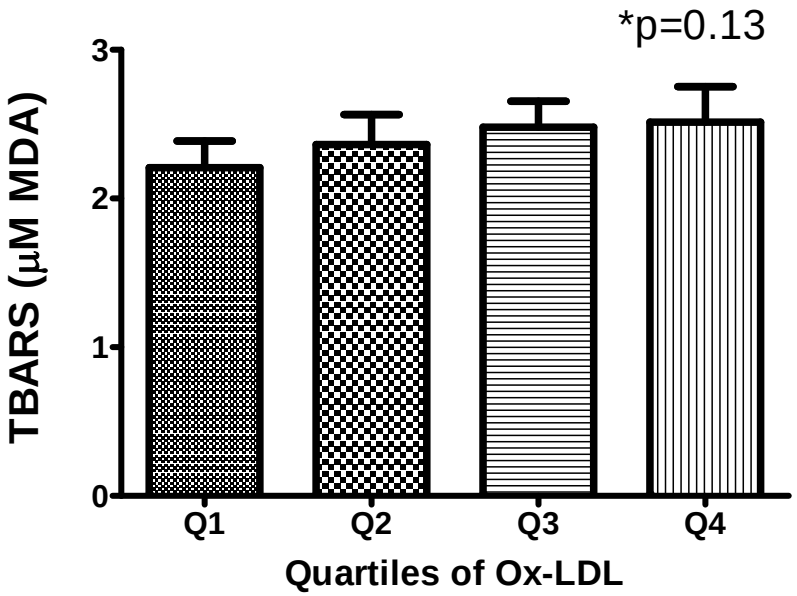


Figure 35 - Mean (±95% CI) baseline TBARS levels by quartiles of Ox-LDL. The mean TBARS levels did not differ between quartiles of Ox-LDL (*p for trend)

Table 16 and Table 17 detail the results of the analyses examining for the significant determinants of TBARS at baseline. The only statistically significant determinant of TBARS in unadjusted analyses was the bicarbonate levels (r=-0.15; p<0.05). The initial backwards stepwise variable selection procedure modelling the baseline TBARS levels, including all of the variables associated with TBARS levels at a significance of p<0.2, identified higher ferritin and HDL levels, lower bicarbonate levels and male sex to be independently associated with increased TBARS levels. A review of the residual versus fitted regression diagnostic plot identified a number of cases with high TBARS levels which may have been influencing the model. A total of 5 cases fulfilled the *a priori* criteria for having an outlying (high) TBARS level (see section 3.17.4) and the variable selection procedure was repeated after excluding these cases. The resultant model, based on 251 cases, identified lower bicarbonate and higher HDL levels to be independently associated with increased TBARS levels; the final model adjusted-R^2 was 0.04.

Table 16 - Determinants of TBARS at baseline: continuous variables.

	n	*r*	*p*
Patient factors			
Age	260	0.03	0.59
Weight	260	-0.06	0.35
Pre-dialysis systolic blood pressure	260	-0.06	0.32
Pre-dialysis diastolic blood pressure	260	-0.06	0.34
Dialysis factors			
Time on renal replacement therapy	260	0.05	0.39
Urea reduction ratio	260	0.02	0.80
Laboratory parameters			
Haemoglobin	260	0.10	0.11
Ferritin	260	0.12	0.06
C-reactive protein	260	-0.10	0.12
Albumin	260	-0.07	0.27
Cholesterol	259	-0.02	0.79
High-density lipoprotein	259	0.12	0.06
Low-density lipoprotein	254	-0.04	0.55
Triglycerides	260	-0.08	0.17
Bicarbonate	260	-0.15	0.02
Calcium	260	-0.03	0.62
Phosphate	260	-0.07	0.25
Parathyroid hormone	248	-0.06	0.32
HbA1c	72	-0.02	0.88

r: Correlation coefficient

Table 17 - Determinants of TBARS at baseline: categorical variables.

			n	TBARS (μM) Mean [±sem]	p
Patient factors:	Sex	Male	156	2.4 [±0.1]	0.17
		Female	104	2.3 [±0.1]	
	Ethnicity	Caucasian	199	2.4 [±0.1]	0.71
		Asian	46	2.5 [±0.2]	
		Black	13	2.3 [±0.2]	
		Other*	2	2.8	
	Smoking history	Never smoked	118	2.3 [±0.1]	0.49
		Current smoker	57	2.3 [±0.1]	
		Ex-smoker	85	2.5 [±0.1]	
	Dialysis access	Fistula	218	2.4 [±0.1]	0.62
		Catheter	38	2.3 [±0.1]	
		Graft*	4	2.3 [±0.2]	
Co-morbidities:	Diabetes	Yes	74	2.3 [±0.1]	0.32
		No	186	2.4 [±0.1]	
	Ischaemic heart disease	Yes	74	2.5 [±0.1]	0.10
		No	186	2.3 [±0.1]	
	Malignancy	Yes	17	2.7 [±0.1]	0.14
		No	243	2.4 [±0.1]	
	Peripheral vascular disease	Yes	71	2.4 [±0.1]	0.99
		No	189	2.4 [±0.1]	
	Left ventricular dysfunction	Yes	24	2.4 [±0.2]	0.92
		No	236	2.4 [±0.1]	
Drugs:	ACEi / A2RBs / DRI	Yes	83	2.4 [±0.1]	0.61
		No	177	2.4 [±0.1]	
	β-blockers	Yes	55	2.5 [±0.1]	0.32
		No	205	2.4 [±0.1]	
	Statins	Yes	147	2.4 [±0.1]	0.51
		No	113	2.4 [±0.1]	
	Aspirin	Yes	117	2.3 [±0.1]	0.30
		No	143	2.4 [±0.1]	
	Clopidogrel	Yes	20	2.3 [±0.2]	0.66
		No	240	2.4 [±0.1]	
	Dipyridamole	Yes	3	2.0 [±0.5]	0.44
		No	257	2.4 [±0.1]	
	Warfarin	Yes	16	2.4 [±0.2]	0.89
		No	244	2.4 [±0.1]	
	Sulphonylureas	Yes	13	2.3 [±0.2]	0.67
		No	247	2.4 [±0.1]	
	Insulin	Yes	54	2.4 [±0.1]	0.74
		No	206	2.4 [±0.1]	

* Excluded from statistical analysis owing to small group size; sem: standard error of the mean. ACEi: Angiotensin converting enzyme inhibitors; A2RBs: Angiotensin II receptor blockers; DRI: Direct renin inhibitors

6.2 Temporal changes in oxidative stress and the effects of vitamin E

6.2.1 12-month changes in Ox-LDL levels

Figure 36 shows the Ox-LDL levels at each study visit and Figure 37 the change in Ox-LDL levels after 12 months. There were no significant differences in the Ox-LDL levels between the groups at baseline (p=0.10) or at 12 months (p=0.35). Comparison of the baseline and 12-month levels within groups revealed no significant difference in the control group (p=0.13) but a statistically significant increase in the VE group (27.6 [4.3-65.4] vs 32.8 [4.3 - 102.4] ng/mL, p=0.02). This test, however, was not significant at the pre-specified 5% level after applying a Bonferroni correction to adjust for the increased familywise type 1 error rate associated with multiple pairwise testing. A comparison of the change in Ox-LDL levels after 12 months did not differ between the groups (p=0.63) (see Figure 37) and a Friedman's test within groups for changes in Ox-LDL levels across the three sampling points was not significant in either the control (p=0.18) or VE (p=0.07) groups.

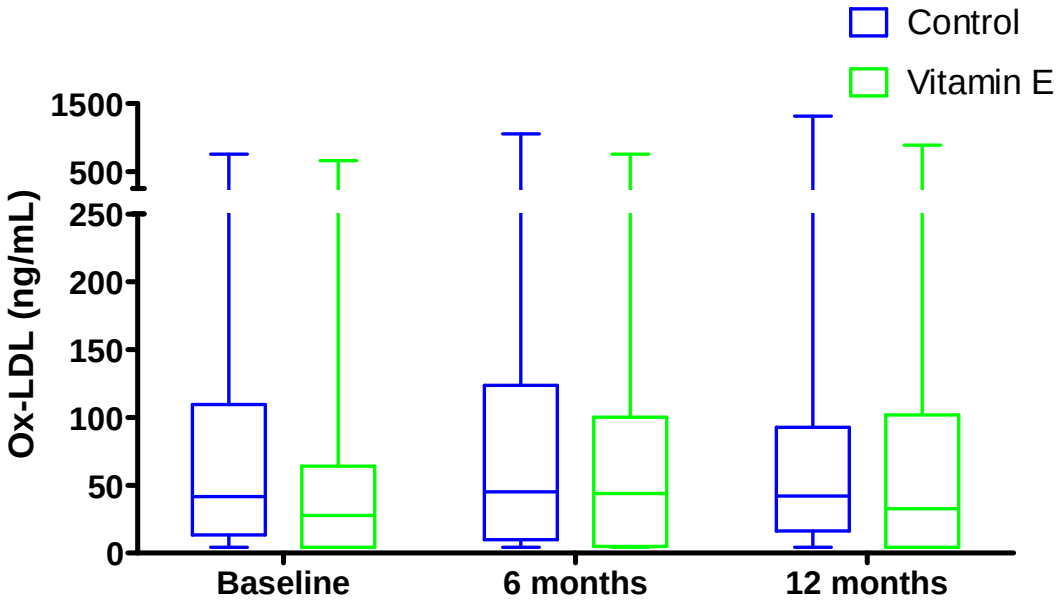


Figure 36 - Comparison of the Ox-LDL levels in patients randomised to the control or Vitamin E membrane.

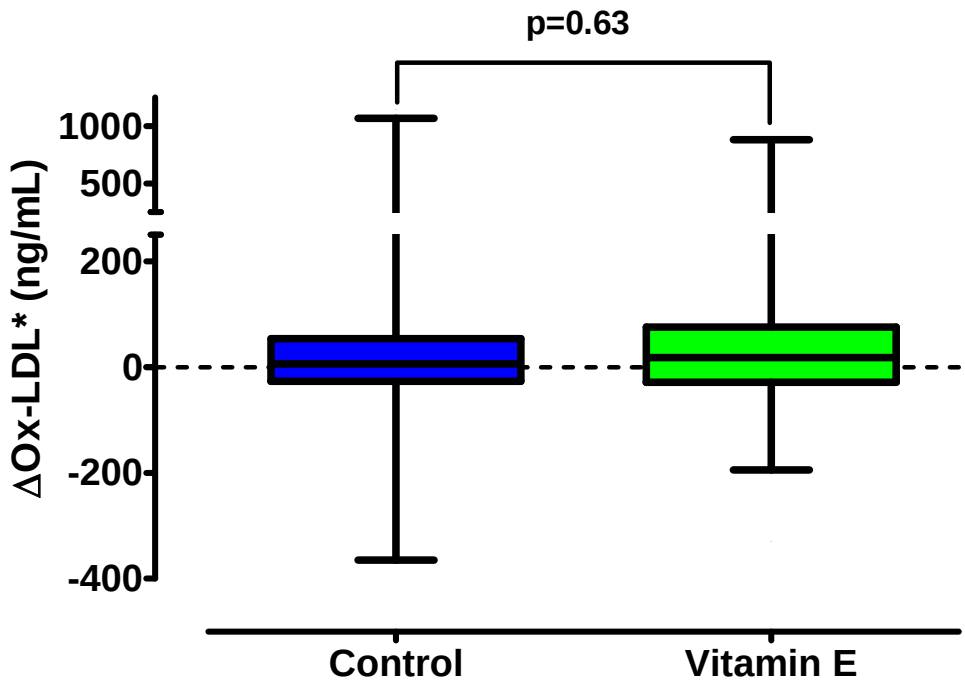


Figure 37 - Comparison of the 12 month change in Ox-LDL levels for patients randomised to the control or Vitamin E membrane.
(*ΔOx-LDL = $Ox\text{-}LDL_{12months}$ - $Ox\text{-}LDL_{baseline}$)

6.2.2 12-month changes in TBARS levels

Figure 38 displays the mean TBARS levels measured at each of the three sampling points for both study groups; levels were similar between groups at baseline (p=0.40) and 12-months (p=0.60). A repeated measures ANOVA of the normally distributed TBARS data found the levels to increase significantly across sampling points (p=0.045) but no effect of study group (p=0.49) or interaction between time and study group (p=0.70) were observed. Comparing the change in TBARS levels between groups similarly found no significant difference (p=0.75) as shown in Figure 39. There was no significant correlation between the 12-month change in Ox-LDL and TBARS levels (r=-0.02, p=0.78).

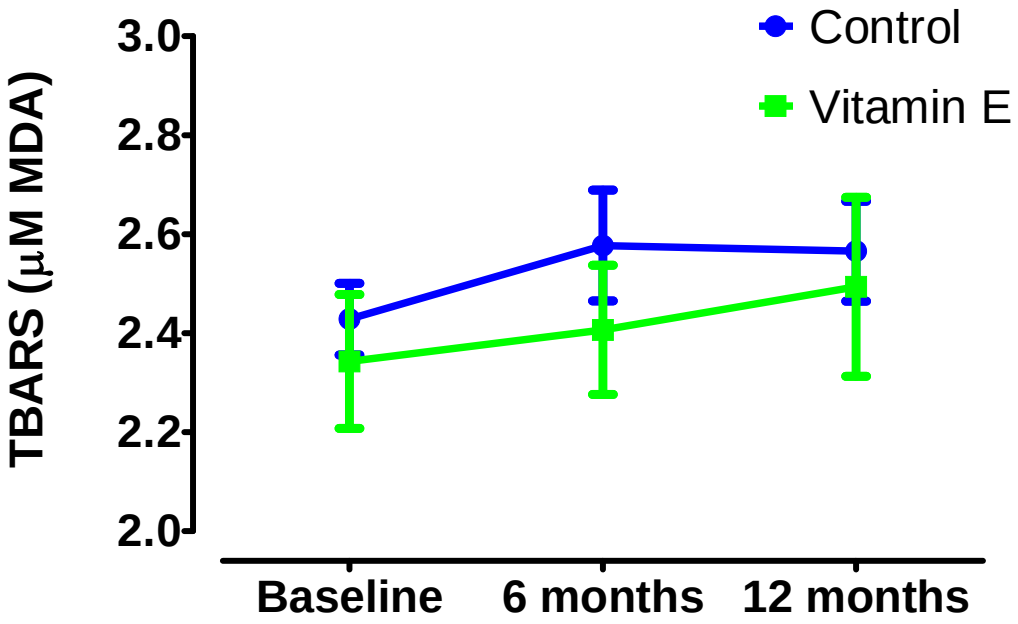


Figure 38 - A graph displaying the mean (±95% CI) TBARS levels at each of the sampling points for the two groups. The levels increased during the study period (p=0.045 for trend) but there was no difference between the two groups.

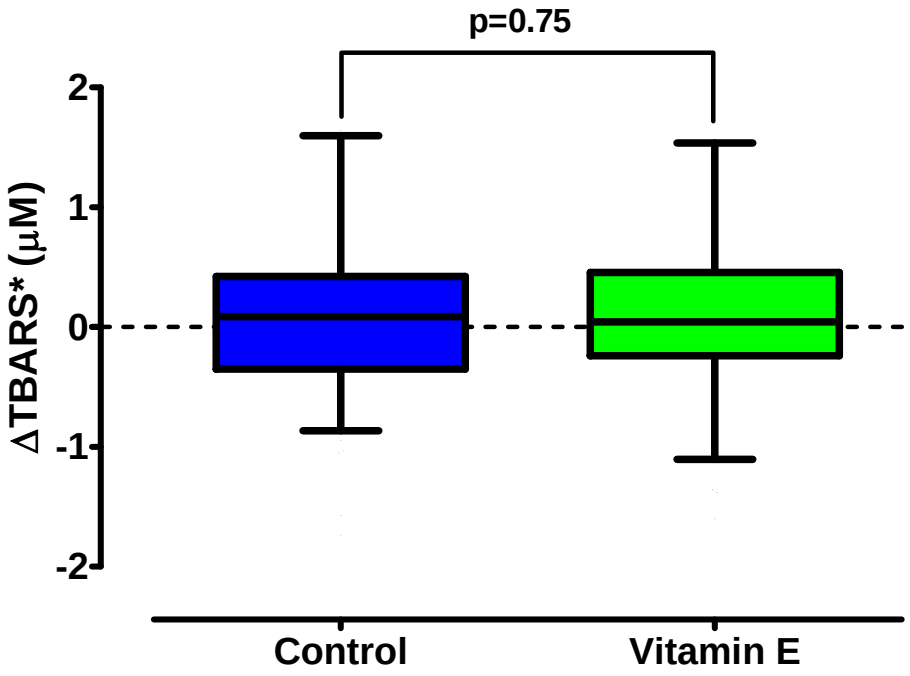


Figure 39 - Comparison of the 12 month change in TBARS levels between patients randomised to the control and vitamin E membranes. There was no significant difference in the 12 month change in TBARS levels between groups. (*ΔTBARS = $TBARS_{12months}$ - $TBARS_{baseline}$).

6.3 Oxidative stress and ESA resistance index

At baseline there was no difference in ERI when patients were compared between quartiles of Ox-LDL (p=0.67) and the ERI was not correlated with TBARS levels (r=0.03, p=0.65). After 12-months there were no significant correlations between the change in ERI and either the change in Ox-LDL or TBARS levels (r=0.01, p=0.84 and r=-0.04, p=0.57 respectively). Given the apparent differential effect of the VE-membrane conditional on the baseline ERI, the oxidative stress biomarkers were similarly examined after dividing the study population into tertiles of ERI at baseline. These analyses revealed no significant differences in the baseline levels of Ox-LDL (p=0.96) or TBARS (p=0.86) between the tertile nor were there any differential effects of the VE-membrane on the 12-month change in Ox-LDL (p>0.44 in all cases) or TBARS (p>0.49 in all cases) within any of the tertiles.

6.4 Discussion

Vitamin E has been widely used as an anti-oxidant owing to its ability to interrupt free radical propagated chain reactions leading to the formation of a tocopheroxyl radical [733]. As blood-membrane interactions are thought to be a major source of oxidative stress in HD patients there is a rationale for bonding VE to the dialysing surface of HD membranes. As discussed in section 1.4.2, oxidative stress appears to be increased in HD patients and may underlie a number of the complications they encounter including ESA-resistance, inflammation and the increased rates of CV disease. In the present study two biomarkers of lipid peroxidation were measured: Ox-LDL and TBARS.

The initial marker of oxidative stress measured in the study samples was Ox-LDL and there were a number of concerns regarding the accuracy and validity of this dataset. Of all the samples measured, 22% were below the lower limit of detection for the assay and 71% had concentrations which fell between the lowest two standards in a region of the standard curve which, by nature of the curve fitting process, was likely to be poorly reproducible between assays. The low QC sample had an Ox-LDL concentration intermediate between the 2nd and 3rd standard, a more reproducible region of the standard curve, yet had an inter-assay CoV of 19%, suggesting the variability in the study samples was likely to be even greater. The accuracy and reproducibility of the assay may have been improved by analysing the study samples at a lower dilution than the manufacturer recommended 1:10 but this option was not considered by the R&D technicians performing the assay nor discussed with the investigator (Dr Simon Lines) as concerns with the dataset only became apparent after all of the samples had been analysed. Due to financial constraints

it was not possible to purchase more ELISA kits to repeat all of the assays at a lower dilution.

In terms of the absolute Ox-LDL levels measured in the present study - it is hard to place these in the context of the published literature. A variety of commercial Ox-LDL ELISA's are available and have been used in published studies although they vary in methodology, with some being sandwich ELISAs and others competitive ELISAs, and in the antigenic specificity of the antibodies used [949]. Furthermore the levels of Ox-LDL reported in seemingly similar HD populations vary greatly [858, 891, 950-956] even in studies using ELISA's from the same manufacturers. Indeed, one study using two different commercial Ox-LDL ELISAs to measure samples from the same patients reached differing conclusions with the two assays highlighting this inconsistency [957]. It is thus difficult to establish whether the apparent low levels of Ox-LDL measured in the study samples were indeed reflective of low levels of oxidative stress or represented an anomaly given the concerns with the assay. Perhaps most relevant to the present study in this regard was the study by Calo *et al*. [891] which measured Ox-LDL in HD patients using the same commercial ELISA kits as used here yet they reported approximately 10-fold higher Ox-LDL levels. Differences between the two studies such as the use of plasma rather than serum, smaller patient numbers, the exclusion of patients with diabetes and the use of low-flux dialysers in the Calo *et al*. [891] study may be responsible for the contrasting results although whether these differences could account for such a large discrepancy is unclear. In order to try and determine whether the apparent low levels of Ox-LDL measured in the present study were a result of methodological problems with the assay or were indeed reflective of low levels of oxidative stress, it was decided to perform an additional measure of oxidative stress and to this end a TBARS assay was developed and utilised.

Unlike the Ox-LDL ELISA the TBARS assay is very non-specific. The use of TBARS as a measure of oxidative stress has been employed for over 30 years [958] and is predicated on the reactivity of TBA towards MDA [959]. Malondialdehyde is a side product of enzymatic arachidonic acid oxygenation and an end product of oxidative lipid peroxidation and it forms a fluorescent red adduct with TBA [959, 960]. The central tenet of the assay is that more lipid peroxidation results in higher MDA levels, and hence the formation of more MDA:TBA adducts, the levels of which can be measured spectrophotometrically or fluorometrically. There are, however, widespread criticisms of the assays with particular reference to its lack of specificity and sensitivity. For example TBA has been shown to react with a variety of compounds, besides MDA, such as sugars, amino acids, bilirubin

and albumin [961]. Despite lipid peroxidation being the most abundant source of MDA, it can also be produced by non-oxidative mechanisms such as heavy metal catalysed degradation of amino acids and sugars [962] or during prostaglandin metabolism [963, 964]. The reaction between TBA and MDA is dependant on a number of factors, chiefly reaction pH and temperature, and in the absence of a standardised methodology, comparing absolute levels between studies may be misleading. However, despite these valid criticisms concerning the assay, it has been, and continues to be, widely reported as an outcome measure in studies examining oxidative stress. Indeed, of the several studies examining the effects of VE coated modified cellulose membranes, three reported the effects on oxidative stress using TBARS as an outcome measure [864, 866, 965], and when the results were pooled in a meta-analysis an overall beneficial effect of these VE membranes on TBARS was observed [884].

As with the Ox-LDL assay, the TBARS levels in the study samples on the whole were low. Again, at the lower concentration ranges where most of the study samples were clustered, the assay was not particularly reproducible with high intra- and inter-assay variabilities. This arose because the best fit regression line through the standards was relatively flat at low concentrations (see Figure 11 and Figure 12) thus small changes in optical absorbance translated into correspondingly larger changes in concentration, as compared to samples intersecting the steeper portions of the graph (i.e. at greater TBARS concentrations). Despite the concerns regarding the lack of standardised methodology for TBARS determination and its non-specific nature, the levels of TBARS in the present study population were broadly in keeping with other published studies of HD patients [332, 405, 866, 966, 967] and the overall low levels were consistent with the Ox-LDL data.

Interestingly there was little agreement between the two markers of oxidative stress both in terms of the associations with other variables at baseline and the change in each parameter after 12 months. Both TBARS and Ox-LDL are putative markers of lipid peroxidation and thus one may expect them to be highly correlated. Although there was no significant difference in the mean TBARS levels when patients were divided into quartiles on the basis of Ox-LDL levels, there was a non-significant trend towards increasing TBARS levels across quartiles of increasing Ox-LDL concentration (see Figure 35). Published studies measuring both have produced varying results with some reporting good agreement [968] and others reporting no correlation [969] or different time courses of response to antioxidant interventions [895]. The Ox-LDL levels in the present study were quantified by an ELISA and are thus specific for molecules possessing the epitope

recognised by the antibodies - MDA-modified apolipoprotein B 100. However Ox-LDL is not a distinct entity as the oxidation of LDL is a complex process leading to oxidative changes in both the lipid and protein components, for example resulting in a number of different changes to apolipoprotein B amino acids and cross-linking, or the formation of aldehydes and ketones within the lipid moiety [970]. It is therefore possible that one specific oxidative modification of Ox-LDL, as recognised by the antibodies used in the ELISA, may not be reflective of the overall oxidative burden. The non-specific nature of the TBARS assay has already been highlighted. Thus an explanation for the lack of agreement between the two measures of oxidative stress measured in the present study may simply be that they are measuring different things, albeit both related to lipid peroxidation. Another explanation for the lack of agreement between these two biomarkers may be that it is a reflection of the poor reproducibility of one or both of the assays such that a true association exists but it was not detected using the methodologies employed here.

Despite consistency in the literature reporting higher TBARS in HD patients compared to healthy controls [332, 405, 966, 967], in line with the widely accepted dogma that oxidative stress is enhanced in HD patients, the literature on Ox-LDL levels is less clear cut with some studies reporting Ox-LDL levels to be higher in HD patients compared to healthy controls [417, 418] with others reporting the converse to be true [954, 955]. The use of different ELISA kits to measure Ox-LDL, as already discussed, may partly explain these discrepancies. Another part of the explanation for these conflicting findings may be the flux of HD membrane used. In the studies reporting higher levels of Ox-LDL in HD patients [417, 418] low-flux dialysers were used whereas the study by Ribeiro *et al*. [955], which reported lower levels in HD patients compared to controls, used high-flux membranes. In another study reporting lower levels in HD patients compared to controls [954] no comment was made on the dialyser flux. Wanner *et al.* [355] previously demonstrated a reduction in Ox-LDL levels after 6 weeks when patients switched from low- to high-flux polysulfone dialysers. More recently, however, Schneider *et al.* [919] reported significant reductions in Ox-LDL levels after 12 months for patients dialysing with both low- and high-flux membranes with no significant difference between the two. The mechanisms by which dialysis with high-flux membranes might lead to a lowering of Ox-LDL levels are not immediately obvious. Several early short term studies, involving relatively small numbers of patients, suggested a benefit of high-flux dialysis in terms of improving the lipid profile in HD patients [971-974], which in turn could theoretically influence Ox-LDL levels. However in all of these studies a low-flux modified cellulose membrane was compared to a high-flux

polysulfone membrane making it hard to attribute the findings to membrane flux over composition. A subsequent randomised controlled trial comparing high and low-flux polysulfone membranes reported no difference in lipid profiles [975]. It thus seems likely that membrane composition was the important factor in the earlier studies reporting improvements in lipid profiles [971-974] rather than permeability characteristics. This makes logical sense as LDL has a molecular weight of between 2.4-3.9 MDa [976, 977] (contrast this with albumin 67 kDa and β2-microglobulin 11 kDa) and thus even high-flux membranes are likely to be impermeable to LDL. There are *in vitro* data that polysulfone can adsorb LDL onto its surface [978], although the contribution of this to overall circulating LDL and Ox-LDL levels are unknown, but this might form part of the explanation through which membrane composition could influence lipid profiles. From the studies presented here it is hard to unpick the individual contributions of membrane composition, flux and time on dialysis to the levels of circulating Ox-LDL levels. It is therefore unclear whether the seemingly low Ox-LDL levels in the present cohort were reflective of low levels of oxidative stress or simply that patients were dialysing with high-flux biocompatible membranes. The potential for differences in dialyser flux or composition to alter oxidative stress levels highlights one of the strengths of the present study in that both groups were dialysed with high-flux polysulfone membranes of similar performance characteristics with the only difference being the presence or absence of the VE coating.

It would therefore appear that the levels of oxidative stress, as measured by Ox-LDL and TBARS, were low in the study cohort. This is important to bear in mind when assessing an anti-oxidant intervention such as the VE-bonded membrane used in the present study. As the levels of oxidative stress at baseline were low in the study cohort, it would potentially be harder to show any improvements with anti-oxidant interventions as compared to cohorts with higher levels of oxidative stress. Furthermore both of the assays used in the present study were not particularly accurate or reproducible over the concentration ranges of analyte present in the majority of study samples, thereby limiting the ability to detect subtle differences between subgroups or changes over time. These factors need to be considered when making inferences from these datasets. Prior to examining the effects of the VE-bonded membrane on Ox-LDL and TBARS levels, the significant determinants of these oxidative stress biomarkers in the baseline dataset were evaluated.

6.4.1 Baseline determinants of oxidative stress biomarkers

A number of published studies have examined various markers of oxidative stress and how they relate to disease in HD patients with particular emphasis on CV disease.

Analysis of the baseline Ox-LDL data in the present study revealed the levels to be higher in patients with a prior history of CV disease which is consistent with studies both in haemodialysis [956] and non-renal populations [979-983]. There are a wealth of mechanistic data on the so called “oxidative modification hypothesis” [412] whereby the atherogenicity of circulating LDL is greatly enhanced by oxidative modification to substantiate the association observed in the present study. Oxidised-LDL has been found to have a role in endothelial injury [984-986], foam cell formation [987, 988] and enhanced expression of pro-inflammatory genes [985, 989, 990] which are all key events in the development of atherosclerotic lesions and ultimately CV disease. The observed correlation between Ox-LDL and comorbidity score, as defined in the present study (section 3.7.1), may reflect that three out of the seven disease domains (ischaemic heart disease, peripheral vascular disease and left ventricular dysfunction) used in the scoring system come under the broad umbrella of CV disease and all were significantly associated with Ox-LDL levels in bivariate analyses.

In contrast with the Ox-LDL levels, there was no association between TBARS and prevalent CV disease. In one of the largest studies published to date examining the association between biomarkers of oxidative stress and CV disease in HD patients, Boaz *et al*. [396] reported higher TBARS levels in patients with prevalent CV disease. Compared with the present study the Boaz *et al.* [396] study consisted of fewer patients (76 vs 260) and included a higher proportion of patients with pre-existing CV disease (58% vs 42% using the criteria given by the authors); no comment was made about the proportion of patients with diabetes other than insulin dependent patients were excluded. Thus differing patient characteristics between the studies may explain the inconsistent findings. However it is hard to completely reconcile the lack of association between TBARS and prevalent CV disease in the present study given the widely accepted association between biomarkers of oxidative stress and CV disease in HD populations [324, 396, 397, 956, 991] and the findings of a significant association with Ox-LDL levels.

The lack of a significant association between Ox-LDL and LDL levels was an unexpected finding. Several studies have reported a positive correlation between Ox-LDL and LDL levels in various patient groups including dialysis patients [955], elderly patients [992], patients with type 2 diabetes [993] and familial hypercholesterolaemia [994] as well as healthy subjects [995, 996]. However other studies, in line with the results from the present study, have reported no correlation between Ox-LDL and cholesterol or LDL levels [997, 998]. An association between Ox-LDL and LDL levels would make biological sense given

that Ox-LDL levels are likely to reflect both levels of oxidative stress as well as the levels of available substrate, i.e. LDL. Reasons for the lack of significant association are not clear but potential mechanisms include differential membrane adsorption or a consequence of dialysis with high-flux dialysis membranes.

Higher TBARS levels were independently associated with higher ferritin levels at baseline. A positive association between oxidative stress and ferritin levels has previously been reported in healthy subjects [999] and patients with type 2 diabetes mellitus [1000]. Iron is stored in the body as ferritin molecules, limiting the toxicity associated with free iron, however iron can be released from ferritin by the action of reducing agents, converting Fe^{3+} to Fe^{2+}, particularly in the setting of low antioxidant levels [1001] as are commonly found in HD patients (see section 1.4.2.2). Free iron has been shown to be involved in the formation of oxygen free radicals *in vivo* [1002], a process which can be attenuated to some extent by iron chelation [1003]. Thus it would appear that ferritin has a role both in terms of limiting iron toxicity and providing a potential source of iron capable of exacerbating oxidative stress [1004] to explain the positive association between TBARS and ferritin levels in the present study. Intravenous iron, frequently administered to HD patients, is another potential source of free iron and has been shown to increase oxidative stress both in HD [366] and chronic kidney disease [1005, 1006] patients. However, there was no correlation between iron dose and ferritin levels nor between iron dose and either of the markers of lipid peroxidation measured in the present study suggesting intravenous iron was not a significant contributor to oxidative stress levels in the study cohort.

A positive association between HDL and oxidative stress, as evidenced by a positive association with TBARS in the present study, has been similarly reported in the general population [1007]. The HDL molecule has antioxidant properties conferred principally by the presence of its constituent antioxidant enzymes, paraoxonase and glutathione peroxidase, and the presence of methionine residues on apolipoproteins [1008-1010]. High-density lipoproteins may also have a role in the extraction of oxidised lipid from LDL [1011, 1012] although no significant associations between Ox-LDL and HDL levels were observed in the present study. Additionally, in the presence of systemic inflammation and oxidative stress, there is evidence that the anti-oxidant capabilities of HDL are reduced [1013, 1014], as has been observed in HD patients [1015]. Thus the finding of a positive association between TBARS and HDL levels may represent up-regulation of HDL synthesis in the face of increased oxidative stress.

The lack of an association between diabetes and oxidative stress in the present study is interesting. Diabetes is widely known to be associated with increased levels of oxidative stress in non-dialysis populations [1016, 1017] particularly in the presence of diabetic complications or poor diabetic control [1018-1023]. In contrast to these studies no associations between diabetes and either of the markers of oxidative stress measured were observed in the present study. This may have arisen because the pro-oxidant stimuli associated with HD and renal failure may mask any additional contribution from the presence of a diabetic state. Contrary to this supposition, Dursun *et al.* [1024] reported the effects of HD and diabetes to be additive in terms of oxidative stress as quantified by TBARS levels and anti-oxidant enzyme activity; however little information on the patient characteristics were provided to permit a meaningful comparison with the findings of the present study.

From an appreciation of the associations in the present dataset, in the context of the published literature, it would appear that both Ox-LDL and TBARS were reflective of oxidative stress levels in the patient cohort, at least to some extent, and that the overall levels were low. With reference to Chapter 5 concerning the anaemia data, low levels of oxidative stress may potentially be a contributory factor to explain the low ESA usage for LTHT patients compared to other UK HD populations in the UK Renal Registry Reports [4, 921] although no data are available to substantiate this supposition. In summary the baseline data are broadly in keeping with the published literature concerning the determinants of Ox-LDL and TBARS in HD patients thus providing an appropriate setting to investigate the effects of a VE-bonded dialysis membrane on these biomarkers of oxidative stress.

6.4.2 Changes in oxidative stress over time and the effects of vitamin E

Analyses of the TBARS data revealed a significant increase in the levels across study visits with no differential effect of the VE-bonded membrane; no such trend was observed with the Ox-LDL levels. The data in Figure 39 would suggest that the TBARS levels only increased in slightly over 50% of patients, rather than it being a universal phenomenon. It is therefore possible that the increasing trend in TBARS levels was driven by a subset of patients with large increases in TBARS levels rather than being reflective of all patients; particularly given the use of parametric statistical testing (ANOVA) rather than rank-based non-parametric methods. It was also possible, however, that the increasing trend was

reflective of enhanced oxidative damaged accrued with time on dialysis. In support of this others have shown a positive correlation between time on HD and increased markers of oxidative damage, such as TBARS levels [1025], and negative correlations with levels of antioxidants, such as ubiquitol and α-tocopherol [332]. However, no negative correlation between time on dialysis and either of the markers of oxidative stress measured in the present study was found making this a less likely explanation for the results observed here. As discussed in the previous section, the prevailing levels of oxidative stress in the study cohort was low at baseline. It was therefore more probable, on subsequent determinations, that the levels would increase rather than decrease further - the corollary of the regression to the mean phenomenon. Thus the observed increase in TBARS levels may have been a consequence of inadvertent sample selection bias, such that patients with low levels of oxidative stress were entered into the study.

Concerning the anti-oxidant potential of VE, a number of studies have examined the effects of VE-coated modified cellulose membranes on biomarkers of oxidative stress. Many of these were included in a systematic review [884] which reported an overall beneficial effect of the VE membranes. The present study aimed to address a slightly different question, namely does VE-coating of the more biocompatible polysulfone membranes confer similar benefits? The principal finding of this study with respect to biomarkers of oxidative stress, or more specifically lipid peroxidation, was that no improvements were observed after 12-months with the VE membrane.

Several studies, of relatively short duration and involving small numbers of patients, have measured the effects of VE-bonded polysulfone membranes on biomarkers of oxidative stress with differing results. Two studies [889, 894] reported no improvements in various biomarkers, including total anti-oxidant capacity, markers of protein oxidation and α- and γ-tocopherol levels, after several months with the VE-bonded polysulfone membrane compared to a similar non VE-bonded equivalent membrane. However two different studies [891, 895], this time measuring different biomarkers of oxidative stress including Ox-LDL, TBARS and ADMA, did report improvements with the VE membrane. An obvious difference between the studies reporting positive and negative effects of the VE membrane was the choice of biomarkers measured. The potential to draw differing conclusions depending on the oxidative stress biomarker measured is typified by the present study which showed a lack of consistency between Ox-LDL and TBARS levels in terms of their significant determinants.

Interestingly the two studies which measured Ox-LDL levels, as measured in the present study [891, 895], both reported a fall in the levels following HD with the VE membrane. In the study by Calo *et al*. [891] it is difficult to interpret the effects of the VE-membrane as patients were switched from a low-flux polysulfone membrane (Dr Lorenzo Calo, personal communication, July 2012) to a high-flux VE-coated polysulfone membrane and the Ox-LDL levels were lower after 12 months compared to baseline values. As already discussed, dialyser flux may influence Ox-LDL levels thus the observed reduction in Ox-LDL levels may be a consequence of increased dialyser flux rather than an effect of the VE-membrane. In the second study Morimoto *et al*. [895] reported improvements in Ox-LDL and TBARS levels although these were determined in LDL fractions, obtained by sequential ultracentrifugation, rather than in whole serum as was performed in the present study. Other important differences between the Morimoto *et al*. study [895] and the present study to potentially explain the apparent differing conclusions include fewer patient numbers (31 vs 261), a higher preponderance of females (52% vs 40%) and patients with diabetes (82% vs 28%) in the published study. Additionally the authors provided no information on dialyser flux or delivered dialysis dose although reported a mean dialyser surface area of 1.55 m^2. The lowest surface area dialyser used in the present study was 1.8 m^2 with a significant number using 2.1 m^2 thus the delivered dialysis dose was likely to be greater in the present study. Theoretically this could alter oxidative stress levels, perhaps as a consequence of greater VE-exposure with the larger surface area membranes, although there are no published data to support this. Additionally the mean haemoglobin level in the Morimoto *et al.* study [895] at baseline was 9.8 g/dL with a mean EPO dose of 5416 IU/week which suggests much higher levels of ESA resistance than in the present study (mean haemoglobin 11.6 g/dL; median EPO dose (calculated by applying correction factor of 200) 4000 IU/week) suggesting fundamental differences in the patient cohorts. As discussed in Chapter 5, the higher ESA-resistance may be a consequence of higher levels of inflammation or oxidative stress and therefore the response to the VE-membrane may be expected to differ between the two study populations.

Thus, from the very small number of patients included in published studies to date, it is not possible to form a consensus on the effect of VE-bonded polysulfone membranes on oxidative stress given the conflicting findings. Additionally the choice of oxidative stress biomarkers, and the methods by which they are measured, appears to be of prime importance. The present study, in view of the number of patients recruited, parallel group design, length of follow-up and the pre-specified oxidative stress endpoint, is well placed to

contribute significantly to this evidence base. From these combined data it would appear that there is no convincing evidence that the use of VE-coated polysulfone membranes results in improvements in Ox-LDL or TBARS levels. A potential corollary of this finding is that the non-VE bonded polysulfone membranes are in fact very biocompatible in terms of their ability to induce oxidative stress - perhaps approaching the limit of what may be achievable with synthetic materials, thus the additional benefits of a VE-coating, unlike that observed with the less biocompatible modified cellulose membranes, are small.

In this chapter the effects of a VE-bonded polysulfone membrane on two biomarkers of oxidative stress, Ox-LDL and TBARS, have been evaluated and no evidence of benefit was observed. One of the putative mechanisms through which VE-bonded membranes might improve ERI, as discussed in the last chapter, is through a reduction in oxidative stress. As there was no apparent improvement in the oxidative stress biomarkers in the current study, the question as to whether improving oxidative stress for patients dialysing with biocompatible polysulfone membranes results in improvements in ESA resistance remains unanswered. Perhaps a more important question, however, is whether the use of the VE-bonded membranes translates into clinical benefits for patients through reductions in CV disease rates, morbidity and mortality. The data with respect to these endpoints are presented and discussed in Chapter 9.

Chapter 7 : Inflammation

There are a wealth of observational data linking increased levels of inflammatory biomarkers, such as CRP and IL-6, with adverse outcomes in HD patients including reduced quality of life [1026], increased ESA resistance [923, 924] and increased mortality, particularly from CV disease [163, 429, 435, 439, 440, 505, 506]. There are also mechanistic data linking various components of the inflammatory cascade with these disease processes such as evidence of a role for CRP [510-513] or complement components [56, 511, 547-549] in atherosclerosis and inflammatory mediators in ESA resistance [147, 156-160, 227, 927, 928]. Despite these observational data, evidence that treating inflammation *per se*, as distinct from ameliorating conditions which contribute to inflammation such as infection, translates into benefits for HD patients is lacking. There is, however, a logic that such approaches may improve outcomes for HD patients. In the present study a number of components of the inflammatory response were measured: CRP, C3, SC5b-9, factor D and properdin levels. The relationships between these biomarkers and the patient characteristics were explored in the baseline dataset. The 12-month longitudinal data were then evaluated to determine if these inflammatory parameters changed over time and to determine if there were any effects of dialysis with a VE-bonded membrane.

7.1 Determinants of baseline inflammation

The associations between the inflammatory markers CRP, C3, SC5b-9, factor D and properdin and the independent variables in the baseline dataset were evaluated for their key determinants as detailed in section 3.17.4.

7.1.1 Determinants of baseline CRP levels

In bivariate analyses, the CRP levels were positively correlated with weight, C3, ferritin and triglyceride levels, negatively correlated with HDL and calcium levels (see Table 18), were higher in patients with a prior history of ischaemic heart disease (see Table 19) and lower in patients receiving renin-angiotensin medications (see Table 20) and in patients in the lowest quartile of SC5b-9 levels (see Table 21). There were no differences in CRP levels when patients were compared on the basis of diabetic status or in patients dialysing with CVCs compared to AVFs (see Table 19). Multivariate regression analysis using a backwards stepwise variable selection procedure and the variables associated with CRP at a significance of $p<0.2$ as described in section 3.17.4, initially identified the independent

predictors of higher CRP levels to be male sex, higher C3, SC5b-9 and ferritin levels, lower albumin levels and that the CRP levels were lower in patients of Black ethnicity. However, review of the residuals versus fitted regression diagnostic plot revealed a highly heteroscedastic distribution of the errors. The variable selection procedure was therefore repeated modelling the logarithm of the CRP levels. In this model, higher C3, SC5b-9 and ferritin levels and lower albumin, calcium and HDL levels were independently predictive of higher CRP levels and the CRP levels were lower in Blacks. This model had an adjusted-R^2 of 0.25, constant error variance and was not influenced by the presence of outliers.

7.1.2 Determinants of baseline C3 levels

The C3 levels were positively correlated with age, weight, cholesterol, triglyceride and CRP levels, negatively correlated with blood pressure and time on dialysis (see Table 18) and were lower in current smokers, Caucasians compared to Asians (see Table 19), in patients receiving renin-angiotensin medications and patients not receiving statins (see Table 20); the levels also differed across quartiles of Ox-LDL (see Table 22). There were no significant associations between C3 levels and the presence of diabetes or CV disease (see Table 19). An initial regression model constructed using a backwards stepwise variable selection procedure and the variables associated with C3 levels at a significance of $p<0.2$ retained the variables age, weight, systolic blood pressure, ethnicity and cholesterol, CRP, LDL, factor D and properdin levels. However this model was influenced by multicollinearity as evidenced by a positive regression coefficient for cholesterol and a negative correlation coefficient for LDL, despite these variables being positively correlated with each other in the dataset ($r=0.90$, $p<0.0001$), and the high VIFs for these variables (5.9 and 6.1 respectively). The LDL / cholesterol ratio was calculated but this was not correlated with the C3 levels with sufficient statistical significance to justify inclusion in the variable selection procedure ($r=-0.07$, $p=0.29$). The modelling procedure was therefore repeated retaining the cholesterol and excluding the LDL levels as cholesterol had the strongest association with C3 levels on bivariate testing (see Table 18). This final model had an adjusted-R^2 of 0.33 and identified the independent predictors of higher C3 levels were increased age and weight, higher levels of CRP, cholesterol, factor D, and properdin levels, lower systolic blood pressure and Asian ethnicity.

7.1.3 Determinants of baseline SC5b-9 levels

Of the samples measured at baseline, 41 (16%) had SC5b-9 levels below the limit of detection for the assay and were assigned a value of 34.4 ng/mL which was the concentration of the lowest standard. The associations between SC5b-9 and the

continuous predictor variables in the dataset were evaluated by comparing them between quartiles of SC5b-9 as detailed in section 3.17.4. In bivariate analyses the quartiles of SC5b-9 were positively associated with factor D and CRP levels and dialysis dose, negatively associated with calcium and HbA1c levels (see Table 21) and the SC5b-9 levels were lower in patients receiving sulphonylureas (see Table 20). There was a non-linear relationship with the albumin levels such that they were higher in the third compared to fourth quartile of SC5b-9 levels (see Table 21). Backwards stepwise ordinal logistic regression modelling of the SC5b-9 quartiles was performed initially including all of the variables associated with SC5b-9 levels at a significance of $p<0.2$ as described in section 3.17.4. The initial model identified sulphonylurea therapy, higher HbA1c and lower factor D levels to be independently associated with lower quartiles of SC5b-9 concentrations. However this model was only based on 72 observations as it was censored for non-diabetics owing to the inclusion of HbA1c levels in the variable list for model selection; the procedure was therefore repeated after excluding this variable. The resultant model again identified sulphonylurea therapy and lower factor D levels to be associated with lower quartiles of SC5b-9 but also retained the CRP levels which had a positive association with the quartiles of SC5b-9. The final model had a pseudo adjusted-R^2 of 0.06 and satisfied the Brant test of parallel regression assumption.

7.1.4 Determinants of baseline factor D levels

In unadjusted analyses the levels of factor D were negatively correlated with LDL, calcium, PTH and TBARS levels (see Table 18), positively associated with SC5b-9 levels (see Table 21) and were lower in patients receiving beta-blockers or renin-angiotensin medications (see Table 20) and in patients with a diagnosis of malignancy (see Table 19). An initial regression model was constructed using a backwards stepwise variable selection procedure and all of the variables associated with factor D levels at a significance of $p<0.2$. This model identified the factor D levels were independently positively associated with higher C3 levels, higher quartiles of SC5b-9, lower bicarbonate and LDL levels and were lower in Asians. Review of the regression diagnostic plots suggested the analyses were influenced by a small number of cases with high factor D levels. A total of 12 cases fulfilled the *a priori* criteria for having an outlying factor D level as described in section 3.17.4 and were therefore excluded from the subsequent analysis. Repeating the variable selection procedure after excluding these cases generated a model which identified lower factor D levels were independently associated with increased calcium, PTH or cholesterol levels or patients receiving renin-angiotensin medications; this final model had an adjusted-R^2 of 0.10.

7.1.5 Determinants of baseline properdin levels

The only statistically significant association with the properdin levels in the unadjusted analyses was a positive correlation with phosphate levels (see Table 18). The initial backwards stepwise variable selection procedure, including all of the variables associated with the baseline properdin levels at a significance of $p<0.2$, rejected all of the variables but was censored for non-diabetics owing to the inclusion of HbA1c levels. The procedure was therefore repeated after omitting HbA1c levels from the variable list and the resultant model retained only C3 levels which were positively associated with properdin levels; the model had an adjusted-R^2 of 0.02. A review of the residual versus fitted regression diagnostic plot identified a number of cases with high properdin levels, including 2 samples with properdin levels above the upper limit of the assay which had been assigned values of 280 µg/mL. A total of 6 cases fulfilled the *a priori* criteria set out in section 3.17.4 for having outlying properdin levels and the modelling procedure was repeated after excluding them from the dataset. The resultant model had an adjusted-R^2 of 0.08 and identified that higher properdin levels were independently associated with lower dialysis dose and albumin levels, higher PTH levels and were lower in patients receiving renin-angiotensin medications.

Table 18 - Determinants of CRP, C3, factor D and properdin levels at baseline: continuous variables.

		CRP		C3		Factor D		Properdin	
	n	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Patient factors									
Age	*260*	0.10	*0.11*	0.18	*<0.01*	0.04	*0.57*	0.02	*0.78*
Weight	*260*	0.14	*0.02*	0.38	*<0.0001*	-0.08	*0.18*	0.09	*0.15*
Pre-dialysis systolic blood pressure	*260*	-0.08	*0.22*	-0.14	*0.02*	-0.04	*0.52*	0.02	*0.77*
Pre-dialysis diastolic blood pressure	*260*	-0.10	*0.11*	-0.19	*0.002*	-0.08	*0.22*	0.01	*0.81*
Dialysis factors									
Time on renal replacement therapy	*260*	0.11	*0.07*	-0.14	*0.03*	0.08	*0.19*	0.00	*0.96*
Urea reduction ratio	*260*	0.04	*0.49*	-0.07	*0.28*	0.10	*0.12*	-0.09	*0.17*
Laboratory parameters									
Haemoglobin	*260*	-0.05	*0.43*	-0.07	*0.25*	0.06	*0.37*	0.08	*0.21*
Ferritin	*260*	0.13	*0.04*	0.11	*0.09*	0.00	*0.99*	-0.02	*0.75*
Albumin	*260*	-0.10	*0.10*	0.04	*0.57*	0.09	*0.16*	-0.11	*0.07*
Cholesterol	*259*	0.02	*0.77*	0.15	*0.02*	-0.12	*0.06*	0.05	*0.44*
High-density lipoprotein	*259*	-0.16	*0.01*	-0.10	*0.09*	-0.06	*0.36*	0.00	*0.96*
Low-density lipoprotein	*254*	0.01	*0.88*	0.10	*0.13*	-0.14	*0.03*	0.05	*0.45*
Triglycerides	*259*	0.14	*0.02*	0.33	*<0.0001*	0.00	*0.94*	0.04	*0.48*
Bicarbonate	*260*	-0.10	*0.12*	-0.10	*0.11*	-0.11	*0.07*	-0.06	*0.33*
Calcium	*260*	-0.17	*<0.01*	0.06	*0.38*	-0.14	*0.02*	0.10	*0.11*
Phosphate	*260*	-0.01	*0.91*	0.00	*0.96*	-0.06	*0.31*	0.15	*0.02*
Parathyroid hormone	*248*	0.10	*0.11*	0.01	*0.91*	-0.13	*0.04*	0.12	*0.06*
HbA1c	72	0.04	*0.75*	0.06	0.62	0.07	0.53	-0.20	0.09
Inflammation biomarkers									
C-reactive protein	*260*	1.00	*1.00*	0.35	*<0.0001*	0.00	*0.95*	-0.07	*0.28*
C3	*260*	0.35	*<0.0001*	1.00	*1.00*	0.11	*0.07*	0.12	*0.06*
Factor D	*260*	0.00	*0.95*	0.11	*0.07*	1.00	*1.00*	-0.03	*0.60*
Properdin	*260*	-0.07	*0.28*	0.12	*0.06*	-0.03	*0.60*	1.00	*1.00*
Oxidative stress									
TBARS	*260*	-0.10	*0.12*	-0.10	*0.15*	-0.13	*0.03*	0.06	*0.33*

r: Correlation coefficient

Table 19 - Determinants of CRP, C3, SC5b-9, factor D and properdin levels at baseline: Patient factors and comorbidities.

		n	CRP (mg/L)	p	C3 (mg/mL)	p	SC5b-9 (ng/mL)	p	Factor D (µg/mL)	p	Properdin (µg/mL)	p
Patient factors:												
Sex	Male	*156*	6.9 [2.7-16.0]	0.17	0.77 [±0.02]	0.88	68.5 [39.8-104.7]	0.74	3.5 [2.9-4.2]	0.65	53.5 [43.9-67.2]	0.51
	Female	*104*	6.2 [1.7-14.0]		0.77 [±0.02]		66.6 [39.7-122.7]		3.3 [2.9-4.2]		55.2 [41.8-74.7]	
Ethnicity	Caucasian	*199*	6.8 [2.4-14.5]	0.052	0.76 [±0.01]	0.04†	66.8 [41.3-112.4]	0.50	3.5 [2.9-4.3]	0.12	53.3 [41.9-68.4]	0.77
	Asian	*46*	7.9 [1.6-21.0]		0.83 [±0.03]		63.4 [38.2-105.4]		3.2 [2.7-3.9]		56.3 [45.0-69.3]	
	Black	*13*	1.9 [0.5-6.7]		0.76 [±0.04]		70.8 [34.4-92.9]		3.6 [3.2-4.1]		54.0 [44.8-64.1]	
	Other*	*2*	5.2 [0.6-9.8]		0.69 [±0.06]		121.1 [86.5-155]		4.0 [3.4-4.5]		118.6 [97.9-139]	
Smoker	Never	*118*	5.6 [1.6-13.8]	0.09	0.77 [±0.02]	0.001‡	68.8 [39.4-122.3]	0.51	3.5 [2.9-4.2]	0.83	55.9 [44.3-73.1]	0.44
	Current	*57*	6.7 [2.1-14.3]		0.70 [±0.02]		57.0 [38.5-95.8]		3.4 [2.9-3.9]		54.0 [39.7-66.3]	
	Ex	*85*	8.3 [3.6-20]		0.82 [±0.02]		67.4 [43.4-101.9]		3.4 [2.8-4.2]		52.2 [43.3-64.7]	
Dialysis access	Fistula	*218*	6.3 [2.0-14.2]	0.28	0.78 [±0.01]	0.17	67.0 [38.8-112.4]	0.78	3.4 [2.9-4.2]	0.70	53.6 [43.2-69.3]	0.58
	Catheter	*38*	9.0 [2.1-19.7]		0.73 [±0.03]		69.1 [48.8-104.3]		3.6 [2.9-4.1]		53.6 [39.6-64.9]	
	Graft*	*4*	16.5		0.74 [±0.07]		58.0 [42.9-70.9]		4.1 [3.5-4.5]		69.3 [49.8-76.6]	
Comorbidities:												
Diabetes	Yes	*74*	6.1 [2.3-16.3]	1.0	0.79 [±0.02]	0.24	60.1 [38.4-104.3]	0.32	3.4 [2.8-4.2]	0.87	55.2 [44.4-77.9]	0.18
	No	*186*	6.9 [2.1-14.5]		0.76 [±0.01]		68.9 [40.1-110.8]		3.4 [2.9-4.1]		53.6 [42.3-67.1]	
IHD	Yes	*74*	9.8 [3.0-22.0]	<0.01	0.80 [±0.02]	0.13	67.2 [40.1-98.2]	0.89	3.5 [2.9-4.3]	0.48	53.5 [41.2-65.7]	0.52
	No	*186*	6.0 [1.8-13.0]		0.76 [±0.01]		67.0 [39.6-111.4]		3.4 [2.9-4.1]		54.3 [43.9-71.1]	
Malignancy	Yes	*17*	8.5 [5.9-15]	0.16	0.76 [±0.04]	0.76	78.6 [38.0-99.9]	0.89	3.0 [2.6-3.6]	0.04	51.2 [40.6-77.0]	0.90
	No	*243*	6.7 [1.9-14.6]		0.77 [±0.01]		66.8 [39.8-109.1]		3.5 [2.9-4.2]		53.8 [43.4-68.1]	
PVD	Yes	*71*	9.0 [3.2-18.1]	0.09	0.78 [±0.02]	0.73	63.7 [39.4-113.9]	0.94	3.5 [3.1-4.4]	0.36	53.5 [41.9-68.1]	0.85
	No	*189*	6.3 [1.8-14.5]		0.77 [±0.01]		67.2 [39.7-106.0]		3.4 [2.9-4.1]		54.0 [43.9-68.8]	
LV dysfunction	Yes	*24*	6.7 [2.2-12.8]	0.71	0.77 [±0.04]	0.73	55.7 [37.7-80.1]	0.15	3.5 [3.0-4.4]	0.36	46.8 [42.7-60.1]	0.14
	No	*236*	6.8 [2.1-16.5]		0.77 [±0.01]		67.3 [40.5-112.0]		3.4 [2.9-4.1]		54.7 [43.9-70.7]	

Data presented as mean [±SEM] or median [IQR]. *Excluded from analysis owing to small group size, †Caucasians vs Asians, ‡Current vs Never and Ex-smokers. IHD: Ischaemic heart disease, PVD: Peripheral vascular disease, LV: Left ventricular.

Table 20 - Determinants of CRP, C3, SC5b-9, factor D and properdin levels at baseline: Medications.

		n	CRP (mg/L)	*p*	C3 (mg/mL)	*p*	SC5b-9 (ng/mL)	*p*	Factor D (µg/mL)	*p*	Properdin (µg/mL)	p
RAS medications	Yes	*83*	5.9 [1.2-10.4]	*0.01*	0.73 [±0.02]	*0.02*	71.3 [38.5-112.4]	*0.80*	3.2 [2.7-3.8]	*0.03*	52.2 [39.9-64.8]	0.08
	No	*177*	7.8 [2.7-18.3]		0.79 [±0.01]		66.4 [39.7-103.2]		3.5 [3.0-4.3]		54.9 [44.2-71.4]	
β-blockers	Yes	*55*	6.9 [1.4-14.4]	*0.69*	0.76 [±0.02]	*0.45*	60.0 [37.8-90.8]	*0.19*	3.2 [2.6-4.1]	*0.047*	51.5 [44.4-64.2]	0.52
	No	*205*	6.7 [2.4-15.9]		0.78 [±0.01]		67.5 [40.0-113.9]		3.5 [3.0-4.2]		54.9 [41.8-72.1]	
Statins	Yes	*147*	7.0 [2.5-14.5]	*0.58*	0.79 [±0.01]	*0.03*	68.7 [44.7-103.5]	*0.46*	3.5 [3.0-4.3]	*0.26*	53.6 [43.9-71.4]	0.54
	No	*113*	6.6 [1.8-16.3]		0.74 [±0.02]		63.7 [38.1-111.8]		3.4 [2.7-4.1]		54.0 [41.1-66.8]	
Aspirin	Yes	*117*	7.2 [2.4-15.0]	*0.77*	0.78 [±0.02]	*0.29*	66.8 [47.5-111.6]	*0.29*	3.5 [3.0-4.4]	*0.11*	53.1 [44.1-66.3]	0.52
	No	*143*	6.7 [1.8-14.5]		0.76 [±0.02]		67.5 [38.2-106.5]		3.4 [2.8-4.0]		54.1 [41.6-71.0]	
Clopidogrel	Yes	*20*	6.7 [2.1-14.5]	*0.43*	0.78 [±0.05]	*0.93*	68.6 [45.4-94.0]	*0.67*	3.4 [2.7-5.4]	*0.87*	49.3 [38.6-64.0]	0.12
	No	*240*	10.1 [2.5-19.5]		0.77 [±0.01]		66.8 [39.4-108.6]		3.4 [2.9-4.1]		54.1 [43.9-70.7]	
Dipyridamole	Yes	*3*	2.8 [1.1-35]	*0.76*	0.75 [±0.04]	*0.81*	77.7 [44.7-107.2]	*0.80*	3.8 [3.4-6.1]	*0.27*	48.3 [39.4-53.3]	0.33
	No	*257*	6.8 [2.1-14.6]		0.77 [±0.01]		66.8 [39.6-107.9]		3.4 [2.9-4.2]		54.0 [43.1-68.8]	
Warfarin	Yes	*16*	10.8 [3.5-17.6]	*0.15*	0.77 [±0.03]	*0.97*	53.3 [38.1-77.3]	*0.21*	3.4 [2.8-3.8]	*0.67*	49.3 [42.5-67.8]	0.55
	No	*244*	6.5 [1.9-14.5]		0.77 [±0.01]		67.3 [40.5-110.3]		3.5 [2.9-4.2]		53.9 [43.5-68.3]	
Sulphonylureas	Yes	*13*	4.3 [1.3-7.5]	*0.12*	0.79 [±0.05]	*0.70*	38.5 [34.4-71.3]	*0.02*	3.5 [2.9-4.4]	*0.94*	60.3 [40.1-82.2]	0.51
	No	*247*	6.9 [2.1-15.3]		0.77 [±0.01]		67.5 [42.5-110.8]		3.4 [2.9-4.2]		53.6 [42.7-68.1]	
Insulin	Yes	*54*	9 [1.9-20.0]	*0.39*	0.78 [±0.02]	*0.73*	66.7 [44.1-117.9]	*0.58*	3.3 [2.7-4.1]	*0.37*	54.3 [44.2-71.4]	0.75
	No	*206*	6.7 [2.2-14.4]		0.77 [±0.01]		67.1 [39.2-106.8]		3.5 [2.9-4.2]		53.7 [42.6-68.2]	

Data presented as mean [±SEM] or median [IQR]. *Excluded from analysis owing to small group size. RAS: Renin-angiotensin system

Table 21 - Determinants of SC5b-9 levels at baseline: analysis of continuous variables across quartiles of SC5b-9.

	Q1	Q2	Q3	Q4	p
n	65	65	65	65	
SC5b-9 (ng/mL)	<39.8	39.8 - 66.8	67.2 - 106.7	>106.7	
Patient factors					
Age (yrs)	62.9 [±2.0]	64.3 [±1.9]	61.5 [±2.1]	64.7 [±2.0]	0.66
Weight (kg)	73.2 [62.2 - 79.0]	73.6 [61.3 - 90]	69.4 [57.5 - 78.4]	69.3 [56.4 - 82.9]	0.30
Pre-dialysis systolic blood pressure (mmHg)	138.3 [±2.8]	135.1 [±3.3]	136.0 [±3.2]	137.4 [±3.0]	0.88
Pre-dialysis diastolic blood pressure (mmHg)	72.8 [±1.6]	70.5 [±1.6]	72.3 [±1.7]	70.3 [±1.6]	0.62
Dialysis factors					
Time on renal replacement therapy (yrs)	3.7 [1.2 - 7.5]	3.0 [1.2 - 6.2]	3.4 [1.5 - 7.0]	4.9 [2.1 - 8.8]	0.11
Urea reduction ratio	0.75 [±0.01]	0.74 [±0.01]	0.74 [±0.01] §	0.77 [±0.01] ‡	0.02
Laboratory parameters					
Haemoglobin (g/dL)	11.7 [±0.2]	11.6 [±0.2]	11.5 [±0.2]	11.6 [±0.1]	0.87
Ferritin (µg/L)	455 [±31]	465 [±26]	495 [±28]	488 [±26]	0.70
Albumin (g/L)	37.5 [±0.6]	37.7 [±0.6]	39.4 [±0.4] §	37.2 [±0.5] ‡	0.02
Cholesterol (mmol/L)	3.9 [±0.1]	4.0 [±0.1]	3.9 [±0.1]	4.0 [±0.1]	0.86
High-density lipoprotein (mmol/L)	1.1 [±0.0]	1.2 [±0.1]	1.2 [±0.0]	1.2 [±0.0]	0.69
Low-density lipoprotein (mmol/L)	2.1 [±0.1]	2.1 [±0.1]	2.1 [±0.1]	2.1 [±0.1]	0.97
Triglycerides (mmol/L)	1.35 [1.0 - 2.0]	1.5 [1.1 - 2.4]	1.5 [1.0 - 2.0]	1.4 [0.9 - 2.1]	0.50
Bicarbonate (mmol/L)	22.0 [±0.3]	22.3 [±0.3]	21.6 [±0.3]	21.9 [±0.3]	0.42
Calcium (mmol/L)	2.43 [±0.02]	2.41 [±0.02]	2.35 [±0.02]	2.36 [±0.02]	0.04
Phosphate (mmol/L)	1.45 [±0.06]	1.53 [±0.06]	1.52 [±0.06]	1.51 [±0.06]	0.85
Parathyroid hormone (pmol/L)	22 [12 - 45]	22 [7 - 37]	23 [11 - 48]	21 [9 - 40]	0.59
HbA1c (%)	8.6 [6.8 - 9.8]	7.1 [6.7 - 8.1]	6.8 [5.7 - 7.4]	6.8 [6.4 - 8.0]	0.049
Inflammatory markers					
C-reactive protein (mg/L)	4.6 [1.4 - 8.7] †‡	8.2 [2.8 - 17.7]*	9.7 [3.5 - 20.4]*	8.3 [1.8 - 18.6]	0.01
C3 (mg/mL)	0.74 [±0.02]	0.79 [±0.02]	0.79 [±0.03]	0.77 [±0.02]	0.29
Factor D (µg/mL)	3.2 [2.7 - 3.7] ‡	3.4 [2.8 - 4.0] ‡	3.5 [2.8 - 4.1]* †	3.9 [3.2 - 4.7]	0.001
Properdin (µg/mL)	53.3 [45.2 - 63.9]	53.6 [44.9 - 65.0]	51.3 [39.9 - 72.5]	57.7 [39.4 - 74.6]	0.94
Oxidative stress					
Ox-LDL (ng/mL)	34.6 [4.3 - 124.8]	33.7 [13.5 - 81.5]	38.2 [16.5 - 61.4]	33.6 [4.3 - 106.4]	0.98
TBARS (µM MDA)	2.4 [±0.1]	2.3 [±0.1]	2.3 [±0.1]	2.5 [±0.1]	0.72

Data presented as range, mean [±SEM] or median [IQR]. The p-value is for significance of omnibus test. Pairwise *post hoc* testing was performed if omnibus test significant with significant differences between quartiles indicated as: vs *Q1, †Q2, ‡Q3 or §Q4.

Table 22 - Comparison of CRP, C3, SC5b-9, factor D and properdin levels between quartiles of Ox-LDL.

	Quartiles of Ox-LDL				
	Q1	**Q2**	**Q3**	**Q4**	***p***
n	65	64	64	64	
Ox-LDL (ng/mL)	<8.9	8.90 - 34.0	36.2 - 96.8	>96.8	
CRP (mg/L)	6.9 [2.9 - 14.9]	6.5 [2.8 - 19.9]	6.8 [1.8 - 13.9]	6.5 [1.7 - 14.3]	0.80
C3 (mg/mL)	0.80 [±0.02]	0.81† [±0.02]	0.72* [±0.02]	0.75 [±0.02]	0.01
SC5b-9 (µg/mL)	37.9 [14.9 - 62.9]	67.9 [45.3 - 97.9]	72.9 [45.0 - 105.6]	63.0 [37.6 - 117.2]	0.92
Factor D (µg/mL)	3.5 [3.0 - 3.9]	3.4 [2.9 - 4.1]	3.4 [2.6 - 4.1]	3.7 [3.2 - 4.4]	0.07
Properdin (µg/mL)	57.2 [46.3 - 68.8]	50.4 [42.7 - 74.3]	54.8 [44.0 - 74.5]	51.8 [41.0 - 63.8]	0.32

Data presented as range, mean [±SEM] or median [IQR]. The p-value is for significance of omnibus test. Pairwise *post hoc* testing was performed if omnibus test significant and significant differences between quartiles indicated: vs *Q2, †Q3

7.2 Temporal changes in inflammation and the effects of vitamin E

Analyses of the longitudinal data were undertaken to investigate the changes in inflammatory markers over time and to evaluate the influence of the VE-bonded membrane as detailed in section 3.17.5.

7.2.1 12-month changes in CRP levels

The CRP levels did not differ between groups at baseline (p=0.29) or 12-months (p=0.84) as shown in Table 23. Similarly, comparison of the 12-month and baseline CRP levels identified no significant differences in either the control (p=0.84) or VE (p=0.25) groups (see Table 23). A repeated measures ANOVA was performed on the log-transformed CRP levels, which approximated a normal distribution, and identified that the CRP levels changed significantly across study visits (p<0.001) but there was no significant effect of study group allocation (p=0.47) nor significant interaction between study group and time (p=0.94) (see Figure 40). Comparing the 12-month change in CRP levels between study groups similarly found no effect of the VE-bonded membrane (p=0.68, see Figure 41).

Table 23 - Analyses of baseline and 12-month data for the inflammatory markers CRP, C3, SC5b-9, factor D and properdin.

		Baseline	12 months	*p**
CRP (mg/L)	Control	7.1 [2.8 - 14.5]	5.2 [1.3 - 17.6]	*0.84*
	Vitamin E	5.6 [1.6 - 17.9]	6.5 [1.7 - 14.2]	*0.25*
	p†	*0.29*	*0.84*	
C3 (mg/mL)	Control	0.76 [±0.01]	0.69 [±0.02]	*<0.0001*
	Vitamin E	0.78 [±0.02]	0.71 [±0.02]	*<0.0001*
	p†	*0.50*	*0.49*	
sC5b-9 (ng/mL)	Control	61.0 [40.5 - 100.6]	57.8 [41.0 - 104.8]	*0.80*
	Vitamin E	67.5 [39.4 - 113.9]	72.5 [44.7 - 123.2]	*0.42*
	p†	*0.46*	*0.36*	
Factor D (µg/mL)	Control	3.5 [3.0 - 4.3]	3.6 [2.9 - 4.2]	*0.38*
	Vitamin E	3.4 [2.7 - 4.1]	3.4 [2.6 - 4.3]	*0.27*
	p†	0.06	0.31	
Properdin (µg/mL)	Control	51.5 [42.1 - 66.1]	49.6 [41.0 - 63.0]	*0.17*
	Vitamin E	56.4 [44.4 - 74.8]	51.5 [40.9 - 68.4]	*0.02*
	p†	*0.07*	*0.60*	

Data presented as mean [±SEM] or median [IQR].
*p-value for baseline vs 12 months; †p-value for between group comparisons

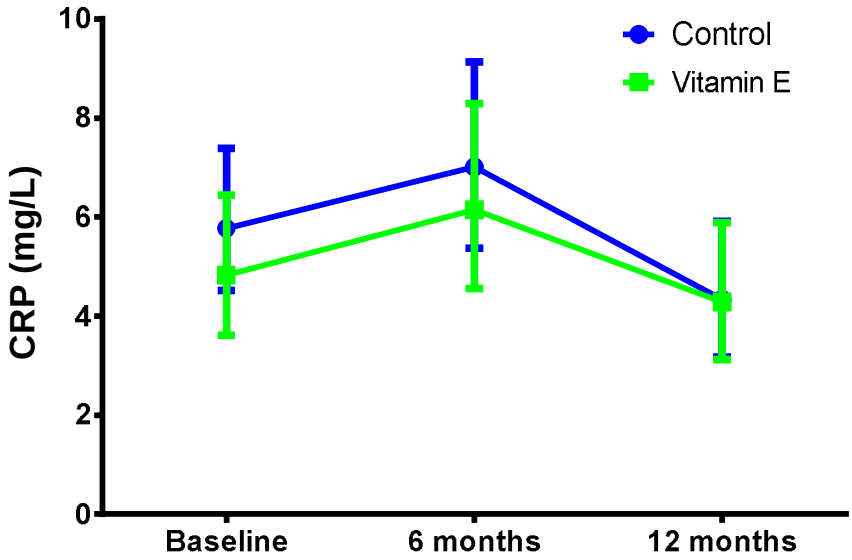


Figure 40 - CRP levels at baseline, 6 and 12 months in each study group. The CRP levels changed significantly across study visits (p<0.001) but there was no effect of study group (p=0.47) nor significant interaction between study group and time (p=0.94).

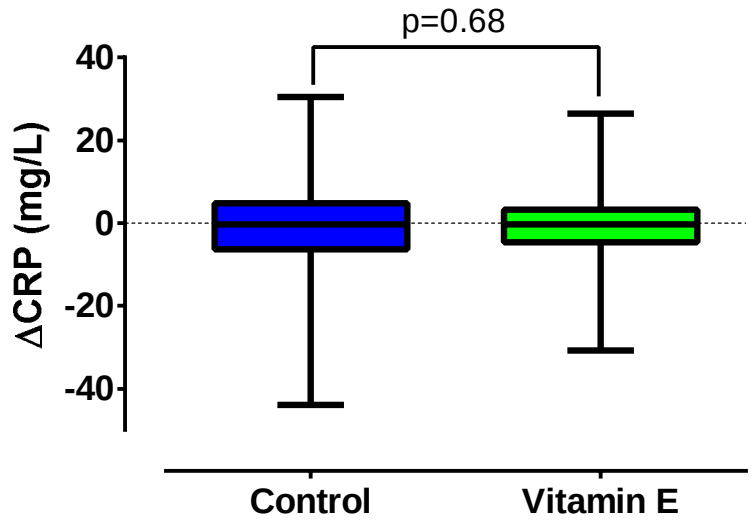


Figure 41 - Comparison of 12-month change in CRP levels between study groups. There was no significant difference in the 12-month change in CRP levels between study groups. ($\Delta CRP = CRP_{12months} - CRP_{baseline}$).

7.2.2 12-month changes in C3 levels

The C3 levels were similar in the control and VE groups at baseline (p=0.50) and 12 months (p=0.49) (see Table 23). The 12-month C3 levels were significantly lower than baseline in both the control (p<0.0001) and VE (p<0.0001) groups (see Table 23). A repeated measures ANOVA, analysing the C3 levels across study visits, identified that the changes in C3 over time were significant (p<0.0001) but there was no significant effect of study group allocation (p=0.38) nor significant interaction between study group and time (p=0.67) as shown in Figure 42. A comparison of the 12-month change in C3 levels between the two study groups found no significant difference (p=0.35, see Figure 43).

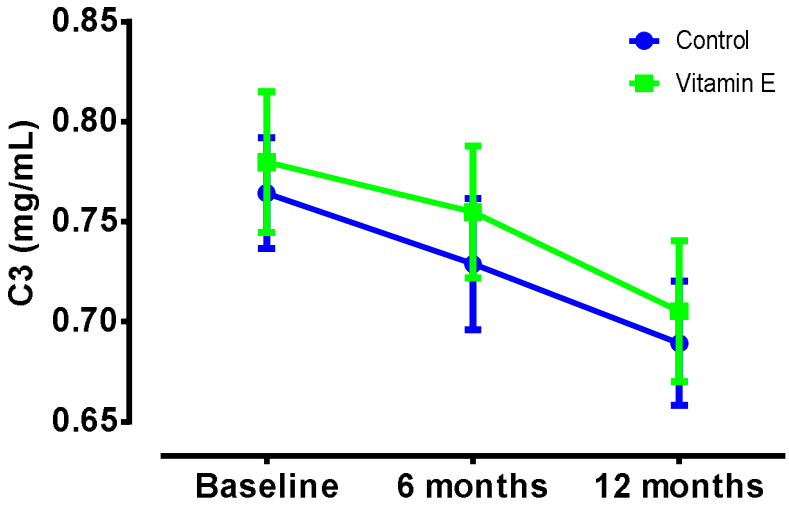


Figure 42 - C3 levels at baseline, 6 and 12 months in each study group. The C3 levels fell significantly across study visits (p<0.0001) but there was no significant effect of study group allocation (p=0.38) nor significant interaction between group and time (p=0.67).

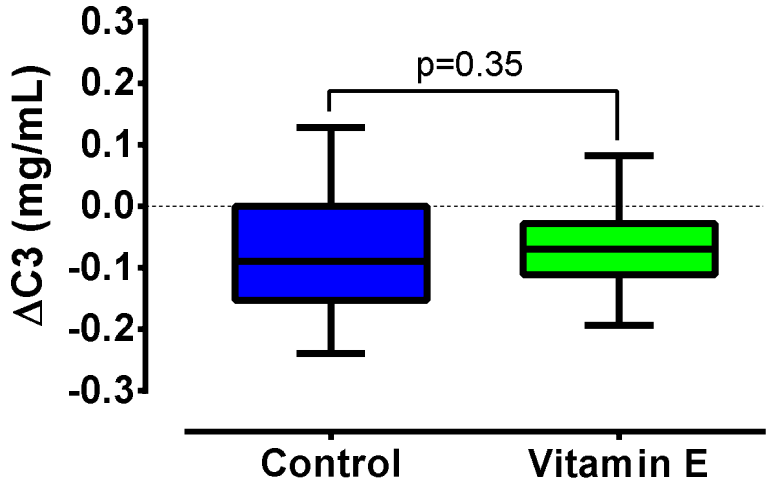


Figure 43 - Comparison of 12-month change in C3 levels between study groups. The C3 levels fell in both groups after 12 months in the majority of patients with no difference between the groups. ($\Delta C3 = C3_{12months} - C3_{baseline}$).

Given the striking finding of a significant fall in C3 levels across study visits, irrespective of the dialyser used, it was decided to explore the relationships between the changes in the modifiable variables correlated with C3 levels at baseline and the change in C3 levels. These variables were weight, blood pressure, cholesterol, triglycerides and CRP levels (see Table 18). The results of the correlation analyses looking for significant associations between the changes in these variables and the change in C3 levels are shown in Table 24. The change in C3 levels was significantly positively correlated with the change in weight, CRP, cholesterol and triglyceride levels. Using the change in these variables to model the change in C3 levels with multivariate regression generated a model with an adjusted-R^2 of 0.16 suggesting that 16% of the variance in the change in C3 levels in the dataset could be attributed to changes in weight and the levels of CRP, cholesterol and triglycerides.

Table 24 - Spearman's rank correlation coefficients for the 12-month change in C3 levels and the 12-month change in each of the variables significantly associated with C3 levels at baseline.

	r	*p*
ΔWeight	0.15	*0.03*
ΔSystolic blood pressure	0.01	*0.86*
ΔDiastolic blood pressure	0.04	*0.60*
ΔCholesterol	0.23	*<0.001*
ΔTriglycerides	0.14	*0.04*
ΔCRP	0.38	*<0.0001*

In each case, the change [Δ] was calculated by subtracting the baseline value from the 12-month value

7.2.3 12-month changes in SC5b-9 levels

As shown in Table 23, there were no significant differences in the SC5b-9 levels between groups at baseline (p=0.46) or 12-months (p=0.36), nor when the 12-month and baseline levels were compared in the control (p=0.80) or VE (p=0.42) groups. Owing to a significant number of the samples analysed having SC5b-9 levels below the lower limit of detection for the assay, it was not possible to transform the data such that it approximated a normal distribution therefore a repeated measures ANOVA was not performed. A Friedman's test examining for significant changes over time was not statistically significant in either the control (p=0.85) or VE (p=0.94) groups. The SC5b-9 levels at each study visit are depicted graphically in Figure 44. Comparison of the 12-month change in the SC5b-9 levels between study groups revealed no significant difference (p=0.48, see Figure 45).

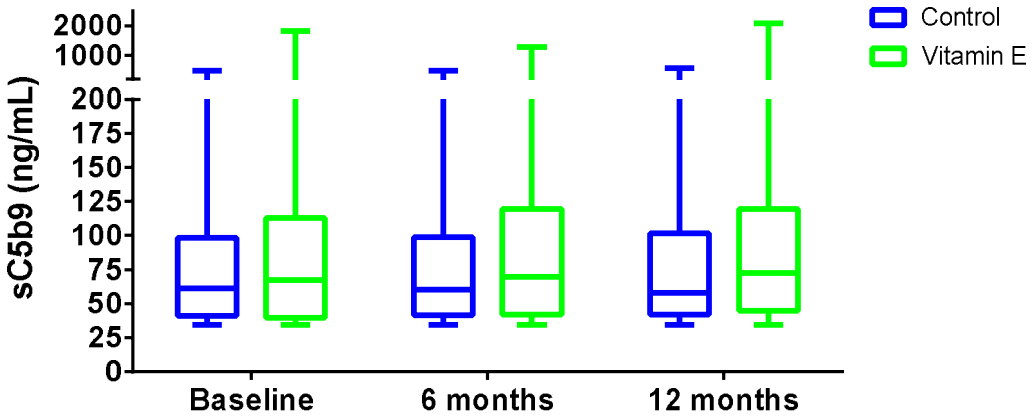


Figure 44 - SC5b-9 levels at baseline, 6 and 12 months in each study group There were no significant inter-group differences nor significant changes in the SC5b-9 levels across study visits.

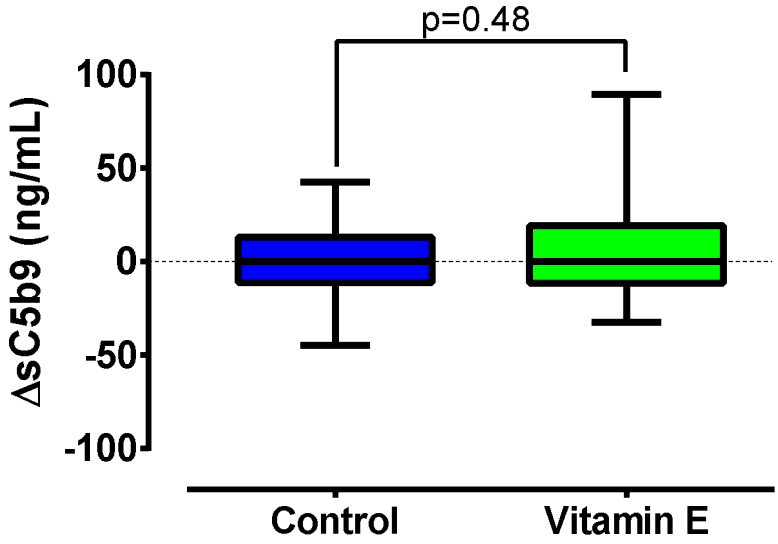


Figure 45 - Comparison of 12-month change in SC5b-9 levels between study groups. There was no significant difference in the 12-month change in SC5b-9 levels between study groups. (ΔSC5b-9 = SC5b-$9_{12months}$ - SC5b-$9_{baseline}$).

7.2.4 12-month changes in factor D levels

As shown in Table 23, there were no significant differences in the factor D levels between the groups at baseline (p=0.06) or 12-months (p=0.31), nor when the 12-month and baseline levels were compared in the control (p=0.38) or VE (p=0.27) groups. A repeated measures ANOVA was performed after transforming the factor D levels by calculating the reciprocals, which approximated a normal distribution, to examine for significant changes in the factor D levels across study visits and to look for any effects of study group allocation (see Figure 46). The factor D levels did not change significantly across study visits (p=0.06) nor was there any significant effect of study group allocation (p=0.13) or significant interaction between study group and time (p=0.44). Comparison of the 12-month change in factor D levels revealed no significant difference between the groups (p=0.92, see Figure 47).

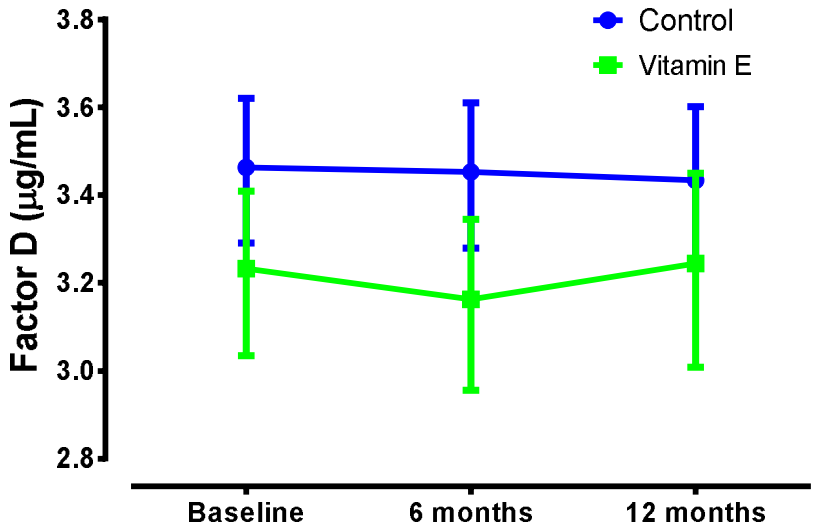


Figure 46 - Factor D levels at baseline, 6 and 12 months in each study group. There were no significant changes in the factor D levels across study visits nor significant effects of study group allocation.

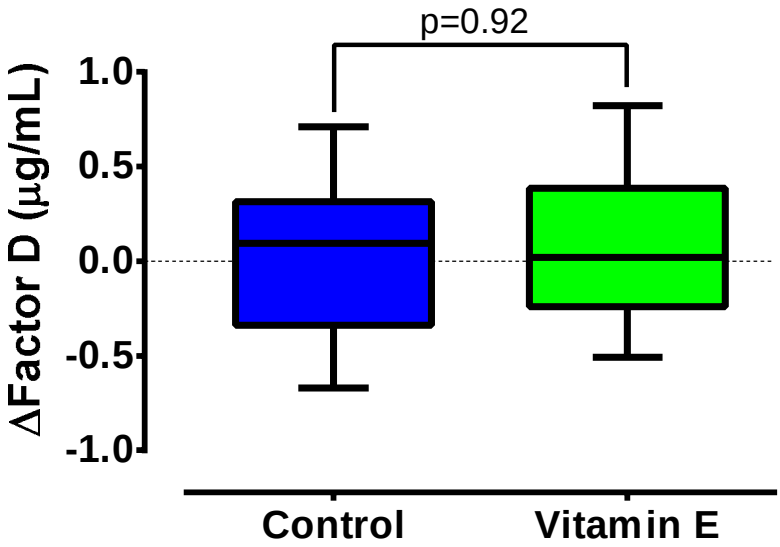


Figure 47 - Comparison of 12-month change in factor D levels between study groups. There was no significant difference in the 12-month change in factor D levels between study groups. (ΔFactor D = Factor $D_{12months}$ - Factor $D_{baseline}$).

7.2.5 12-month changes in properdin levels

As shown in Table 23, there was no difference when the properdin levels were compared between study groups at baseline (p=0.07) or 12-months (p=0.60). Within group comparisons of the baseline and 12-months levels, however, revealed a significant reduction in properdin levels in the VE group (p=0.02) but no significant changes in the control group (p=0.17). A repeated measures ANOVA was performed on the log transformed properdin levels, which approximated a normal distribution, to examine for significant changes in the properdin levels across study visits and to look for any inter-group differences (see Figure 48). This analysis revealed the changes in properdin levels across study visits to be non-significant (p=0.08) and there was no significant effect of study group allocation (p=0.22) nor significant interaction between study group and time (p=0.11). A comparison of the 12-month change in properdin levels revealed no significant difference between the two groups (p=0.38, see Figure 49).

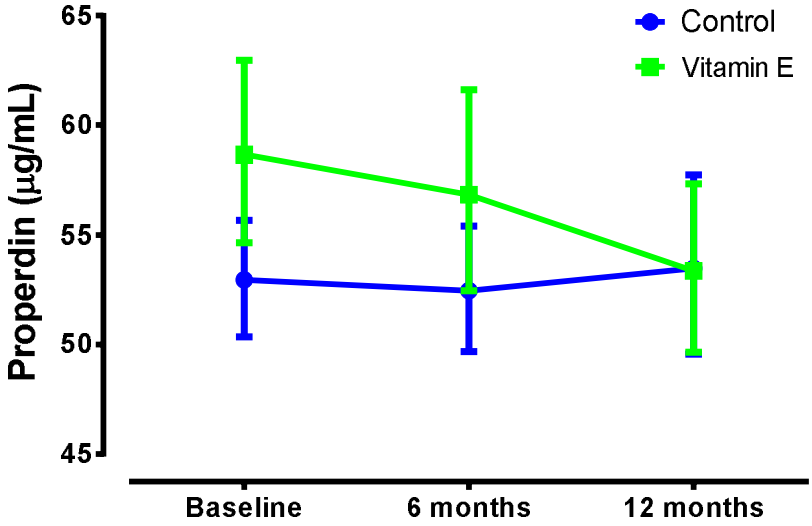


Figure 48 - Properdin levels at baseline, 6 and 12 months in each study group. There were no significant changes in the properdin levels across study vistis nor significant effects of study group allocation.

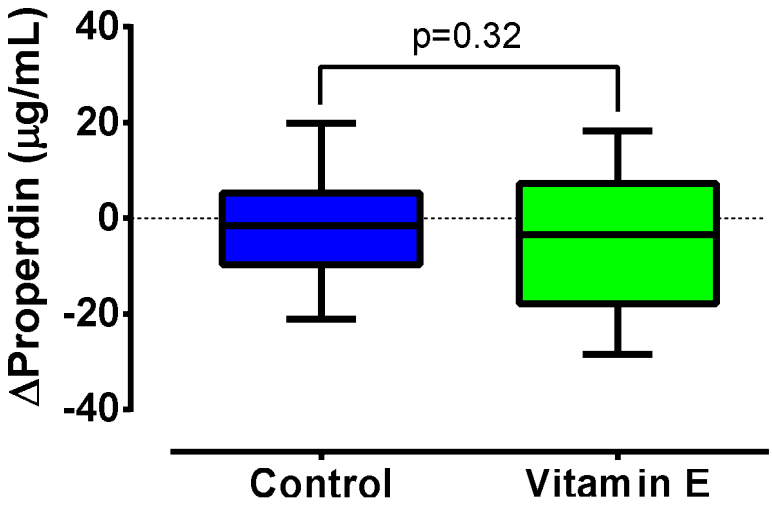


Figure 49 - Comparison of 12-month change in properdin levels between study groups. There was no significant difference in the 12-month change in properdin levels between study groups. (ΔProperdin = $Properdin_{12months}$ - $Properdin_{baseline}$).

In summary, the principal findings concerning the 12-month inflammatory marker datasets were significant reductions in C3 levels over 12-months, irrespective of dialyser used, and a possible effect of the VE-membrane in lowering properdin levels. This latter finding, however, was only evident on pairwise comparison of the baseline and 12-month properdin levels in the VE group; both a Friedman's analysis, examining for significant changes in properdin levels across all 3 study visits in the VE group, and a between group comparison of the 12-month changes in properdin levels were not statistically significant. Taking these findings together, it would seem unlikely that dialysis with the VE-bonded membrane had a properdin-lowering effect.

7.2.6 Inter-relationships of the 12-month changes in the inflammatory markers

In addition to examining the changes in the inflammatory markers over the 12-month study period and the effects of the VE membrane, the associations between the changes in the inflammatory markers were evaluated. To this end the Spearman's rank correlation coefficients were determined for the 12-month changes in each of the inflammatory variables (see Table 25). From these data it can be seen that the 12-month change in CRP levels was positively correlated with changes in both the C3 and SC5b-9 levels and there was a weaker, but significant, positive correlation between the 12-month changes in C3 and properdin levels. In terms of associations between the changes in the markers of inflammation and oxidative stress measured, the only statistically significant finding was a negative correlation between the changes in SC5b-9 and Ox-LDL levels (r= -0.16, p=0.02).

Table 25 - Spearman's correlation coefficients for the 12-month change in each of the inflammatory markers.

	ΔC3	**ΔSC5b-9**	**ΔFactor D**	**ΔProperdin**
ΔCRP	0.38*	0.37*	-0.05	-0.03
ΔC3		0.13	-0.04	0.14†
ΔSC5b-9			-0.03	-0.09
ΔFactor D				-0.04

In each case, the change [Δ] was calculated by subtracting the baseline value from the 12-month value.*p<0.0001, †p<0.05

7.3 Inflammation and ESA resistance index

To evaluate the associations between the inflammatory markers and the ERI at baseline, the Spearman's correlation coefficients for the ERI and CRP, C3, factor D and properdin levels were determined. The only statistically significant finding was a positive correlation between CRP and ERI as reported in Chapter 5 (see also Table 26); there was no association between ERI and quartiles of SC5b-9 (p=0.14).

Table 26 - Spearman's rank correlation coefficients between baseline levels of ERI and CRP, C3, factor D and properdin.

	r	***p***
CRP	0.15	*0.02*
C3	-0.09	*0.16*
Factor D	-0.07	*0.28*
Properdin	0.04	*0.55*

To further characterise the associations between inflammation and ERI in the study population, correlation analysis between the change in ERI and the change in each of the inflammatory markers was performed. From these analyses there were no significant associations between the 12-month changes in ERI and any of the biomarkers of inflammation measured (Table 27).

Table 27 - Correlation coefficients between the change in ERI and the change in each of the measured biomarkers of inflammation.

	r	*p*
ΔCRP	0.10	*0.15*
ΔC3	-0.03	*0.63*
ΔSC5b-9	0.08	*0.25*
ΔFactor D	-0.01	*0.83*
ΔProperdin	0.06	*0.39*

In each case, the change [Δ] was calculated by subtracting the baseline value from the 12-month value.

Given the findings of an apparent differential effect of the VE membrane on ERI depending on the baseline ERI, as detailed in Chapter 5, it was decided to examine the inflammatory markers after dividing patients into tertiles of ERI at baseline. In these analyses, the CRP levels were significantly higher for patients in the highest tertile of ERI compared to the middle and lowest tertiles ($p<0.01$ in both cases, see Table 11). The levels of C3 were significantly higher for patients in the lowest tertile of ERI compared to the middle tertile (0.79 [±0.02] mg/mL versus 0.73 [±0.02], $p=0.03$); the pairwise comparisons between the other tertiles were not statistically significant ($p>0.08$ in both cases). The SC5b-9, factor D and properdin levels at baseline did not differ between tertiles of ERI at baseline ($p>0.4$ in all cases). To examine for a differential effect of the VE membrane on the inflammatory markers within tertiles of ERI, these 12-month change in each of the inflammatory markers were compared between groups after stratifying patients into tertiles of ERI. In these analyses, there was no differential effect of the VE membrane on the change in the levels of CRP ($p>0.42$ in all tertiles), C3 ($p>0.44$ in all tertiles), factor D ($p>0.20$ in all tertiles) or SC5b-9 ($p>0.41$ in all tertiles). Pairwise testing revealed an apparent difference in the 12 month change in properdin levels for patients in the highest tertile of ERI dialysing with the VE membrane compared to the control membrane (-9.6 [-18.6 - 3.5] versus 1.9 [-7.1 - 9.0] respectively, $p=0.02$) although this became non-significant at the pre-specified 5% level after applying the Bonferroni correction for multiple pairwise testing as described in section 3.17.4. There was no differential effect of the VE membrane on the 12-month change in

properdin levels for patients in the lowest and middle tertiles of ERI (p=0.70 and p=0.86 respectively).

7.4 Discussion

As already highlighted, increased levels of inflammation are associated with a number of problems encountered by chronic HD patients such as ESA resistance [923, 924] and increased mortality rates, particularly from CV disease [163, 429, 435, 439, 440, 505, 506]. Although there is no direct evidence that reducing levels of inflammation improves patient outcomes, such approaches seem logical. One such intervention may be the use of VE which has been shown to have anti-inflammatory properties [741-745] as outlined in section 1.7.1.2. In this chapter, the effects of switching prevalent HD patients to 12-months dialysis with a VE-bonded polysulfone membrane on the levels of CRP, C3, SC5b-9, factor D and properdin were examined. Prior to investigating the effects of the VE-membrane, the key determinants of each of these inflammatory markers in the baseline dataset were evaluated.

7.4.1 Baseline determinants of inflammation biomarkers

C-reactive protein has been the most commonly studied marker of inflammation in HD patients to date, likely owing to the widespread use of CRP measurements in routine clinical practice. C-reactive protein is the archetypal measure of the acute phase response with plasma concentrations capable of increasing by up to 1000-fold [428, 1027]. In the present study the baseline CRP levels were independently associated with the positive acute phase reactants ferritin and C3 and the negative acute phase reactant albumin as might be expected. A previous study [1028] reported no significant correlation between C3 and CRP levels in a cohort of 103 prevalent HD patients without significant inflammation, although the nephelometric assay they used to measure CRP had a detection limit of 6 mg/L unlike the more sensitive assay used in the present study, to possibly explain their failure to detect a positive association between these two acute phase reactants. The levels of CRP were higher in patients with a prior history of IHD in unadjusted analyses in line with previous studies demonstrating an association between CRP levels and carotid atherosclerosis in HD patients [447], for example, and the well established link between increased levels of inflammation and both CV and all-cause mortality in this patient group [163, 429, 434, 435, 439-441, 504-506]. However, there was no independent association between a prior history of IHD and the baseline CRP levels in multivariate regression analysis suggesting that the association evident in the unadjusted analyses could be accounted for by the other factors retained in the final model. The levels of CRP tended to

be higher in patients dialysing via CVCs compared to AVFs although the difference was not statistically significant; this differs from several published studies in which CVC usage has been associated with increased levels of CRP [456, 929, 1029]. The lack of association in the present study may reflect that the study population was selected on the basis of CRP<50 mg/L at baseline, thereby excluding 11% of LTHT chronic dialysis patients (see Figure 24) and potentially diluting any associations between inflammation and CVC usage which may be apparent in unselected HD populations. There was no association between CRP levels and diabetic status in the present study, consistent with the findings of others [456, 1030], which differs from data obtained in non-renal populations in which diabetes is associated with elevated CRP levels [1031]. These discrepancies may reflect that the pro-inflammatory stimuli of HD and renal failure mask any additional contributions from the presence of the diabetic state.

A wealth of data from non-renal populations have demonstrated elevated C3 levels in patients with prevalent CV disease [532-536, 539, 1032] and diabetes [652, 1033, 1034], however, no such associations were evident in the present study of HD patients. The finding of higher C3 levels in Asians mirrors the findings from studies of ethnicity and complement levels in non-renal patients [1035, 1036]. The independent association of weight and C3 levels observed in the present study has similarly been reported both in obese individuals [1037] and apparently healthy adolescents [1038] not on dialysis. This association is likely explained by the fact that C3 and IL-6 are secreted by adipose tissue [1039] with serum C3 levels correlating with the amount of visceral and subcutaneous tissue [1040] which is greater in obese individuals. This association with body fat is also likely to explain the independent association between C3 and cholesterol levels in the present study as has been reported by others [536, 1032, 1039].

There are little or no published data in the modern treatment era concerning the levels of the alternative complement pathway components in HD patients, their determinants or clinical correlates. This represents one of the novel aspects of the present study and highlights the exploratory nature of many of the analyses performed herein. Patients receiving renin-angiotensin medications were noted on bivariate analysis to have lower CRP, C3 and factor D levels and on multivariate analysis to have lower factor D and properdin levels. Angiotensin II has a number of pro-inflammatory actions on the vascular wall, including the production of ROS, inflammatory cytokines and adhesion molecules [1041], thus medications which either block the formation of angiotensin II (ACE-inhibitors, direct renin-inhibitors) or prevent it from binding to its receptor (angiotensin II receptor

antagonists) are likely to ameliorate these inflammatory processes, potentially explaining the findings of the present study. C-reactive protein levels have been extensively studied in this respect with a large number of studies in non-HD populations demonstrating reductions in CRP levels for patients commencing renin-angiotensin medications [1042]. Complement components have been subjected to much less investigation although there are data to suggest that C3 levels may also be lowered by these medications in patients not on dialysis [683], in line with the finding of lower levels in patients receiving RAS-medications in the present study.

In terms of the interplay between oxidative stress and inflammation, the significant associations at baseline were lower factor D levels in patients with higher TBARS levels and a non-linear relationship between C3 and Ox-LDL levels. There are no published data examining the relationship between factor D and markers of oxidative stress in dialysis patients. Previous studies in HD patients, in contrast to the present one, have demonstrated a positive association between the levels of inflammation and oxidative stress [327, 332, 423-425]. There are a number of reasons to explain why increased levels of inflammation and oxidative stress frequently co-exist in HD patients. These include clustering of disease states which predispose to inflammation and oxidative stress, such as diabetes, and the ability of HD therapy to contribute to both of these processes as detailed in sections 1.4.2 and 1.5.1. Inflammation and oxidative stress are also mechanistically linked, for example through the enzyme myeloperoxidase which is activated during HD [333] and has both oxidative [426] and inflammatory actions, particularly affecting nitric oxide bioavailability in the vascular wall [1043]. Conversely oxidative stress can enhance inflammation through upregulation of the transcription factor NFκB, increasing production of pro-inflammatory cytokines such as IL-6 and acute phase proteins such as CRP [1044], and by reducing the availability of thiol groups on proteins [382, 386, 427] as a consequence of the reduction in albumin levels as part of the acute phase response [428]. The complement system is also linked to oxidative stress as Ox-LDL has been shown to bind C3a *in vivo* [430] and ROS such as hydrogen peroxide have been shown to directly activate C5 via a non-enzymatic mechanism [431]. Further evidence of a synergistic relationship between complement and oxidative stress comes from animal models of ischaemia-reperfusion injury whereby complement activation is attenuated by the addition of anti-oxidants, such as captopril which possesses sulfhydryl groups [432, 433]. There are therefore a number of aspects of both inflammation and oxidative stress which are linked. The absence of significant associations in the present study may reflect the low levels of inflammation and oxidative stress present in the study

cohort or the choice of biomarkers. Another explanation may be the use of pre-dialysis blood samples. If dialysis therapy is the principal driver of inflammation and oxidative stress in these patients, the measurement of pre-dialysis biomarker levels may not necessarily reflect the inflammatory and oxidative burden associated with HD treatment.

At baseline the ERI was significantly positively correlated with CRP levels which is consistent with the well established association between inflammation and ESA resistance [922-925]. A number of mechanisms underpin this association, as discussed in section 1.3.2, including suppressive effects of inflammatory cytokines on erythroid progenitor cells [158, 159], reduced red blood cell lifespan [227, 927], blunted EPO response [160] and functional iron deficiency [147, 156, 157, 928]. There were no significant associations between ERI and the complement components measured. Previous work has shown the erythrocytes of HD patients to be particularly susceptible to complement mediated lysis [227] although in the present study there was no association between SC5b-9 levels and ERI at baseline, nor any correlation between the changes in these two parameters, suggesting complement mediated red blood cell destruction may be a minor contributor to anaemia in this study cohort.

7.4.2 Changes in inflammatory markers over time and the effects of vitamin E

In addition to examining the baseline dataset for the significant determinants of the various inflammatory markers measured, the longitudinal data were analysed both to assess the effects of the VE membrane and to explore the inter-relationships of the changes over time in the inflammatory biomarkers. There were no significant effects of the VE membrane on any of the inflammatory markers measured after 12 months. Pairwise comparison of the baseline and 12-month properdin levels revealed a significant decrease in those patients dialysing with the VE membrane but there were no differences between the groups at baseline or 12 months nor when the changes in properdin levels were compared between groups. It is therefore possible that the apparent significant decrease in properdin levels observed in the VE group was a product of multiple statistical testing rather than a true effect of the VE membrane, i.e. a type 1 statistical error. A previous study employing proteomics has demonstrated that properdin is adsorbed onto the surface of polysulfone membranes during dialysis [1045], although the influence of VE-coating membranes on the adsorption of properdin has not been studied, and the extent to which dialyser adsorption contributes to circulating properdin levels in HD patients is unclear. Enhanced

membrane adsorption of properdin in the presence of a VE-coating may, therefore, be a potential mechanism to explain the observation in the present study although no evidence for this phenomenon exists.

The most striking observation from the longitudinal data was the progressive reduction in C3 levels across study visits, coupled with the negative correlation between C3 levels and the number of years on dialysis. The change in C3 levels was correlated with the change in weight, cholesterol, triglyceride and CRP levels over the 12-month study period although further analysis indicated that the change in these factors only explained 16% of the variance in the change in C3 levels, suggesting the importance of unmeasured factors. A unifying explanation for these observations, therefore, remains enigmatic. Possible factors which could explain the fall in C3 levels include dialyser adsorption [1045, 1046] or complement consumption and it is likely that both of these factors are contributory. The levels of C3 doubtless decrease during complement activation although may not be a sensitive indicator of activation given the relatively small proportion of circulating C3 which is cleaved [1047-1049]. The molecular weight of C3 is 185 kDa ruling out dialytic losses. Regarding the possible contribution of complement activation to the decline in C3 levels, it is difficult to make inferences based on the relative levels of the biomarkers measured in the present study. As described in section 1.5.2, C5b-9 is one of the end products of activation of the complement cascade and, in the present study, the pre-dialysis levels of SC5b-9 were measured. There was no association between the levels of C3 and SC5b-9 at baseline but what this means in terms of the degree of complement activation is unclear. For example low SC5b-9 levels may be a consequence of low levels of complement activation, with reduced synthesis, or reflect high levels of complement activation with consumption of the terminal complement components. Blood-membrane interactions are a major stimulus for complement activation in HD patients so the pre-dialysis SC5b-9 levels measured in the present study are likely to represent the nadir. The SC5b-9 molecule has a short half life of approximately 40 minutes [1050] thus the predialysis levels are likely to be more reflective of inter-dialytic SC5b-9 clearance and / or redistribution rather than dialysis associated complement activation. Furthermore, the fate of activated complement components to form either the membrane attack complex C5b-9 or the soluble complex SC5b-9, as measured in the present study, is dependent on a number of factors important among which are the circulating levels of vitronectin (also known as protein S) and clusterin (also known as apolipoprotein J). These molecules bind the C5b-7 complex, preventing its insertion into lipid membranes, and limit the polymerisation of C9 necessary for membrane attack complex formation [485, 498, 499]

thus lower levels of vitronectin or clusterin may be expected to result in the formation of relatively less SC5b-9. Both vitronectin and clusterin have been shown to be adsorbed onto the surface of dialysis membranes [1045, 1046, 1051] and, in the case of vitronectin, correlate negatively with the length of time on dialysis [1052]. This suggests that the profile of SC5b-9 generation following complement activation in HD patients, particularly during dialysis, may differ markedly from complement activation occurring in non-HD patients or during the inter-dialytic period, and highlights the pitfalls of using pre-dialysis SC5b-9 levels as a marker of complement activation in this setting. The levels of factor D increased across quartiles of SC5b-9 levels suggesting that increased SC5b-9 levels may reflect enhanced complement activity. This has a biological plausibility as factor D is the rate limiting enzyme of the alternative pathway [228] and therefore higher levels are likely to be permissive for greater SC5b-9 generation. An alternative explanation for the fall in C3 levels across study visits, the negative correlation between C3 levels and time on dialysis, and the positive correlation between the change in C3 levels and the change in weight may be that they reflect protein-energy malnutrition. Protein-energy malnutrition is very common among HD patients, with the degree of malnutrition correlating with the length of time of dialysis dependency [1053]. Studies of severely malnourished children [1054, 1055] and guinea pigs [1056] have demonstrated reductions in the levels of C3. It remain unclear, however, whether lesser degrees of malnutrition could result in reduction in C3 levels or whether C3 levels only fall in the setting of severe malnutrition. No data were available on nutritional status of the HD patients to corroborate this theory.

The foregoing discussion presented here highlights some of the difficulties inherent in studying complement activation *in vivo*, particularly with the biomarkers measured here, as low levels of a particular component may represent increased complement activation (i.e. increased consumption), reduced complement activation (i.e. reduced formation of downstream components), increased clearance or reduced synthesis. Furthermore, the circulating levels of certain components or complexes, as already highlighted with SC5b-9, are dependent on a number of factors besides simply the extent of complement activation. These complexities may go some way to explaining why there was no significant association in the present study between the principle substrate of the complement cascade, C3, and one of the downstream products of complement activation SC5b-9.

In the present study, no convincing reductions in the levels of inflammation were observed with the VE-bonded membranes. The anti-inflammatory effects of VE-bonded polysulfone membranes have only been studied in relatively small numbers of patients to date.

Mandolfo *et al*. [889] followed 16 patients dialysing with CVC's for 12-months in a cross-over study design and reported no effects of the VE-bonded polysulfone membrane on the levels of CRP or IL-6. In a larger study of 62 HD patients, Panichi *et al*. [893] reported significant reductions in both CRP and IL-6 levels after 6 months for patients dialysing with a VE-bonded polysulfone membrane and no significant changes in patients dialysing with a comparable non-VE bonded polysulfone membrane. The VE and comparator membranes were both low-flux, contrasting with the high-flux membranes used in the present study, although membrane flux has previously been shown not to influence CRP and IL-6 levels [355, 919, 1057]. Analysis of the change in CRP after 6 months in the present study found no significant differences between the groups and no reduction in levels with the VE membrane (p=0.78). The baseline CRP levels in the present study were similar to those reported by Panichi *et al.* [893] suggesting the apparent discrepant findings could not be attributed to vastly different levels of inflammation in the two study populations. Interestingly Panichi *et al*. [893], despite demonstrating reductions in both ERI and inflammation after 6 months with the VE membrane, reported no correlation between the changes in these two variables suggesting that the improvements in ERI may not have been directly attributable to reductions in inflammation. This, combined with the similar findings in the present study regarding the absence of association between changes in inflammation and ERI, suggests that strategies aimed at reducing inflammation may only have a limited impact on improving ESA resistance in the subset of HD patients without significant inflammation.

In the present study a number of markers of inflammation and components of the complement system were measured. With the exception of CRP, there is very little published data on the determinants or significance of these markers in HD patients. One of the novel aspects of the present study was the measurement of a number of components of the alternative complement pathway, including serial measurements over time. The most significant findings with regards to the inflammatory markers were a reduction in C3 levels over the 12 month study period, irrespective of study group allocation, and a negative association with the number of years on dialysis. Data from the general population have consistently shown elevated C3 levels to be associated with CV disease [533, 534, 536, 538-540] and CV disease is the leading cause of death in HD patients [11], with rates far exceeding those seen in the general population [12]. The findings with respect to C3, therefore, are on the face of it hard to reconcile as reductions in C3 levels may be expected to translate into improved CV outcomes. This may not necessarily follow, however, as the spectrum of CV disease seen in HD patients differs from that seen in the

general population both at the morphological level, with increased vascular calcification [56-58] and reduced myocardial capillary density [1058], and at the phenotypic level with more deaths attributable to complications of cardiac failure, such as arrhythmias, than to atherosclerotic disease [14, 92, 1059-1062] (see section 1.2.3). Much of the mechanistic data concerning the role of complement in CV disease pertains to atherosclerosis [511, 547-549] which may be a less prominent feature of CV disease in HD patients. Infections are also common in HD patients and are a leading cause of death after CV disease [4]. Patients with deficiencies in complement C3 are prone to infections [1063] thus reductions in C3 levels, as observed in the present study, may increase patients susceptibility to infections. Analyses of the complement levels with regards to the risks of CV events and infective episodes in the study patients is provided in Chapter 9.

As already highlighted, no beneficial effects of the VE-bonded membranes on markers of inflammation were observed in the present study. The majority of the anti-inflammatory activities of VE relate to cellular aspects of inflammation, such as the expression of cell surface adhesion molecules [737, 738] and leukocyte adhesion [739, 740], thus the ability of VE to influence complement activation may be limited. One of the first studies of the VE-bonded polysulfone dialysers, using *in vitro* techniques, found no difference in the level of complement activation, as measured by C3a, C4a and C5a levels, between VE-bonded and unbonded polysulfone membranes suggesting that the addition of a VE coating on the dialyser surface may have a limited impact on complement activation [885]. Thus it is perhaps not surprising that there was no effect of switching to a VE-bonded dialysis membrane on the levels of the alternative complement components measured in the present study.

In Chapter 5, a beneficial effect of the VE-bonded membrane in reducing ERI for patients with higher levels of ESA-resistance was observed. The only significant finding when the levels of the inflammatory markers were compared across tertiles of ERI at baseline was higher CRP levels in the group of patients in the highest tertile of ERI. Although the levels of CRP and the ERI were significantly positively correlated, the changes in each of these variables over 12-months were not correlated. This suggests that inflammation may contribute to ESA resistance, but reducing inflammation in a subset of non-inflamed prevalent HD patients, as were included in the present study, may have a limited impact on improving ESA resistance. Himmelfarb *et al*. [227] have previously reported on the increased erythrocyte deposition of C5b-9 in HD patients, compared to controls, suggesting complement activation may play a role in the shortened red blood cell lifespan

of HD patients. Although it is hard to infer the degree of complement activiation from the levels of the biomarkers measured in the present study, the absence of any association between the complement levels and the ERI, both in terms of the levels at baseline and the changes over 12 months, may mean that the complement system is only a minor contributor to anaemia in non-inflamed HD patients dialysing with biocompatible dialysis membranes as were studied here.

In conclusion, no effects of the VE-membrane on the markers of inflammation measured in the present study were observed. Given this finding, the question of whether measures aimed at improving generalised inflammation translate into improved ESA resistance remains unanswered. The absence of an association between changing levels of inflammation and ERI, both in the present study and elsewhere [893], might suggest that lowering inflammation would only have a limited impact on improving ESA resistance, particularly in patients with already low levels of inflammation. There are little or no published data in HD patients in the modern treatment era examining the levels of the alternative complement pathway components, their determinants or their biological associations. The current study, therefore, represents the largest series of HD patients with serial measurements of the alternative complement pathway components to date and thus will contribute to understanding in this area. In particular, the findings of a decline in C3 levels over 12 months and the negative association between C3 levels and time on dialysis merits further investigation. In addition to the ERI, oxidative stress and inflammation endpoints discussed so far, all patients had samples taken for analysis of fibrin clot structure - another putative marker of CV risk which has only been studied in small numbers of dialysis patients.

Chapter 8 : Fibrin clot structure and function

Fibrin clots obtained from numerous patient groups, including patients with IHD [644, 648, 649, 681, 708], diabetes [654, 655, 719, 720], PVD [714, 715], abdominal aortic aneurysms [713], chronic heart failure [718] and cerebrovascular disease [711, 712], are denser and more resistant to fibrinolysis than fibrin clots derived from healthy controls. Similar findings have also been described in the small number of end-stage renal failure patients studied to date including patients undergoing chronic peritoneal dialysis [645] and HD [646]. Furthermore, in the study of HD patients [646], those patients who went on to have a fatal CV event formed clots which had a shorter lag time and were denser and more resistant to fibrinolysis. Thus it appears that an altered clot phenotype may be both reflective of prevalent CV disease and predictive of future risk. In this chapter the determinants of fibrinogen levels and the *ex vivo* fibrin clot parameters were evaluated in the study cohort. The longitudinal data were then analysed to study the impact of dialysis with the VE-bonded membrane on the clot characteristics, to determine if the fibrin clot phenotype changed over time and to explore the associations between changing clot characteristics and changing levels of inflammation and oxidative stress.

8.1 Assay data used for analyses

Samples for analysis of fibrin clots were available for 714 of the 715 study visits. No fibrin clot data were obtained from 7.6% of the samples from the clotting assay and 7.3% of the samples from the fibrinolysis assays because there was insufficient clot formation or fibrinolysis to allow accurate determination of the clot parameters described in section 3.15. A review of the samples which did not yield assay data revealed a high proportion were obtained from patients dialysing via CVCs compared to fistulas or grafts (clotting assay: 83% vs 7%, fibrinolysis assay 79% vs 7%; $p<0.0001$ both cases). Of the samples obtained from patients dialysing via CVCs, data were only obtained in 50% of the clotting assays and 54% of the fibrinolysis assays. Warfarin therapy also influenced the assays as, of the samples obtained from patients receiving warfarin, only 73% yielded clotting assay results and 82% fibrinolysis assay results. Considering these data together, 100% of the samples with no clotting assay data and 90% of the samples with no fibrinolysis assay data were obtained from patients either dialysing via a CVC and / or who were receiving warfarin. Additionally in 6 samples, obtained from 4 patients, there was a complete absence of fibrinolysis of the formed clot after 9 hours thus it was not possible to calculate any of the fibrinolysis parameters. There were therefore data available from 660 clotting assays and 662 fibrinolysis assays (see Figure 50).

Given that high proportions of samples obtained via CVCs or from patients receiving warfarin yielded no assay data, the samples from these patients which did yield data were evaluated and found to differ significantly from the remaining samples (see Table 28 and Table 29). It was therefore decided to exclude all samples obtained from patients dialysing via CVCs or receiving warfarin from further analyses. This resulted in a complete fibrin clot dataset for 578 samples which were analysed in more detail (see Figure 50).

Table 28 - Clot characteristics of samples obtained from patients on warfarin.

	Warfarin		
	Yes	**No**	**p**
Clotting assay parameters			
n	33	627	
Lag time (s)	539 [514 - 593]	519 [464 - 599]	0.09
Maximum absorbance (au)	0.558 [±0.017]	0.539 [±0.006]	0.43
Clot rate ($x10^{-4}s$)	2.6 [1.8 - 2.9]	4.1 [3.2 - 4.9]	<0.0001
Fibrinolysis assay parameters			
n	37	625	
Lag time (s)	621 [557 - 864]	594 [521 - 695]	0.07
Maximum absorbance (au)	0.272 [±0.020]	0.410 [±0.006]	<0.0001
Clot rate ($x10^{-4}s^{-1}$)	2.0 [1.1 - 3.4]	4.9 [3.8 - 6.2]	<0.0001
$Lys50_{t0}$ (s)	2811 [2382 - 3233]	2176 [1994 - 2498]	<0.0001
$Lys50_{lag}$ (s)	1989 [1677 - 2561]	1567 [1398 - 1859]	<0.0001
$Lys50_{MA}$ (s)	767 [644 - 858]	728 [598 - 910]	0.61
Lysis rate ($x10^{-4}s^{-1}$)	-1.2 [-1.5 - -0.8]	-1.2 [-1.7 - -0.7]	0.54
Lysis area	427 [267 - 613]	565 [390 - 869]	<0.01

Data presented as mean [±SEM] or median [IQR]. au: Absorbance units

Table 29 - Clot characteristics of samples obtained via central venous catheters.

	Central venous catheter sample		
	Yes	**No**	**p**
Clotting assay parameters			
n	45	615	
Lag time (s)	626 [488 - 967]	519 [465 - 589]	<0.0001
Maximum absorbance (au)	0.538 [±0.023]	0.540 [±0.006]	0.92
Clot rate ($x10^{-4}s$)	2.7 [1.8 - 4.2]	4.1 [3.2 - 4.9]	<0.0001
Fibrinolysis assay parameters			
n	49	613	
Lag time (s)	837 [660 - 1508]	590 [519 - 672]	<0.0001
Maximum absorbance (au)	0.308 [±0.026]	0.409 [±0.006]	<0.0001
Clot rate ($x10^{-4}s^{-1}$)	2.6 [1.1 - 4.7]	4.9 [3.8 - 6.2]	<0.0001
$Lys50_{t0}$ (s)	2906 [2318 - 3835]	2173 [1995 - 2465]	<0.0001
$Lys50_{lag}$ (s)	2028 [1580 - 2693]	1560 [1398 - 1846]	<0.0001
$Lys50_{MA}$ (s)	863 [657 - 1112]	722 [598 - 897]	<0.01
Lysis rate ($x10^{-4}s^{-1}$)	-1.1 [-1.5 - -0.5]	-1.2 [-1.7 - -0.7]	0.18
Lysis area	405 [277 - 577]	575 [394 - 877]	<0.001

Data presented as mean [±SEM] or median [IQR]. au: Absorbance units

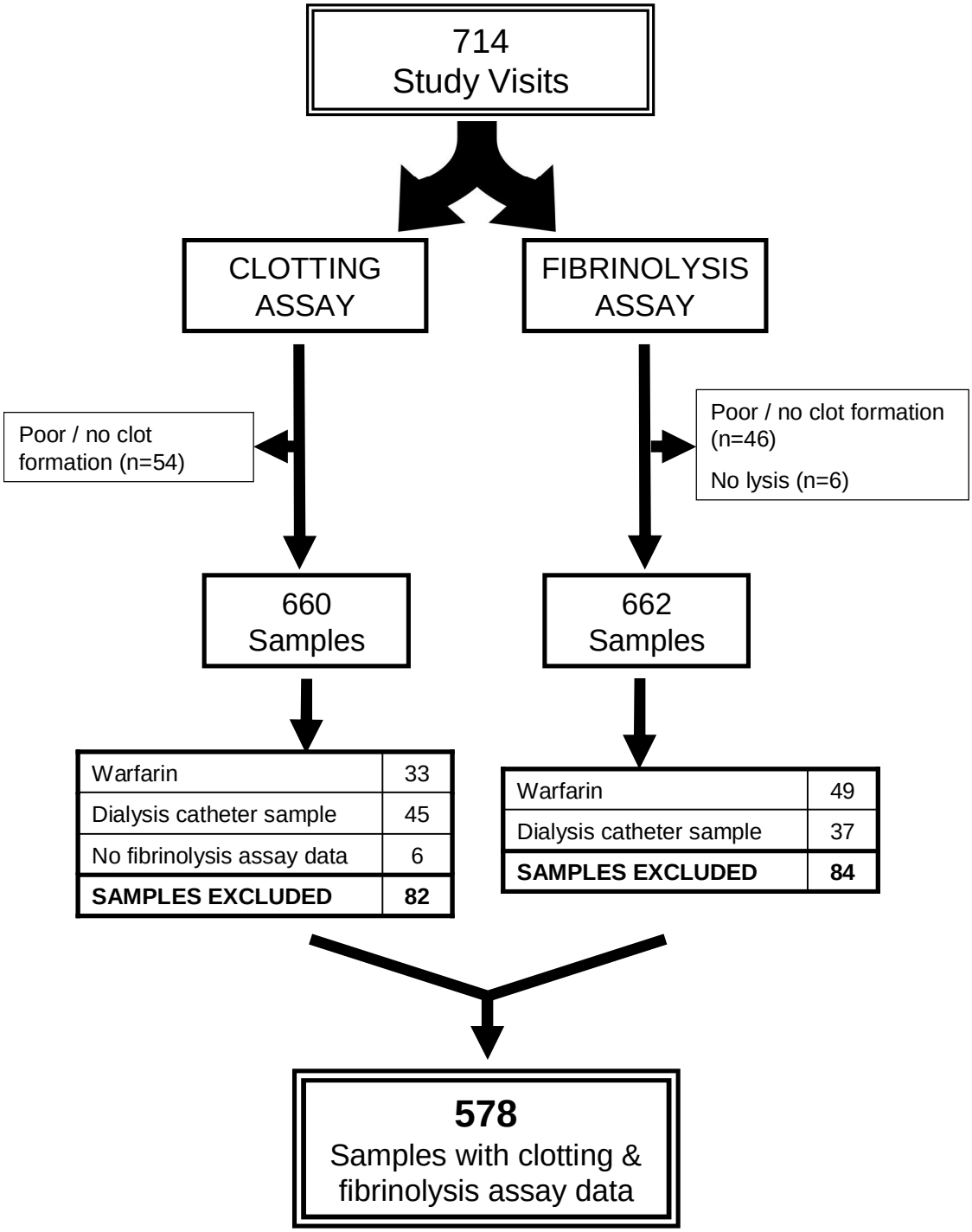


Figure 50 - Breakdown of fibrin clot structure samples assayed, excluded and subsequently used for analyses. Samples for fibrin clot structure analyses were available from 714 study visits. A significant proportion of the samples obtained from patients dialysing via central venous catheters or receiving warfarin yielded no assay data. The final dataset, which was subsequently analysed in more detailed, comprised clotting and fibrinolysis assay data from 578 patient samples.

8.2 Determinants of baseline fibrinogen levels and fibrin clot parameters

Given the relatively large number of variables measured in the clotting and fibrinolysis assays, the inter-relationships between the variables were examined. Table 30 details the correlation coefficients for the parameters which were measured in both the clotting and the fibrinolysis assays (i.e. lag time, maximum absorbance and clot rate). The shared parameters were positively correlated between the two assays and the clot rate was positively correlated with the maximum absorbance and negatively correlated with the lag times in both assays.

Table 30 - Correlation matrix for shared clotting (C) and fibrinolysis (L) assay variables.

	Lag (L)	Max Abs (C)	Max Abs (L)	Clot rate (C)	Clot rate (L)
Lag (C)	0.58*	-0.24*	-0.31*	-0.35*	-0.42*
Lag (L)		-0.26*	-0.30*	-0.33*	-0.33*
Max Abs (C)			0.84*	0.41*	0.53*
Max Abs (L)				0.69*	0.82*
Clot rate (C)					0.72*

*p<0.0001

Table 31 details the correlation coefficients for the fibrin clot parameters measured in the fibrinolysis assay. All of the measures of fibrinolysis (i.e. $Lys50_{t0}$, $Lys50_{lag}$, $Lys50_{MA}$, lysis rate and lysis area) were positively correlated. The lag times tended to be longer and the final clot turbidity lower in the fibrinolysis compared to the clotting assay (see Figure 51).

Table 31 - Correlation matrix for fibrinolysis assay variables.

	Max Abs	Clot rate	$Lys50_{t0}$	$Lys50_{lag}$	$Lys50_{MA}$	Lysis rate	Lysis area
Lag	-0.30*	-0.33*	0.31*	-0.07	-0.09	0.00	-0.25**
Max Abs		0.82*	0.22*	0.36*	0.53*	-0.07	0.77*
Clot rate			-0.05	0.08	0.49*	0.00	0.65*
$Lys50_{t0}$				0.89*	0.65*	0.45*	0.54*
$Lys50_{lag}$					0.75*	0.48*	0.70*
$Lys50_{MA}$						0.59*	0.89*
Lysis rate							0.47*

*p<0.0001, **p<0.01

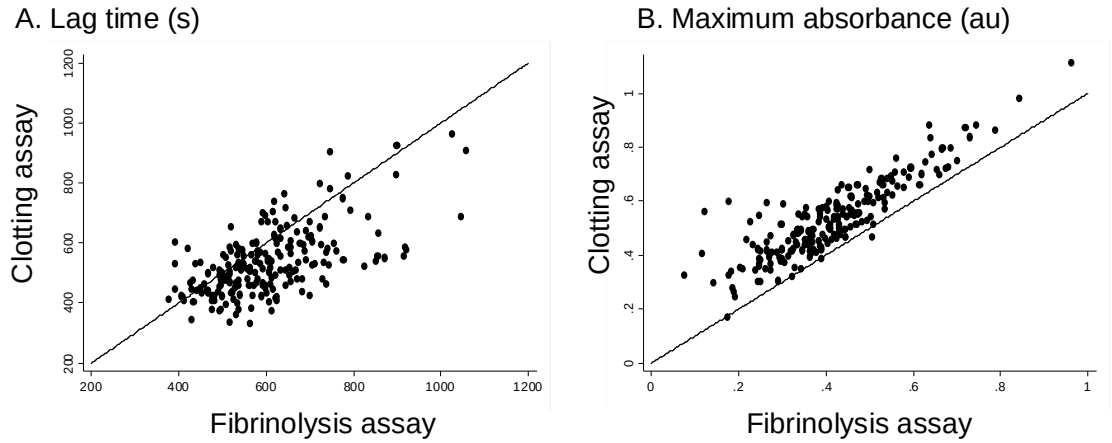


Figure 51 - Scatter plots of A. lag times and B. maximum absorbancies derived from the clotting and fibrinolysis assays. (Line indicates equivalence). From these graphs lag times tended to be shorter and the maximum absorbance greater in the clotting assay

Based on these results, the fibrin clot parameters selected for more detailed analyses were the lag time and maximum absorbance from the clotting assay and the $Lys50_{t0}$ times from the fibrinolysis assay. A more detailed explanation of the rationale for selecting this subset of variables is presented in the discussion (section 8.6.1). For the fibrinogen levels and each of the fibrin clot assay parameters selected for further analysis, the significant determinants in the baseline dataset were evaluated prior to examining the changes in these variables across study visits and the effects of dialysis with the VE-bonded dialysis membrane.

8.2.1 Determinants of baseline fibrinogen levels

Samples with fibrinogen levels greater than 4.5 g/L were not quantified, 11% of the baseline dataset, and were assigned a value of 4.5 g/L as detailed in section 3.9. The continuous predictor variables were compared across quartiles of fibrinogen concentrations as described in section 3.17.4. In bivariate analyses the fibrinogen levels were positively associated with weight (see Table 32), CRP, C3, ferritin and triglyceride levels (see Table 33), were higher in patients receiving aspirin, in ex-smokers compared to patients who had never smoked (see Table 34) and were significantly associated with bicarbonate levels (see Table 33). Backward stepwise ordinal logistic regression modelling of the quartiles of fibrinogen levels was performed including all of the variables associated with fibrinogen levels at a significance of $p<0.2$ as described in section 3.17.4. The resultant model identified that higher CRP and C3 levels, shorter time on dialysis and being a current smoker were independently associated with higher fibrinogen levels. The final model had an adjusted-R^2 of 0.13 and satisfied the Brant test of parallel regression.

Table 32 - Determinants of baseline fibrinogen levels: analysis of continuous variables by quartiles of fibrinogen (patient and dialysis factors).

	Quartiles of fibrinogen concentration				
	Q1	***Q2***	***Q3***	***Q4***	***p***
n	64	64	64	64	
Fibrinogen (g/L)	<3.4	3.4-3.7	3.8-4.1	>4.1	
Patient factors					
Age (yrs)	59.4 [±2.2]	64.6 [±2.2]	62.9 [±2.0]	66.1 [±1.6]	0.11
Weight (kg)	66.4 [58.9 - 74.9] †‡	69.3 [55.9 - 83.0]	73.7 [62.4 - 91.5]*	76.3 [63.7 - 88.2]*	<0.01
Pre-dialysis SBP (mmHg)	139.4 [±2.9]	139.6 [±3.3]	134.9 [±2.6]	133.8 [±3.4]	0.40
Pre-dialysis DBP (mmHg)	74.5 [±1.9]	72.6 [±1.8]	70.2 [±1.3]	68.8 [±1.5]	0.07
Dialysis factors					
Time on RRT (yrs)	5.0 [1.7 - 10.4]	3.6 [1.4 - 7.8]	4.0 [1.2 - 6.6]	3.3 [1.2 - 3.3]	0.14
Urea reduction ratio	0.76 [±0.01]	0.76 [±0.01]	0.76 [±0.01]	0.73 [±0.01]	0.26

Data presented as range, mean [±SEM] or median [IQR]. For variables with significant omnibus test statistic across quartiles, pairwise testing was performed after applying a Bonferroni correction to the α-level. Significant differences on pairwise testing between quartiles indicated as: vs *Q1, †Q3, ‡Q4. SBP/DBP: Systolic / Diastolic blood pressure; RRT: Renal replacement therapy.

Table 33 - Determinants of baseline fibrinogen levels: analysis of continuous variables by quartiles of fibrinogen (laboratory parameters).

	Quartiles of fibrinogen concentration				
	Q1	***Q2***	***Q3***	***Q4***	***p***
Laboratory parameters					
Haemoglobin (g/dL)	11.4 [±0.2]	11.8 [±0.1]	11.5 [±0.2]	11.6 [±0.2]	0.34
Ferritin (µg/L)	458 [±26]	452 [±27]	440 [±25]§	548 [±32]‡	0.02
C-reactive protein (mg/L)	2.5 [0.4 - 8.5]‡§	4.3 [1.7 - 10.9]§	8.3 [3.5 - 14.6]*§	16.8 [6.9 - 38.8]*†‡	<0.001
C3 (mg/mL)	0.68 [±0.02]‡§	0.74 [±0.02]§	0.80 [±0.03]*	0.86 [±0.02]*†	<0.0001
SC5b-9 (ng/mL)	63.1 [37.0 - 108.5]	62.9 [38.0 - 116.3]	67.0 [44.8 - 113.5]	71.5 [50.9 - 93.1]	0.75
Factor D (µg/mL)	3.3 [2.9 - 4.1]	3.7 [3.1 - 4.4]	3.5 [3.1 - 4.1]	3.2 [2.6 - 4.0]	0.06
Properdin (µg/mL)	52.9 [42.7 - 67.3]	53.2 [44.1 - 67.3]	53.6 [41.4 - 66.0]	55.6 [44.7 - 72.6]	0.64
Albumin (g/L)	38.2 [±0.5]	38.2 [±0.6]	37.4 [±0.5]	38.1 [±0.6]	0.69
Cholesterol (mmol/L)	3.9 [±0.1]	4.1 [±0.1]	3.9 [±0.1]	4.0 [±0.1]	0.53
High-density lipoprotein (mmol/L)	1.2 [±0.1]	1.2 [±0.0]	1.1 [±0.0]	1.1 [±0.0]	0.22
Low-density lipoprotein (mmol/L)	2.0 [±0.1]	2.2 [±0.1]	2.1 [±0.1]	2.1 [±0.1]	0.46
Triglycerides (mmol/L)	1.2 [0.9 - 2.0]§	1.4 [0.9 - 2.1]	1.4 [1.1 - 2.0]	1.8 [1.2 - 2.4]*	0.01
Bicarbonate (mmol/L)	22.4 [±0.3]	21.6 [±0.3]	22.5 [±0.3]§	21.4 [±0.3]‡	<0.01
Calcium (mmol/L)	2.37 [±0.02]	2.41 [±0.02]	2.38 [±0.02]	2.40 [±0.02]	0.57
Phosphate (mmol/L)	1.50 [±0.06]	1.41 [±0.06]	1.53 [±0.06]	1.59 [±0.07]	0.20
Parathyroid hormone (pmol/L)	18.9 [7.9 - 34.3]	22.5 [9.4 - 39.4]	21.4 [13.6 - 35.8]	28.3 [11.6 - 54.1]	0.20
HbA1c (%)	7.5 [5.9 - 9.5]	7.5 [6.1 - 9.2]	7.0 [6.6 - 7.8]	7.3 [6.2 - 9.0]	0.88
Ox-LDL (ng/mL)	38.4 [12.9 - 117.7]	33.7 [4.3 - 96.8]	42.7 [17.1 - 112.3]	28.6 [5.8 - 54.1]	0.30
TBARS (µM MDA)	2.5 [±0.1]	2.3 [±0.1]	2.3 [±0.1]	2.4 [±0.1]	0.51

Data presented as mean [±SEM] or median [IQR]. For variables with significant omnibus test statistic across quartiles, pairwise testing was performed after applying a Bonferroni correction to the α-level. Significant differences on pairwise testing between quartiles indicated as: vd *Q1, †Q2, ‡Q3, §Q4.

Table 34 - Determinants of baseline fibrinogen levels: categorical variables.

			Fibrinogen (g/L)		
			n	Median [IQR]	p
Patient factors:	Sex	Male	103	3.8 [3.4 - 4.3]	0.55
		Female	153	3.7 [3.4 - 4.1]	
	Ethnicity	Caucasian	196	3.7 [3.4 - 4.1]	0.40
		Asian	46	3.9 [3.3 - 4.3]	
		Black	12	3.5 [2.7 - 4.1]	
		Other*	2	2.2 [1.1 - 3.3]	
	Smoking history	Never smoked	118	3.7 [3.3 - 4.1]	0.04†
		Current smoker	56	3.8 [3.4 - 4.1]	
		Ex-smoker	82	3.9 [3.5 - 4.4]	
Co-morbidities:	Diabetes	Yes	74	3.9 [3.4 - 4.1]	0.76
		No	182	3.7 [3.4 - 4.1]	
	Ischaemic heart disease	Yes	74	3.8 [3.5 - 4.3]	0.08
		No	182	3.7 [3.3 - 4.1]	
	Malignancy	Yes	16	4.0 [3.4 - 4.4]	0.16
		No	240	3.7 [3.4 - 4.1]	
	Peripheral vascular disease	Yes	71	3.8 [3.4 - 4.1]	0.72
		No	185	3.7 [3.4 - 4.2]	
	Left ventricular dysfunction	Yes	23	3.7 [3.4 - 4.1]	0.92
		No	233	3.8 [3.4 - 4.2]	
Drugs:	ACEi / A2RBs / DRI	Yes	81	3.7 [3.3 - 4.1]	0.22
		No	175	3.8 [3.4 - 4.2]	
	β-blockers	Yes	54	3.8 [3.3 - 4.3]	0.96
		No	202	3.8 [3.4 - 4.1]	
	Statins	Yes	146	3.8 [3.4 - 4.3]	0.30
		No	110	3.7 [3.4 - 4.1]	
	Aspirin	Yes	116	3.8 [3.4 - 4.3]	0.04
		No	140	3.7 [3.3 - 4.1]	
	Clopidogrel	Yes	20	3.8 [3.2 - 4.5]	0.96
		No	236	3.8 [3.4 - 4.1]	
	Dipyridamole	Yes	3	3.7 [3.7 - 3.8]	0.92
		No	253	3.8 [3.4 - 4.2]	
	Warfarin	Yes	15	3.9 [3.6 - 4.3]	0.21
		No	241	3.7 [3.4 - 4.1]	
	Sulphonylureas	Yes	13	3.9 [3.3 - 4.2]	0.87
		No	243	3.7 [3.4 - 4.1]	
	Insulin	Yes	54	3.9 [3.3 - 4.2]	0.49
		No	202	3.7 [3.4 - 4.1]	

*Excluded from statistical analysis owing to small group size; †Never vs Ex-smokers. ACEi: Angiotensin converting enzyme inhibitors; A2RBs: Angiotensin II receptor blockers; DRI: Direct renin inhibitors

8.2.2 Determinants of baseline lag times

In bivariate analyses the lag times were positively correlated with the APTT and factor D levels, negatively correlated with the PTH and CRP levels (see Table 35), shortened across quartiles of increasing fibrinogen concentration and differed between quartiles of Ox-LDL (see Table 38). Multivariate regression analysis indicated that the lag times were longer in patients with lower levels of fibrinogen, higher APTTs or receiving clopidogrel therapy; the adjusted-R^2 for the model was 0.10. The residual versus predicted plot revealed one outlying case, repeating the stepwise variable procedure after excluding this case retained the fibrinogen and APTT variables in the model but excluded clopidogrel (model adjusted-R^2=0.08).

8.2.3 Determinants of baseline maximum absorbance

In unadjusted analyses the maximum absorbance was positively associated with fibrinogen, CRP, C3 and triglyceride levels, age, weight and PT and negatively correlated with blood pressure (see Table 35). It was also higher in patients with a history of ischaemic heart disease, in ex-smokers compared to patients who had never smoked (see Table 36) and differed across quartiles of SC5b-9 concentration (see Table 38). On multivariate regression analysis there were independent positive associations between maximum absorbance and fibrinogen, CRP, C3 and PT levels and negative associations with triglyceride levels, TBARS and diastolic blood pressure. Despite the high inter-correlation of a number of the predictor variables (i.e. fibrinogen, CRP, C3; $r>0.35$, $p<0.0001$ in all cases) inspection of the variance inflation factors (VIFs) revealed no evidence of multicollinearity (max VIF 2.1) suggesting this was not influencing the model significantly. Two samples had an outlying value for maximum absorbance and repeating the variable selection procedure after excluding these cases had no effect on the variables retained in the final model. The initial regression model, i.e. including the complete dataset, had an adjusted-R^2 of 0.78. Constructing a model including only fibrinogen concentration had an adjusted-R^2 of 0.70 suggesting that fibrinogen concentration was the major determinant of the maximum absorbance, explaining 70% of the variance.

8.2.4 Determinants of baseline $Lys50_{t0}$ times

The $Lys50_{t0}$ times were positively associated with C3, CRP, factor D, triglyceride, albumin and HbA1c levels, negatively associated with diastolic blood pressure and TBARS levels (see Table 35) and were longer in women, patients without a diagnosis of malignancy (see Table 36), patients receiving aspirin or clopidogrel therapy (see Table 37), and differed

between quartiles of fibrinogen concentration (see Table 38). The initial backwards stepwise variable selection procedure rejected all of the variables but was only based on 56 patients owing to the inclusion of HbA1c which was only measured in patients with diabetes. The procedure was therefore repeated after omitting this variable and the resultant model retained only triglyceride and HDL levels. Both variables had positive regression coefficients suggesting the model was influenced by multicollinearity given the strong negative inter-correlation of these variables on bivariate analysis ($r=-0.52$, $p<0.0001$). The cholesterol / HDL ratio was therefore calculated and added to the variable list for model selection in place of the individual variables. (The cholesterol / HDL ratio was significantly positively correlated with the $Lys50_{t0}$ times ($r=0.15$, $p=0.03$) justifying its inclusion in the variable selection procedure). The resultant model (adjusted-R^2=0.03) indicated that $Lys50_{t0}$ times were longer in patients receiving aspirin and shorter in patients receiving renin-angiotensin medications. Entering the cholesterol / HDL ratio and the triglyceride levels, either individually or together, into the stepwise variable selection procedure resulted in the same model. A review of the residuals versus fitted regression diagnostic plot identified a number of cases with extreme $Lys50_{t0}$ times and 9 cases fulfilled the *a priori* criteria for being outliers (section 3.17.4); the analyses were therefore repeated after excluding these cases. This final model identified that the $Lys50_{t0}$ times were positively associated with factor D, albumin, triglyceride and CRP levels, were lower in men, and were negatively associated with haemoglobin and TBARS levels. This model had an adjusted-R^2 of 0.20, constant error variance and no evidence of multicollinearity. The improvement in the model adjusted-R^2 and the retention of different variables by the stepwise selection procedure suggested that the initial regression model was influenced by the cases with outlying $Lys50_{t0}$ times.

Table 35 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$ times at baseline: continuous variables.

		Lag time		*Maximum absorbance*		*$Lys50_{t0}$*	
	n	***r***	***p***	***r***	***p***	***r***	***p***
Patient factors							
Age	*205*	0.07	0.32	0.14	0.04	0.05	0.43
Weight	*205*	-0.04	0.53	0.25	<0.001	0.08	0.28
Pre-dialysis systolic blood pressure	*205*	0.11	0.12	-0.14	0.04	-0.04	0.62
Pre-dialysis diastolic blood pressure	*205*	-0.05	0.44	-0.24	<0.001	-0.15	0.03
Dialysis factors							
Time on renal replacement therapy	*205*	-0.02	0.83	-0.12	0.10	-0.06	0.36
Urea reduction ratio	*205*	-0.01	0.90	-0.06	0.43	-0.06	0.36
Laboratory parameters							
Haemoglobin	*205*	-0.03	0.62	-0.12	0.09	-0.14	0.050
Ferritin	*205*	-0.03	0.69	0.13	0.06	0.00	0.97
Albumin	*205*	0.04	0.60	-0.01	0.84	0.21	<0.01
Cholesterol	*205*	-0.09	0.21	0.00	0.95	0.13	0.06
High-density lipoprotein	*205*	0.06	0.37	-0.09	0.18	-0.09	0.19
Low-density lipoprotein	*202*	-0.13	0.07	0.01	0.88	0.04	0.60
Triglycerides	*205*	-0.02	0.83	0.14	0.045	0.24	<0.001
Prothrombin time	*203*	0.00	0.97	0.16	0.02	0.09	0.18
Activated partial thromboplastin time	*203*	0.20	<0.01	0.13	0.06	0.06	0.36
Bicarbonate	*205*	0.06	0.38	-0.12	0.08	-0.02	0.75
Calcium	*205*	-0.01	0.92	-0.01	0.94	-0.07	0.32
Phosphate	*205*	-0.10	0.15	0.03	0.65	0.02	0.73
Parathyroid hormone	*195*	-0.18	0.01	0.08	0.30	-0.01	0.88
HbA1c	*58*	0.04	0.75	-0.06	0.65	0.36	<0.01
Oxidative stress							
TBARS	*205*	0.02	0.77	-0.13	0.06	-0.24	<0.001
Inflammation							
C-reactive protein	*205*	-0.14	0.04	0.57	<0.0001	0.20	<0.01
C3	*205*	0.05	0.52	0.44	<0.0001	0.28	<0.0001
Factor D	*205*	0.21	<0.01	-0.08	0.25	0.23	<0.01
Properdin	*205*	0.07	0.31	0.07	0.35	-0.11	0.13

r: correlation coefficient

Table 36 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$: categorical variables (Patient factors and Comorbidities).

		n	Lag time (s)	*p*	Maximum Absorbance (au)	*p*	$Lys50_{t0}$ (s)	*p*
Patient factors								
Sex	Male	130	512 [460 - 595]	*0.61*	0.532 [±0.013]	*0.98*	2145 [1938 - 2322]	*0.03*
	Female	75	532 [461 - 583]		0.532 [±0.016]		2168 [2035 - 2528]	
Ethnicity	Caucasian	155	518 [461 - 583]	*0.64*	0.533 [±0.011]	*0.09*	2149 [1971 - 2425]	*0.99*
	Asian	39	513 [462 - 599]		0.554 [±0.025]		2106 [1990 - 2427]	
	Black	10	574 [423 - 706]		0.444 [±0.045]		2147 [1957 - 2359]	
	Other*	1	589		0.424		1897	
Smoking history	Never	92	534 [462 - 599]	*0.40*	0.513 [±0.014]	*0.01*[†]	2148 [2012 - 2434]	*0.30*
	Current	42	513 [459 - 550]		0.508 [±0.018]		2072 [1884 - 2367]	
	Ex-smoker	71	507 [451 - 584]		0.571 [±0.018]		2153 [2022 - 2403]	
Co-morbidities								
Diabetes	Yes	59	535 [469 - 589	*0.28*	0.531 [±0.016]	*0.94*	2168 [2022 - 2463]	*0.33*
	No	146	513 [452 - 589]		0.533 [±0.012]		2140 [1944 - 2422]	
IHD	Yes	61	534 [474 - 590]	*0.29*	0.570 [±0.018]	*0.01*	2176 [2058 - 2472]	*0.07*
	No	144	513 [451 - 589]		0.516 [±0.011]		2137 [1945 - 2365]	
Malignancy	Yes	12	485 [421 - 529]	*0.13*	0.586 [±0.036]	*0.18*	1986 [1931 - 2081]	*0.04*
	No	193	519 [461 - 592]		0.529 [±0.010]		2153 [1989 - 2428]	
PVD	Yes	57	538 [471 - 586]	*0.26*	0.534 [±0.018]	*0.91*	2153 [1987 - 2434]	*0.75*
	No	148	512 [453 - 589]		0.531 [±0.012]		2146 [1960 - 2424]	
LV dysfunction	Yes	16	500 [451 - 605]	*0.84*	0.545 [±0.036]	*0.70*	2076 [1932 - 2267]	*0.34*
	No	189	518 [461 - 588]		0.531 [±0.010]		2149 [1981 - 2426]	

Data presented as mean [±SEM] or median [IQR]. *Excluded from statistical analysis owing to small group size; [†]Never vs Ex-smokers. IHD: Ischaemic heart disease; PVD: Peripheral vascular disease; LV: Left ventricular

Table 37 - Determinants of lag time, maximum absorbance and $Lys50_{t0}$: categorical variables (Drugs).

		n	Lag time (s)	*p*	Maximum Absorbance (au)	*p*	$Lys50_{t0}$ (s)	*p*
Drugs								
ACEi / A2RBs / DRI	Yes	68	504 [461 - 608]	*0.54*	0.509 [±0.016]	*0.10*	2100 [1963 - 2291]	*0.12*
	No	137	532 [459 - 588]		0.543 [±0.012]		2172 [1980 - 2461]	
β-blockers	Yes	40	504 [442 - 570]	*0.27*	0.560 [±0.028]	*0.17*	2164 [1979 - 2512]	*0.65*
	No	165	520 [462 - 592]		0525 [±0.010]		2146 [1976 - 2410]	
Statins	Yes	120	525 [461 - 585]	*0.44*	0.535 [±0.012]	*0.73*	2152 [2013 - 2499]	*0.18*
	No	85	513 [447 - 596]		0.528 [±0.017]		2138 [1947 - 2341]	
Aspirin	Yes	103	528 [462 - 583]	*0.95*	0.550 [±0.014]	*0.07*	2218 [2022 - 2528]	*<0.01*
	No	102	513 [458 - 599]		0.514 [±0.013]		2096 [1941 - 2269]	
Clopidogrel	Yes	15	536 [480 - 702]	*0.14*	0.553 [±0.041]	*0.55*	2400 [2139 - 2481]	*0.046*
	No	190	518 [460 - 586]		0.530 [±0.010]		2143 [1957 - 2404]	
Dipyridamole	Yes	3	554 [522 - 651]	*0.30*	0.521 [±0.017]	*0.89*	2101 [2056 - 2145]	*0.64*
	No	202	518 [461 - 589]		0.532 [±0.010]		2148 [1970 - 2426]	
Sulphonylureas	Yes	11	532 [476 - 569]	*0.89*	0.492 [±0.033]	*0.33*	2044 [1980 - 2283]	*0.34*
	No	194	518 [460 - 590]		0.543 [±0.010]		2148 [1970 - 2427]	
Insulin	Yes	41	533 [469 - 602]	*0.20*	0.542 [±0.021]	*0.61*	2168 [2019 - 2493]	*0.40*
	No	164	517 [457 - 586]		0.529 [±0.011]		2145 [1955 - 2412]	

Data presented as mean [±SEM] or median [IQR]. ACEi: Angiotensin converting enzyme inhibitors; A2RBs: Angiotensin II receptor blockers; DRIs: Direct renin inhibitors

Table 38 - Analysis of fibrin clot variables by quartiles of A. fibrinogen, B. Ox-LDL and C. SC5b-9 concentrations at baseline.

Quartile	*n*	Lag time (s)	Maximum absorbance (au)	$Lys50_{t0}$ (s)
A. Fibrinogen (g/L)				
Q1: <3.4	*51*	571 [480-635]	0.382 [±0.009]	2109 [1939-2300]
Q2: 3.4-3.7	*52*	533 [491-583]	0.484 [±0.008]	2125 [1952-2280]
Q3: 3.8-4.1	*50*	518 [436-584]	0.554 [±0.008]	2121 [1977-2433]
Q4: >4.1	*50*	472 [433-542]	0.710 [±0.016]	2257 [2074-2536]
p		<0.01	<0.0001	0.046
B. Ox-LDL (ng/mL)				
Q1: <8.9	*52*	516 [466-634]	0.539 [±0.018]	2177 [2013-2529]
Q2: 8.9-34.0	*54*	524 [457-580]	0.535 [±0.021]	2154 [1944-2542]
Q3: 36.3-96.8	*48*	482 [434-562]	0.541 [±0.021]	2089 [1939-2226]
Q4: >96.8	*48*	544 [480-619]	0.517 [±0.018]	2180 [1977-2429]
p		0.047	0.82	0.10
C. SC5b-9 (ng/mL)				
Q1: <40.3	*53*	505 [462-563]	0.498 [±0.020]	2065 [1933-2349]
Q2: 40.3-66.8	*50*	539 [455-590]	0.522 [±0.018]	2153 [2027-2462]
Q3: 67.2-105	*46*	521 [458-605]	0.584 [±0.023]	2227 [2032-2426]
Q4: >105	*56*	521 [459-593]	0.531 [±0.018]	2146 [1968-2503]
p		0.61	0.02	0.21

Data presented as mean [±SEM] or median [IQR]; p is for omnibus test statistic

8.3 Temporal changes in fibrinogen levels and fibrin clot parameters and the effects of vitamin E

As outlined in section 8.1 fibrin clot data was only analysed for a subset of the study patients, principally due to the exclusion of patients receiving warfarin or dialysing via a CVC. A comparison of the baseline characteristics between study groups for this subset of patients with fibrin clot structure and function assays data are shown in Table 39 and Table 40. Within this patient subset, those patients dialysing with the VE-bonded membranes tended to have been on dialysis for less time, have higher post dialysis weights, lower factor D levels and were receiving a lower dose of dialysis.

Table 39 - Comparison of baseline characteristics between study groups for the subset of patients with fibrin clot structure and function assays data.

	Control	Vitamin E	p
N	99	106	
Sex			0.61
Male	61 (62%)	69 (65%)	
Female	38 (38%)	37 (35%)	
Age (yrs)	64.8 [±1.5]	63.3 [±1.6]	0.50
Ethnicity			0.79
White	74 (75%)	81 (76%)	
Asian	20 (20%)	19 (18%)	
Black	4 (4%)	6 (6%)	
Other	1 (1%)	0	
Smoking history			0.46
Never smoked	40 (40%)	52 (49%)	
Current smoker	22 (22%)	20 (19%)	
Ex-Smoker	37 (37%)	34 (32%)	
Time on renal replacement therapy (yrs)	4.3 [1.8-9.1]	3.2 [1.2 - 5.9]	0.03
Pre-dialysis systolic blood pressure (mmHg)	134 [±2.3]	138 [±2.4]	0.16
Pre-dialysis diastolic blood pressure (mmHg)	70 [±1.2]	72 [±1.2]	0.35
Post dialysis weight (kg)	68.4 [56.0-78.9]	73.4 [61.6-87.3]	0.02
Dialysis dose (urea reduction ratio)	0.77 [±0.01]	0.75 [±0.01]	0.03
Co-morbidity n (%)			
Diabetes	24 (24%)	35 (33%)	0.17
Ischaemic heart disease	34 (34%)	27 (25%)	0.17
Peripheral vascular disease	29 (29%)	28 (26%)	0.65
Left ventricular dysfunction	6 (6%)	10 (9%)	0.44
Malignancy	5 (5%)	7 (7%)	0.43
Systemic collagen disease	7 (7%)	3 (3%)	0.20
Drugs at baseline n (%)			
ACEi / A2RB	30 (30%)	38 (36%)	0.40
B-blockers	17 (17%)	23 (22%)	0.41
Statins	58 (59%)	62 (58%)	0.99
Aspirin	47 (47%)	56 (53%)	0.44
Clopidogrel	7 (7%)	8 (8%)	1.00
Dipyridamole	0	3 (3%)	0.25
Sulphonylureas	4 (4%)	7 (7%)	0.54
Insulin	19 (19%)	22 (21%)	0.78

Data presented as mean [±SEM] or median [IQR] unless stated. A2RB: Angiotensin II receptor blocker; ACEi: Angiotensin converting enzyme inhibitors.

Table 40 - Comparison of the baseline biochemistry, oxidative stress and inflammation parameters between study groups for the subset of patients with fibrin clot structure and function assays data.

	Control	Vitamin E	p
Biochemistry			
Albumin (g/L)	37.7 [±0.5]	37.7 [±0.4]	0.99
Calcium (mmol/L)	2.36 [±0.02]	2.40 [±0.02]	0.15
Phosphate (mmol/L)	1.45 [±0.05]	1.51 [±0.05]	0.38
Parathyroid hormone (pmol/L)	20.4 [13.2-41.2]	22.3 [9.0-42.5]	0.87
Bicarbonate (mmol/L)	22.0 [±0.3]	22.1 [±0.2]	0.60
Cholesterol (mmol/L)	3.9 [±0.1]	4.0 [±0.1]	0.33
Low-density lipoprotein (mmol/L)	2.0 [±0.1]	2.1 [±0.1]	0.26
High-density lipoprotein (mmol/L)	1.2 [±0.0]	1.2 [±0.0]	0.97
Triglycerides (mmol/L)	1.4 [1.1-2.0]	1.4 [0.9-2.2]	0.54
Oxidative stress markers			
Ox-LDL (ng/mL)	38.2 [12.5-112]	27.0 [4.3-58.4]	0.12
TBARS (µM MDA)	2.4 [±0.1]	2.4 [±0.1]	0.60
Inflammatory markers			
CRP (mg/L)	6.9 [3.0-14.1]	5.5 [1.4-14.5]	0.27
C3 (mg/mL)	0.77 [±0.02]	0.79 [±0.02]	0.35
SC5b-9 (ng/mL)	61.0 [39.5-111]	67.5 [38.8-120]	0.54
Factor D (µg/mL)	3.6 [3.1-4.4]	3.4 [2.6-4.1]	0.02
Properdin (µg/mL)	51.1 [43.9-67.1]	56.7 [43.7-74.4]	0.12

Data presented as mean [±SEM] or median [IQR]

At baseline, there were no significant differences in the fibrinogen levels or fibrin clot characteristics between the two study groups (see Table 41).

Table 41 - Comparison of fibrinogen levels and fibrin clot parameters between study groups at baseline.

	Control	Vitamin E	p
Fibrinogen (g/L)	3.8 [3.4-4.1]	3.7 [3.4-4.1]	0.83
Lag time (s)	519 [468-589]	515 [450-591]	0.66
Maximum Absorbance (au)	0.526 [±0.015]	0.537 [±0.013]	0.59
$Lys50_{t0}$ (s)	2136 [1954-2403]	2157 [1988-2443]	0.47

Data presented as mean [±SEM] or median [IQR]. au: absorbance units

8.3.1 12-month changes in fibrinogen levels

As shown in Figure 52, there were no differences in the fibrinogen levels between the groups at baseline (p=0.83) or at 12-months (p=0.26), nor when the baseline and 12-month levels were compared in the control (p=0.82) or VE (p=0.21) groups. The 12-month changes in the fibrinogen levels were compared between groups and found not to differ

(p=0.32; see Figure 53). The between group comparison of the 12-month change in fibrinogen levels remained non-significant after adjusting for post-dialysis weight and time on dialysis which were the two significant determinants of the fibrinogen levels which differed between the study groups at baseline.

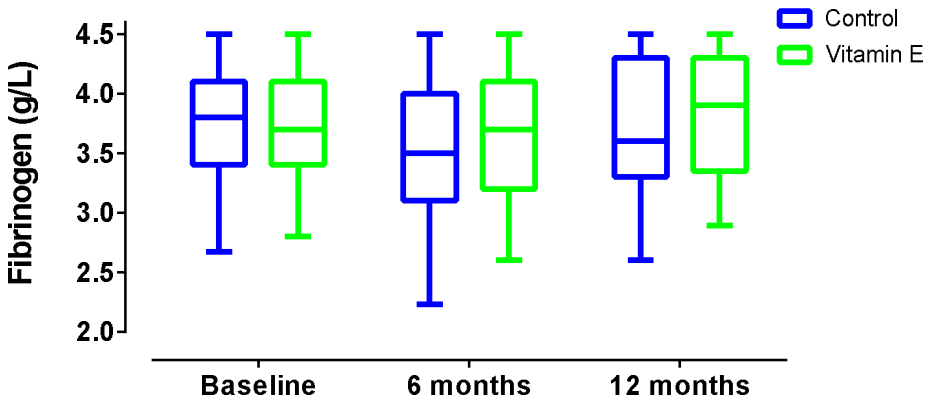


Figure 52 - Fibrinogen levels at baseline, 6 and 12 months. There were no significant inter-group differences nor significant changes in the fibrinogen levels across study visits.

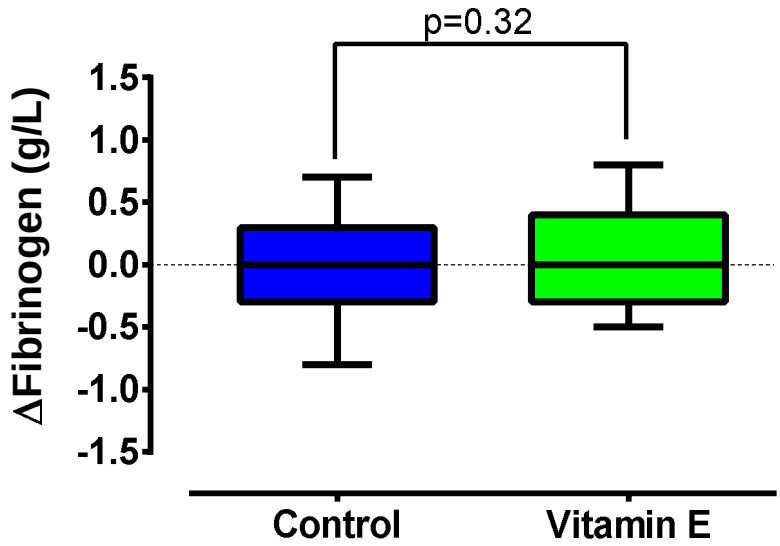


Figure 53 - Comparison of 12-month change in fibrinogen levels between study groups. There was no difference in the 12-month change in fibrinogen levels between study groups. (ΔFibrinogen = $Fibrinogen_{12months}$ - $Fibrinogen_{baseline}$).

8.3.2 12-month changes fibrin clot parameters

Repeated measures ANOVA identified no significant trends nor a significant effect of study group when lag times (p=0.21 and 0.92 respectively), maximum absorbance (p=0.10 and 0.23 respectively) and $Lys50_{t0}$ (p=0.46 and 0.42 respectively) were analysed across study visits (see Figure 54). Comparisons of the 12-month changes in each of these parameters

between study groups revealed no statistically significant differences (Figure 54). The absence of a significant difference between study groups in the 12-month change in each of the fibrin clot paramters shown in Figure 54 persisted in analyses which were adjusted for the factors which differed between the groups at baseline (i.e post dialysis weight, years of dialysis dependency, factor D levels and dialysis dose; p>0.27 in all cases).

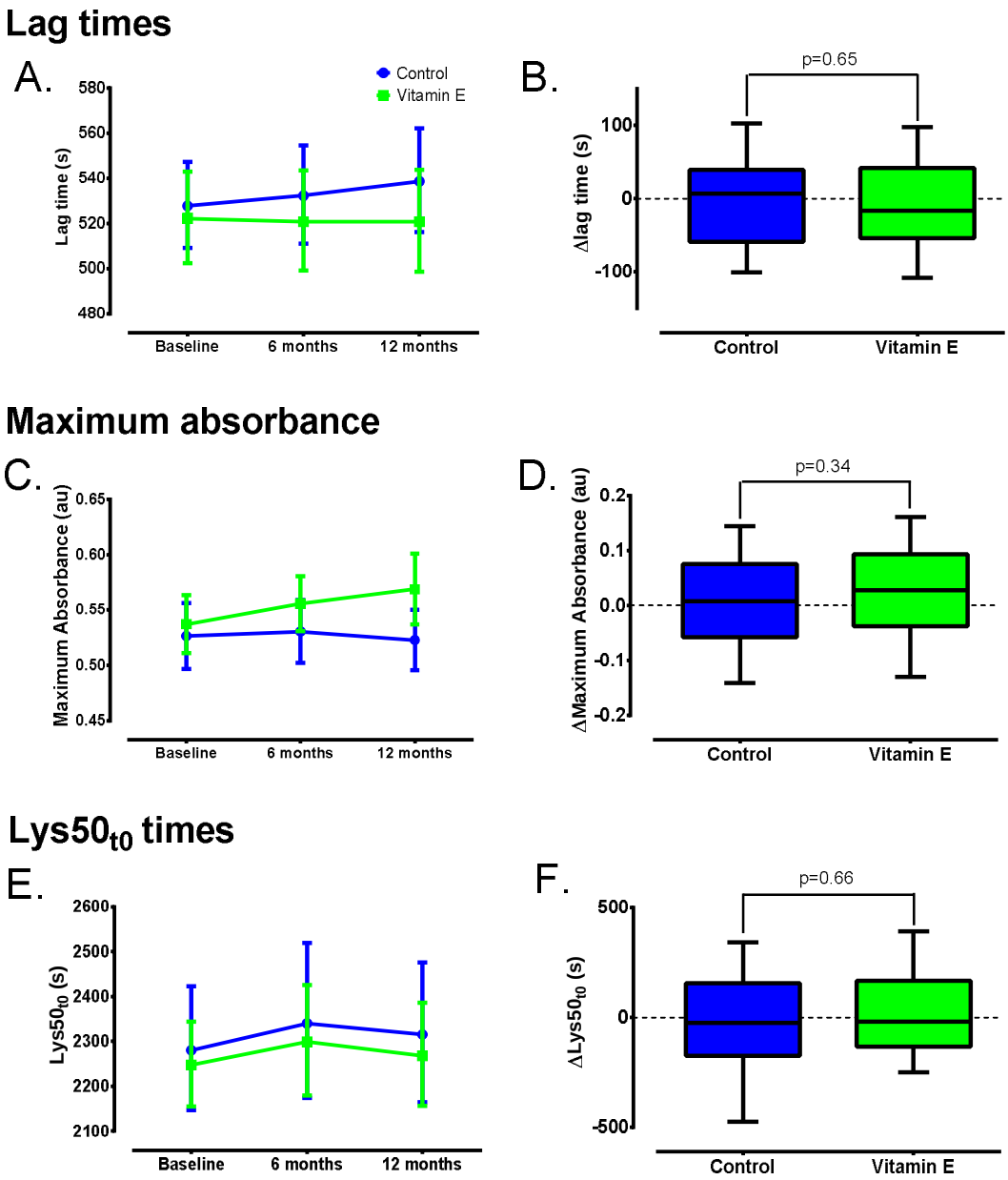


Figure 54 - Comparisons of the fibrin clot parameters between study groups across the three study visits and the change in each parameter after 12 months. There were no significant changes over time nor effects of study group allocation on the change in (A) lag times, (C) maximum absorbance or (E) $Lys50_{t0}$ times across study visits. Comparing the 12-month change in each of these parameters between study groups similarly found no significant differences (B,D and F). ($\Delta variable = variable_{12months} - variable_{baseline}$).

Given that there was no effect of the VE membrane on the fibrinogen levels or fibrin clot characteristics, the relationships between the changes in the fibrinogen levels and the fibrin clot parameters, and the changes in the biomarkers of oxidative stress and inflammation were evaluated in the whole dataset i.e. after combining the two study groups.

8.4 Changes in fibrinogen levels and fibrin clot parameters in relation to changes in oxidative stress

The Spearman rank correlation coefficients for the 12-month change in each of the biomarkers of oxidative stress measured in the present study and the change in fibrinogen levels and the fibrin clot parameters are detailed in Table 42. The only statistically significant finding was a positive correlation between the changes in Ox-LDL levels and lag times.

Table 42 - Spearman's rank correlation coefficients for changes in oxidative stress markers, fibrinogen and fibrin clot parameters.

	ΔFibrinogen	**ΔLag time**	**ΔMaximum absorbance**	**ΔLys50$_{t0}$ times**
ΔOx-LDL	-0.04	0.23**	-0.03	-0.09
ΔTBARS	-0.12	0.03	-0.08	-0.10

In each case Δv*ariable* was defined as $variable_{12months}$ - v$ariable_{baseline}$. *p<0.05, **p<0.01.

8.5 Changes in fibrinogen levels and fibrin clot parameters in relation to changes in inflammation

The Spearman's rank correlation coefficients for the 12-month change in each of the inflammatory biomarkers measured, including fibrinogen levels, and the fibrin clot parameters are shown in Table 43. Reductions in the lag times were associated with increasing fibrinogen, CRP and SC5b-9 levels. The maximum absorbance increased with increases in the levels of fibrinogen, CRP, C3 and SC5b-9. Lengthening of the Lys50$_{t0}$ times were associated with increases in the C3 and SC5b-9 levels. There were strong correlations between the changes in fibrinogen levels and the changes in CRP, C3 and SC5b-9 levels. Given that the fibrinogen levels were an important determinant of the fibrin clot phenotype, partial correlation analysis was undertaken to determine if the changes in

the inflammatory markers were associated with changes in the fibrin clot parameters after adjusting for the change in fibrinogen levels. In these adjusted analyses, there were significant associations between the change in maximum absorbance and the change in both CRP (partial correlation coefficient=0.47, p<0.001) and C3 (partial correlation coefficient=0.19, p<0.05) levels. The remainder of the statistically significant findings in the unadjusted bivariate analyses become non-significant when the analyses were adjusted for the change in fibrinogen levels.

Table 43 - Spearman's rank correlation coefficients for changes in inflammation markers, fibrinogen and fibrin clot parameters.

	ΔFibrinogen	**ΔLag time**	**ΔMaximum absorbance**	**ΔLys50$_{t0}$ times**
ΔFibrinogen	(1.00)	-0.20**	0.73**	0.10
ΔCRP	0.41**	-0.25**	0.55**	0.15
ΔC3	0.40**	-0.12	0.44**	0.16*
ΔSC5b-9	0.23**	-0.16*	0.31**	0.18*
ΔFactor D	-0.02	-0.08	0.00	-0.03
ΔProperdin	-0.06	0.06	-0.08	-0.01

In each case Δ*Variable* was defined as $Variable_{12months}$ - $Variable_{baseline}$. *p<0.05, **p<0.01

8.6 Discussion

Despite the relatively large number of studies examining fibrin clot characteristics in different patient groups, only one published study to date has examined them in chronic HD patients [646]. Furthermore, only a small proportion of published studies have performed serial measurements of fibrin clot structure following an intervention [683, 1064]. In the present study several markers of inflammation, oxidative stress and components of the complement cascade were measured permitting a detailed evaluation of the key determinants of fibrin clot structure and function in HD patients. In addition, the effects of changes in the levels of inflammation and oxidative stress on the fibrin clot characteristics were determined to establish if interventions targeted at improving the levels of inflammation and oxidative stress may have the potential to influence the fibrin clot phenotype. One such potential intervention, the use of VE bonded dialysis membranes, was evaluated here.

Fibrin clot analysis was performed on 714 samples although a significant number of assays yielded no results because there was insufficient clot formation or fibrinolysis to enable measurement of the clot parameters. A large proportion of the samples without assay data were obtained from patients dialysing via CVCs or receiving warfarin. Analyses comparing these samples with the remainder of the dataset revealed significant differences in the fibrin clot characteristics (see Table 28 and Table 29) therefore these samples were excluded from the dataset prior to further analyses. It is likely that some or all of the samples obtained from patients dialysing via CVC's were contaminated with heparin to explain the different clot characteristics. To prevent catheter thrombosis all CVCs were locked between dialysis sessions with concentrated heparin solution (5000 units/mL, typical locking volume 1.5-1.7 mL/lumen) in line with the LTHT renal unit policy. At the start of the HD treatment, the locking solution was aspirated from the CVC and the patient connected to the HD circuit. Prior to obtaining the blood sample for analysis, blood was drawn through the CVC for approximately 30 seconds until the column of blood reached the bubble trap (see 3.7.4). It is likely, however, that these samples still contained trace quantities of heparin, possibly due to contamination of the blood as it was drawn through the CVC or as a result of systemic dissipation of small amounts of the locking solution when handling the dialysis catheter prior to aspiration. The majority of the anticoagulant effect of heparin is as a result of its binding to anti-thrombin III (AT) [1065]. The heparin-AT complex then inactivates a number of components of the coagulation pathway, including Xa, IXa, XIa and XIIa, and most importantly it binds and inactivates thrombin [1065]. Thus heparin contamination could potentially explain the longer lag times and slower clotting rates in the assays performed on samples obtained from CVCs compared to AVFs.

A number of samples obtained from patients receiving warfarin yielded no assay results and the This carboxylation permits calcium-dependent conformational changes thereby facilitating interactions with phospholipid membranes which are necessary for their physiological activity [769]. Vitamin K dependent proteins include the coagulation factors II, VII, IX and X and the regulatory proteins C and S [769]. These factors are all upstream of thrombin in the clotting cascade and thus warfarin may be anticipated to have minimal effects in the assay as exogenous thrombin is added. However, owing to the small quantities of exogenous thrombin added, intra-assay fibrin clot formation relies on the generation of endogenous thrombin, via the positive feedback amplification loop described in 1.6.1.3, to explain the inhibitory effects observed with warfarin. More samples obtained from patients on warfarin yielded data from the fibrinolysis assay compared to the clotting

assay. On reviewing the individual sample data, this situation arose because a number of the samples obtained from patients receiving warfarin did form a delayed clot which was not fully formed during the 1 hour clotting assay but was evident on the 10 hour fibrinolysis assay.

8.6.1 Fibrin clot variables selected for further analyses

As detailed in Table 30 and Table 31, several of the fibrin clot parameters were highly inter-correlated consistent with the original report of this assay [721]. The lag phase tended to be shorter and the maximum absorbance greater in the clotting compared to the fibrinolysis assay as shown in Figure 51. This was because the reaction mix in the fibrinolysis assay contained tPA therefore clot formation and breakdown were occurring concurrently. In both assays, the clot rate was positively correlated with the maximum absorbance and negatively correlated with the lag phase, and increasing fibrinogen concentrations were associated with shorter lag times and the formation of denser clots. These findings reflect that fibrin clot structure is influenced to a large degree by the kinetics of fibrin polymerisation [639]. Faster rates of fibrin polymerisation, as occur with higher fibrinogen concentrations [642, 643], result in the formation of clots comprised of thinner fibres which are denser with smaller intrinsic pores in both *in vitro* purified fibrinogen [641-643] and *ex vivo* plasma-based [644-649] systems. All of the measures of fibrinolysis were positively correlated (Table 31) and the variability in the fibrinolysis parameters was greater than the variability in the clotting parameters, as detailed in section 3.15, which is likely related to the fact that clot formation and breakdown were occurring at the same time in the fibrinolysis assays thereby increasing the variability in the derived clot parameters. Furthermore, fibrinolysis appears to be more unpredictable as it progresses, possibly due to variations in the way the clot breaks apart to reveal plasminogen binding sites. Such variation is evident on review of the absorbance-time graphs and explains why the variables which measure the whole of the clot lysis, i.e. lysis rate and lysis area, were subject to the greatest intra- and inter-assay variability. This also provides the rationale for using the time to 50% lysis, termed the $Lys50_{t0}$ time in the present study, as a marker of fibrinolysis susceptibility in line with other studies [646, 647, 655, 679, 681, 683, 688, 715, 718, 721, 1064, 1066]. All of the lysis measures, with the exception of lysis rate, were positively correlated with the maximum absorbance reflecting the known association between denser clots and fibrinolytic resistance [726, 1067-1069]. This association arises due to reduced access of plasminogen to its binding sites on fibrinogen in denser clots, i.e. steric effects, and the increased clot stability conferred by the incorporation of greater amounts of fibrinogen per unit volume.

After evaluating the inter-relationships of the clotting and fibrinolysis assay parameters in the baseline dataset, the variables selected for more detailed analyses were the lag time and maximum absorbance from the clotting assay and the $Lys50_{t0}$ times from the fibrinolysis assay. These variables are the measures of clot formation and fibrinolysis which are most frequently reported in the literature, allowing better contextualisation of the results from the present study. Furthermore the maximum absorbance and the $Lys50_{t0}$ times were the most reproducible clot parameters in the present study (see section 3.15), and in previous experience with the assay [721], thereby potentially facilitating the detection of smaller differences between subgroups. Additionally, with respect to the maximum absorbance and lag times, these have been mapped to specific clot characteristics or aspects of clot formation, such as a positive association between clot turbidity, i.e. maximum absorbance, and the fibrin fibre mass-length ratio [1070, 1071] and the lag phase representing the time taken for protofibril formation prior to lateral aggregation [639, 1072], permitting a better understanding of the processes being indirectly measured. It is important to note that many of the studies examining the associations between clot turbidity and clot architecture (e.g. fibre thickness, porosity) have been performed using purified fibrinogen. The relationships between these clot properties and turbidimetric measurements in plasma samples differ markedly from those observed in the purified fibrinogen systems [1070, 1071], largely as a result of the presence of plasma proteins, such as albumin, fibronectin and ATIII in the former influencing fibrin clot assembly [1073]. Also, although they may be related [647, 648, 709, 715], clot turbidity measured in the present study and clot permeability measured in a number of other studies reflect different aspects of fibrin clot structure with permeability reflecting pore size and turbidity the fibre density [1070, 1074, 1075]. Discrepancies between the two measures can arise because the fibrin network is comprised of a major network, comprising thicker fibres, and a minor network comprising thinner fibres. Changes in the minor network may impact little on the clot turbidity but have a profound effect on clot permeability [1074-1076], an effect which appears to be more pronounced in plasma compared to purified fibrinogen systems [1070]. Analysing the maximum absorbance and lag phase from the clotting assay rather than the fibrinolysis assay, theoretically at least, seems more logical as the former is not affected by the addition of tPA, which increases the lag time and reduces the maximum absorbance as shown in Figure 51, allowing a better appreciation of the factors influencing clot formation. The majority of studies looking at measures of clot density have done so by examining clots formed in the absence of tPA.

8.6.2 Baseline determinants of fibrinogen levels and fibrin clot parameters

In both bivariate and multivariate analyses, there were strong positive associations between fibrinogen levels and the inflammatory markers CRP and C3 reflecting that all three are positive acute phase reactants [428]. There was no difference in fibrinogen levels between the sexes in contrast to studies of non-renal populations which have consistently reported higher fibrinogen levels in women [702, 707]. Part of the explanation for this is thought to be an artefact due to greater dilution of citrated blood samples in men, who tend to have higher haematocrits, as studies using dry dipotassium edetate as an anticoagulant, thus avoiding any sample dilution, have reported no sex difference in fibrinogen levels [1077]. In the present study there was no significant difference in the packed cell volume between males and females (36.7 [±0.3] vs 35.7 [±0.4] % respectively; $p=0.08$) which may explain the similar fibrinogen levels between sexes. Although no linear association with age was observed across the quartiles of fibrinogen concentration, patients in the highest quartile were significantly older than those in the lowest quartile ($p<0.05$) which is consistent with the observation that fibrinogen levels increase with age [702]. Increased fibrinogen levels have been associated with both prevalent [1078] and incident [690] CV disease, including studies of HD patients [45, 435, 646, 1079], but no association with prevalent CV disease was observed in the present study. Again this may reflect that patients with higher levels of inflammation, and therefore likely higher CV disease risk and burden, were excluded from study participation in contrast to the cited studies which had no such exclusion criteria. There was no difference in the fibrinogen levels when study patients were compared on the basis of diabetic status. The literature is mixed in this respect with two large reports of pooled data from the Fibrinogen Studies Collaboration reporting differing conclusions with one finding no association [707] and the other suggesting higher levels in patients with diabetes [690]; albeit both studies were in non-HD patients.

In addition to examining fibrinogen levels, a number of parameters relating to fibrin clot formation and breakdown were evaluated in the present study. Several studies have reported positive associations between markers of inflammation, such as CRP, C3 and fibrinogen, and clot turbidity and fibrinolysis times and negative associations with lag times [648, 652, 655, 681, 683, 686, 688, 709, 715, 718, 719, 1080] as were found on bivariate analyses in the present study. The associations between the clot parameters and fibrinogen levels were expected, reflecting that fibrin is the major component of clots.

Indeed, with respect to the maximum absorbance, 70% of the variance in this parameter was explained by the fibrinogen levels alone, with only modest improvements in the regression model adjusted-R^2 following the addition of the other variables retained by the variable selection procedure (i.e. C3, CRP, triglyceride, TBARS levels, diastolic blood pressure and prothrombin time), highlighting the importance of fibrinogen levels in determining the clot density. However, given that raised inflammatory markers and hyperfibrinogenaemia tend to co-exist as part of the acute phase response [428], it is hard to assess the individual contributions of the inflammatory markers to the fibrin clot phenotype. Both C3 and CRP were retained in the multivariate regression model of maximum absorbance, along with fibrinogen, and CRP was independently associated with $Lys50_{t0}$ times. Taking these findings together would tend to suggest that alterations in the fibrin clot phenotype in the setting of inflammation, particular clot density and fibrinolysis susceptibility with reference to the present study, may arise through mechanisms additional to increases in fibrinogen levels. For example several studies have demonstrated that C3 is a component of clots [682, 685, 686], the addition of C3 in both *in vitro* and *ex vivo* systems has been shown to impede fibrinolysis in a dose-dependent manner [652, 686] and data from the present study and elsewhere [688] have shown a positive association between C3 levels and fibrinolysis times. These data suggest a role for complement, or more specifically C3, in determining fibrinolytic susceptibility. C-reactive protein has been shown to bind fibrinogen [684], and thus may potentially alter fibrin network assembly, although more recent data suggest that CRP is not a component of plasma derived clots [652]. Increased factor D levels were independently associated with longer lysis times in the present study, however there are no previously published studies examining this association. It is not clear whether factor D may have a direct functional role in reducing fibrinolytic susceptibility, as is postulated for C3, or whether increased factor D levels are reflective of, or permissive for, enhanced complement activity which itself influences fibrinolysis. Factor D is the rate limiting enzyme of the alternative pathway and the alternative pathway is important for amplifying the complement response following activation by any of the three pathways [493], therefore the association between factor D levels and fibrinolysis times in the present study may simply be a surrogate for complement activation. An association between increased factor D levels and increased complement activation is supported by the positive association between factor D and SC5b-9 levels in the baseline dataset (see Table 21). Somewhat counter to this argument was the lack of association between SC5b-9 levels and fibrinolysis times, even in unadjusted analyses, although the difficulties in making inferences concerning the degree

of complement activation on the basis of the relative levels of the biomarkers measured in the present study has been discussed in section 7.4.2.

It would seem, therefore, that the data in the present study would support a role for complement components in determining the fibrin clot phenotype. This association has a biological basis given that there is cross-talk between components of the complement cascade and both the haemostatic and fibrinolytic cascades [1081-1083]. This cross-talk includes shared activators and inhibitors, for example the ability of FXIIa to activate C1q and therefore the classical complement pathway, and C1 esterase inhibitor to inhibit complement activation, the contact coagulation system (kallikrein and FXIIa), FXI, thrombin, tPA and plasmin [496]. Thrombin provides an important link between coagulation and complement as thrombin has been shown to have C5 convertase activity [571], be an agonist for the PKC-dependent pathway of decay accelerating factor regulation [572] and to cleave factor H forming a monocyte chemotactic factor [573]. With reference to the present study, SC5b-9 has been shown to inhibit platelet aggregation *in vivo* [1084]. There are therefore a number of fibrinogen-independent mechanisms through which inflammation, or more specifically complement activation, may influence fibrin clot assembly and hence the clot structure.

In addition to the aforementioned associations between inflammatory markers and fibrin clot structure, similar clot characteristics (i.e. denser clots, shorter lag times, increased fibrinolytic resistance) have also been observed in patients with CV disease [644, 648, 649, 681, 708, 715], including those on HD [646]. In the present study a prior history of ischaemic heart disease was associated with the formation of denser clots on bivariate analysis but there were no significant associations with lag or fibrinolysis times which is consistent with the small body of literature concerning fibrin structure in HD patients [646]. There was no influence of diabetic status on any of the fibrin clot parameters identified for detailed analyses and, with respect to fibrinolysis times, this differs from the published data in non-renal patients. The few studies of non-renal patients examining *ex vivo* clots in patients with diabetes have reported no differences in lag times and maximum absorbance [688, 719] when compared to non-diabetic controls, as was found in the present study, but increased fibrinolysis times in diabetic patients [652, 655, 688]. A number of mechanisms have been put forward to explain the reduced fibrinolytic susceptibility of fibrin clots formed from patients with diabetes including post-translational modifications of the fibrinogen molecule, such as glycation or oxidation [1085-1088], increased PAI-1 levels [1089-1093]

and increased incorporation of C3 into the clot [652]. Despite no difference in the fibrinolysis times between diabetics and non-diabetics in the present study, HbA1c levels were positively correlated with fibrinolysis times, as has been reported in purified fibrinogen systems [720, 1094], suggesting a possible role of fibrinogen glycation in fibrinolytic resistance. The absence of a significant association between diabetes and fibrinolytic resistance in the present study, contrasting with those studies already cited [652, 655, 688], may have arisen because the effects of uraemia or dialysis therapy on the fibrin clot phenotype (see [645, 646]) are greater than that of diabetes such that any additional contribution of the diabetic state is masked.

There was an independent positive association between fibrinolysis times and triglyceride levels. Several studies have demonstrated a strong correlation between the PAI-1 and triglyceride levels [703, 1095-1098] thus the triglyceride levels may have been a proxy for PAI-1 levels in this analysis. Since PAI-1 is also a positive acute phase reactant [428] this represents another fibrinogen-independent mechanism through which inflammation may contribute to fibrinolytic resistance. The initial regression model of $Lys50_{t0}$ times retained the HDL levels and recent work has demonstrated a number of HDL-associated proteins, including serum amyloid P and the apolipoproteins A-I, E, J and A-IV, are present in fibrin clots derived from plasma and purified fibrinogen [1099] suggesting HDL may influence the clot phenotype. The finding of longer lysis times in women is at odds with the higher levels of PAI-1 [1096, 1100-1102] and homocysteine [1103, 1104] (which is associated with fibrinolytic resistance [1105]) reported in men suggesting that if a true sex difference in fibrinolytic susceptibility exists, it is likely due to other factors, such as fibrinogen levels (although these did not differ between the sexes in the present study) or the levels of sex hormones for example.

The finding of a negative independent association between haemoglobin levels and fibrinolysis times was an unexpected finding and it was unlikely to represent a mechanistic association given the absence of significant amounts of haemoglobin or red blood cells in the plasma samples analysed. The haematocrit and the haemoglobin level were highly significantly positively correlated in the baseline dataset ($r=0.90$, $p<0.0001$), and substituting the haematocrit for the haemoglobin level in the stepwise regression variable selection procedure modelling $Lys50_{t0}$ times resulted in the retention of the same variables, including haematocrit in place of haemoglobin. Plasma samples obtained from patients with higher haemoglobin levels (and hence haematocrit) would be relatively more

diluted by the citrate anticoagulant thus the association with $Lys50_{t0}$ times in the present study may represent a dilution effect.

Both the clot density and fibrinolysis times were negatively and independently associated with TBARS levels. Studies using oxidatively modified fibrinogen have demonstrated a prolongation of the clotting time and a reduced final clot turbidity [670, 671] but no effect on lysis susceptibility [671]. Studies measuring $PGF_{2\alpha}$ as a marker of oxidative stress have reported inverse associations with clot permeability, no association with clot turbidity, and a positive association with lysis times in patients with acute coronary syndromes [648], active rheumatoid arthritis [680] and HD patients [646]. The apparent discrepant findings of a negative association between oxidative stress (i.e. TBARS) and fibrinolysis in the present study and a positive association between oxidative stress (i.e. $PGF_{2\alpha}$) and fibrinolysis times in several published studies [646, 648, 679, 680] are hard to reconcile. They may, on the one hand, simply reflect the different methods employed for measuring the fibrin clot phenotype. The fibrinogen molecule is particularly susceptible to oxidative modification [1106] and, depending on the profile and extent of the oxidative modifications, it is possible that differing effects on the fibrin clot phenotype may be observed. However such radical differences between the studies so as to produce opposite results, particularly given one of the studies was on HD patients [646], seems improbable. The oxidative stress markers measured in the present study differed from those in the published studies although all are putative markers of lipid peroxidation and may therefore be expected to produce similar findings; although such a supposition was not the case for the two biomarkers of lipid peroxidation measured in the present study. The finding in the present study that increased TBARS levels were associated both with lower clot densities and shorter lysis times, given the positive correlation of these two fibrin clot variables in the dataset (see Table 31), combined with the significantly larger dataset compared to the previously cited studies, suggests that the observed associations in the present study may be real rather than a chance finding from multiple statistical testing.

8.6.3 Changes in fibrin clot parameters over time and the effects of vitamin E

Cardiovascular disease in HD patients has been associated with increased levels of oxidative stress [396, 404-406] and inflammation [163, 429, 435, 439, 506] and both of these factors have been shown to influence fibrin clot characteristics, as discussed in the previous sections, providing the rationale for studying the effects of anti-inflammatory and

anti-oxidative interventions on the fibrin clot phenotype. The small numbers of published studies evaluating VE-bonded polysulfone HD membranes have reported improvements in markers of inflammation [893] and oxidative stress [891, 895] in some, but not all [889, 894], cases. The present study examined the impact of the VE-bonded membrane on fibrin clot structure and found no effects on the lag time, maximum absorbance or $Lys50_{t0}$ times. These findings are perhaps not surprising as the putative anti-inflammatory and anti-oxidant properties of the VE membranes, the mechanisms through which the VE membrane may alter fibrin clot structure, were not evident in the present study.

The vast majority of published data regarding fibrin clot structure are observational with very few studies performing serial measurements. An intervention study by Gajos *et al.* [1064] examined the impact of the anti-oxidant omega-3 polyunsaturated fatty acid (n-3 PUFA) on fibrin clot structure, in combination with standard pharmacotherapy, in patients with stable coronary artery disease undergoing percutaneous coronary intervention and stent implantation. The levels of the oxidative stress biomarker $PGF_{2\alpha}$ were positively correlated with lysis times at baseline, consistent with a number of studies [646, 648, 679, 680], and after 1-month treatment with n-3 PUFA there were significant reductions in both the $PGF_{2\alpha}$ levels and lysis times compared to patients treated with placebo. In the present study, no changes in the levels of oxidative stress were observed in patients dialysing with the VE membrane nor were changes in either of the oxidative stress markers measured correlated with changes in fibrinolysis times. Despite Gajos *et* al. [1064] reporting reductions in the levels of oxidative stress (i.e. $PGF_{2\alpha}$) and fibrinolysis times the two may not be mechanistically linked given the pleiotropic actions of PUFA with regards to coagulation, in addition to their anti-inflammatory and anti-oxidative properties [1107], which include effects on the levels of fibrinogen, the levels or activity of a number of clotting factors (e.g. FVII, FVIII and vWF) [1108, 1109] and reductions in plasminogen activator inhibitors [1110]. Only some of these potentially confounding factors were measured in the study and none were adjusted for in the analyses of fibrinolysis times.

Rajzer *et al*. [683] examined the effects of anti-hypertensive medications on fibrin clot structure and reported reductions in C3 levels, improved fibrin clot permeability and reduced lysis times after 6 months commensurate with lower blood pressures. Reductions in C3 levels over 12 months were observed in the present study (see Figure 42) but, despite changes in C3 levels being positively correlated with changes in the maximum absorbance and $Lys50_{t0}$ times (see Table 43), no corresponding reductions in the clot density or fibrinolysis times were observed at the study population level. This may reflect

the dispersion of values in the fibrin clot parameters such that the statistical analyses used for comparing them across study visits were too insensitive to detect any changes.

In the present study, there were significant associations between the changes in lag times and the change in fibrinogen, CRP and SC5b-9 levels, the changes in maximum absorbance and the change in fibrinogen, CRP, C3 and SC5b-9 levels and the change in $Lys50_{t0}$ times and the change in C3 and SC5b-9 levels (see Table 43). These findings would suggest that interventions which alter the levels of inflammation may have the potential to influence the clot phenotype. Given the fact that fibrinogen levels were significantly associated with all of the fibrin clot parameters on bivariate analyses (see Table 38) and that the CRP and C3 levels also increased across the quartiles of fibrinogen concentration (see Table 32) and, furthermore, the changes in fibrinogen levels were positively correlated with changes in the CRP and C3 levels (see Table 43), it is hard to discern the additional contribution of changes in inflammation, over and above changes in the fibrinogen levels, in altering fibrin clot phenotype. Fibrinogen, CRP and C3 are all acute phase reactants [428] and hence all tend to increase during inflammation. To try and better understand the effects of inflammation, separate from any changes in fibrinogen levels, on the fibrin clot phenotype partial correlation analyses for the changes in the inflammatory markers and the changes in the fibrin clot parameters were carried out while adjusting for the changes in fibrinogen levels. The results of these adjusted analyses found that changes in either CRP or C3 levels were positively associated with changes in the clot density, suggesting an effect of inflammation on the clot density independent of fibrinogen levels. Whether these reflect a role for inflammation *per se* or a specific effect of the CRP or C3 molecules is unclear. An i*n vitro* study examining the effect of adding C3 to a purified fibrinogen system, for example, reported no effects of C3 on clot density [686], suggesting the observations in the present study may not be a direct effect of increased C3 levels. It is known that several other acute phase proteins, such as haptoglobin and α1-antitrypsin for example, are also present in clots [686, 1099]. The incorporation of these other molecules into the fibrin clot may represent a fibrinogen-independent mechanisms through which inflammation can lead to the formation of denser clots. Despite the identification of a large number of inflammatory mediators present within fibrin clots [1099], for many the effects of different levels of incorporation on the clot phenotype have not been elucidated and therefore this mechanism remains conjecture at present. Data from the present study would suggest that inflammation is associated with a tendency to form clots which are denser and more resistant to fibrinolysis, the phenotype associated with CV disease [645-648, 654, 655, 679, 681, 688, 708-712, 714-723], and that reducing inflammation may be

associated with reductions in clot density and fibrinolytic resistance. These data would therefore support the idea that interventions aimed at reducing inflammation may have 'favourable' effects on the fibrin clot phenotype. Although the published observational body of data points to an association between an adverse clot phenotype and CV disease, it remains to be seen if altering the fibrin clot structure to a more 'favourable' phenotype, i.e. less dense and less resistant to fibrinolysis, would translate into improved CV outcomes.

It still remains unclear whether elevated fibrinogen levels and alterations to the fibrin clot phenotype are simply reflective of CV risk mediated through other pathways, e.g. inflammation or oxidative stress, or lie on the causal pathway of CV disease. One way to try and address the question of whether hyperfibrinogenaemia and alterations to the fibrin clot phenotype are mechanistically linked to CV disease has been through the study of patients with dysfibrinogenaemias or FXIII polymorphisms, for example, which result in alterations to the fibrin clot structure or fibrinogen levels in the absence of potentially confounding factors such as inflammation. In these settings, the association between an adverse clot phenotype, elevated fibrinogen levels and CV disease are less clear cut.

Against a mechanistic link between hyperfibrinogenaemia and CV disease several studies [1111-1113], including a meta-analysis [1114], have shown that polymorphisms in the fibrinogen gene which are associated with higher fibrinogen levels are not linked to increased rates of CV disease, data which contrasts with large observational series reporting a positive association [690, 1078], including patients on HD [45, 701]. Fibrinogen undoubtedly has a central role in vascular disease given that fibrin is a prominent component of atheromatous plaques [1115, 1116], particularly advanced lesions [1117], and fibrinogen and its degradation products have been shown to be involved in a number of processes implicated in atherosclerosis including effects on vascular tone and endothelial permeability through the binding of ICAM-1 [1118-1120], smooth muscle cell chemotaxis [1121] and proliferation [1122-1124], leukocyte migration [1125], LDL adsorption [1126] and roles in foam cell formation [1127] and platelet activation [1128]. However, despite this, evidence that these processes are enhanced in the presence of increased fibrinogen levels are lacking. Studies evaluating the pharmacological lowering of fibrinogen levels using fibrates, for example, have been largely disappointing with respect to CV disease prevention [1129, 1130] although fibrates have a number of pleiotropic effects besides lowering fibrinogen levels such as alterations to the lipid profile [1131]. In addition to the processes discussed here, the prominent role of fibrinogen levels in determining the structure and function of the fibrin clot have been demonstrated in the

present study and elsewhere [641-649] and this may represent another mechanism through which hyperfibrinogenaemia could contribute to CV disease.

In support of an association between an adverse clot phenotype and an increased risk of CV disease are studies of patients with thrombophilic dysfibrinogenaemia who form fibrin networks characterised by thin fibres, reduced permeability and increased resistance to fibrinolysis [1132-1136]. The presence of the Thr312Ala polymorphism in the α-fibrinogen chain has been associated with venous thrombosis [1137] and post stroke mortality in patients with atrial fibrillation [1138]. The fibrin networks derived from purified fibrinogen are more rigid and comprised of thicker fibres in patients homozygous for the Ala312 allele although lag times, clot turbidity and permeability appear to be unaffected [1139] perhaps suggesting an attenuation in the association between fibrin clot phenotype and CV risk. An argument against a mechanistic link between fibrin clot structure and CV disease is the observation that patients with the Val34Leu polymorphism in the FXIII A-subunit form fibrin clots which are less permeable [1140], a characteristic associated with CV disease in a number of studies [644, 646, 648, 649, 715, 718], yet these patients have a lower risk of CV disease [1141-1144]. By way of an explanation for this apparent paradox Lim *et al.* [1145] suggested a moderating effect of fibrinogen concentrations such that reductions in clot permeability with increasing fibrinogen concentrations are more pronounced with the "higher" risk Val34 allele compared to the "lower" risk Leu34 allele. The corollary being that this FXIII polymorphism may only be protective against CV disease at higher fibrinogen concentrations.

With regards to fibrin clot structure it is important to recognise that fibrin clots formed *ex vivo*, and particularly those formed from purified fibrinogen as analysed in a number of the studies cited, differ markedly from the fibrin clots formed *in vivo*. For example, Gersh *et al.* [1146] demonstrated that the incorporation of erythrocytes into fibrin clots, as occurs physiologically, resulted in thicker fibrin fibres and changes in the elastic and viscous modulus' although no effect on clot permeability was observed. It is therefore not clear exactly how the clot characteristics measured in the laboratory relate to the *in vivo* fibrin clot phenotype. Clot formation *in vivo* requires an initiating event, such as a ruptured coronary artery atherosclerotic plaque in the case of a myocardial infarction, prior to the formation of a fibrin clot. Given that clot formation is a relatively late or downstream event in the evolution of CV disease, it is difficult to see mechanistically how it could influence risk. It is conceivable that denser more fibrinolysis-resistant clots *in vivo* may be associated with worse outcomes, particularly if occlusive thrombi are preceded by smaller

thrombotic events, the clinical consequences of which will likely be mitigated by more rapid fibrinolytic clearance. Susceptibility to fibrinolysis could therefore theoretically influence outcomes with more resistant clots resulting in increased tissue ischaemia or necrosis following a thrombotic event, such as a stroke or myocardial infarction. Rapidity of clot formation or clot density may have a bearing on whether a clot is occlusive or non-occlusive, or on the thrombus size following atherosclerotic plaque rupture, to potentially explain a mechanistic link between the measured clot parameters, such as lag times and maximum absorbance, and clinical outcomes. In summary, it is unclear whether altered fibrin clot properties have a mechanistic role in CV disease or whether, as seems to be more likely, they represent a form of summation biomarker reflecting a number of different factors, for example inflammation, oxidative stress, PAI-1 levels, fibrinogen concentration, glycaemic control, drug therapy to name a few, and that the CV risk is mediated through these factors and simply reflected in the fibrin clot phenotype.

With regard to the present study, there was no evidence that switching prevalent HD patients to a VE-bonded dialysis membrane for a period of 12-months had any effect on the fibrin clot structure. However, the question as to whether interventions which reduce oxidative stress or inflammation can impact on the fibrin clot structure remains unanswered. The two interventional studies which showed favourable changes in fibrin clot parameters commensurate with reductions in oxidative stress [1064] and inflammation [683], combined with the findings of the present study that reductions in markers of inflammation were associated with favourable changes in the fibrin clot phenotype, offers some hope that improving levels of inflammation and / or oxidative stress may be potential strategies for altering the clot phenotype. Whether these alterations would translate into improved CV outcomes remains to be answered.

The present study represents the largest study of fibrin clot structure in HD patients allowing a detailed analysis of the determinants of the various clot parameters in this patient group. The large number of patients studied permitted the use of multivariate regression techniques to identify the independent determinants of the fibrin clot parameters given the obvious overlap between several of the variables used in the analyses. These data will add considerably to the small body of published literature concerning fibrin clot structure in HD patients [646]. As has been discussed already certain fibrin clot characteristics, such as increased clot density and fibrinolytic resistance, have been associated with prevalent CV disease [644, 648, 649, 681, 708, 711, 712, 714, 715, 718, 1147] and, in the few studies which have evaluated it, incident disease [646] and

disease progression [715]. The next logical question is whether CV outcomes could be improved by interventions which, either directly or indirectly, manipulate fibrin clot properties. Developing such interventions can only come about after an appreciation of the key determinants of fibrin clot structure as presented here.

Chapter 9 : Clinical outcomes and mortality

In this chapter the data on patient mortality, CV events, non-infective dialysis access events and infective episodes were analysed for the study participants. The relationships between the baseline variables and the various clinical outcomes were considered in addition to analysing the influence of dialysis with the VE-bonded membranes. This was undertaken using a combination of Kaplan-Meier analyses and Cox-regression as detailed in section 3.17.6. The proportions of study patients experiencing at least one clinical event and the total number of clinical events encountered are provided in Table 44. While acknowledging that the overall event rates for the most part were low, and that the present study was not powered to detect significant differences in the clinical endpoints considered, an overview of the baseline factors associated with each of these outcomes is provided here.

Table 44 - Breakdown of clinical events encountered by study patients.

	Number of patients (%)	Number of events
Mortality	15 (5.8%)	15
Cardiovascular events	33 (12.7%)	41
Hospital admission with infection	52 (20%)	70
Non-infective access events	67 (25.8%)	114

9.1 Baseline predictors of mortality

During the 12-month study period 15 (5.8%) patients died. The main causes of death were acute coronary syndromes (n=6, 40%) and sepsis (n=6, 40%); 3 of the 6 patients who died of sepsis had a respiratory focus of infection. The causes of death in the remaining three patients were haemorrhagic stroke, hyperkalaemic cardiac arrest secondary to HD non-attendance and overwhelming sepsis with an intercurrent acute coronary syndrome in a patient with advanced prostate cancer. There were no significant differences in the 12-month mortality when patients were compared on the bases of sex (p=0.10), ethnicity (p=0.84), smoking status (p=0.24) or dialysis access (p=0.45). Mortality was significantly higher for patients with ESRF secondary to renovascular disease (see Figure 55).

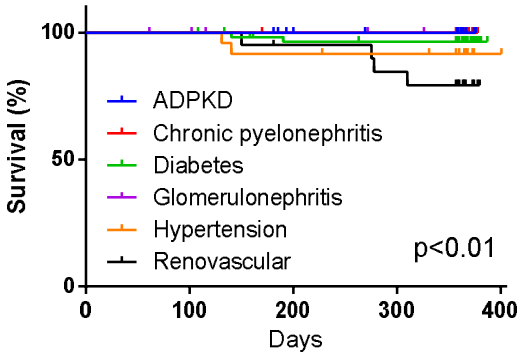


Figure 55 - Patient mortality categorised by aetiology of renal failure. Patients with ESRF secondary to renovascular disease had significantly higher mortality.

Kaplan-Meier analyses, after dividing the continuous variables age, weight, blood pressure, dialysis vintage and dialysis dose into tertiles at baseline did not reveal any evidence of non-linear associations with mortality. Univariate Cox-regression analyses were therefore performed with each of these variables and no significant associations were observed (see Table 45). Considering the patient comorbidities, there were no significant differences in the 12-month mortality when patients were compared on the bases of diabetic status (p=0.18) or the presence of IHD (p=0.71), PVD (p=0.27), malignancy (p=0.32) or left ventricular dysfunction (p=0.70) at baseline.

Table 45 - Univariate Cox-regression hazard ratios for patient characteristics and mortality.

	Hazard ratio [95% CI]	p
Age (per 5 years)	1.10 [0.92 - 1.31]	0.29
Weight (per kg)	0.99 [0.86 - 1.13]	0.85
Systolic blood pressure (per mmHg)	1.00 [0.98 - 1.02]	0.81
Diastolic blood pressure (per mmHg)	0.99 [0.95 - 1.03]	0.55
Time on dialysis (per year)	1.02 [0.93 - 1.11]	0.37
Urea reduction ratio (per 1%)	1.02 [0.95 - 1.10]	0.51

As detailed in section 3.17.6, Kaplan-Meier analyses were performed after categorising the continuous anaemia, oxidative stress, inflammation and fibrin clot structure and function variables into tertiles to examine for non-linear relationships. There were no evidence of non-linear relationships with the exception of the association between SC5b-9 levels and mortality. Comparing patients on the basis of tertiles of SC5b-9 at baseline revealed no patient deaths for patients in the middle tertile, 6 deaths in the lower tertile and 9 in the highest tertile (p=0.01); i.e. a U-shaped relationship between SC5b-9 levels and mortality

(see Figure 56). Given this non-linear relationship, it was not possible to calculate the Cox-regression hazard ratio for the SC5b-9 levels; the hazard ratios for the remainder of the variables are shown in Table 46. In these analyses, the statistically significant findings were the positive associations between ERI and TBARS levels and the risk of mortality. Comparing the mortality between the two study groups revealed no effect of dialysis with the VE-bonded membranes (see Figure 57).

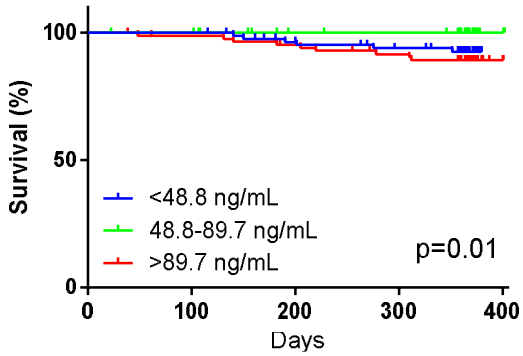


Figure 56 - Patient survival by baseline tertiles of SC5b-9. There were no deaths for patients in the middle tertile of SC5b-9 levels.

Table 46 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and mortality.

	Hazard ratio [95% CI]	p
Anaemia		
Haemoglobin (per g/dL)	1.05 [0.70-1.56]	0.82
ERI (per IU/wk/g/dL Hb)	1.07 [1.01-1.13]	0.02
Oxidative stress		
Ox-LDL (per ng/mL)	1.00 [1.00-1.00]	0.43
TBARS (per µM)	2.08 [1.24-3.48]	<0.01
Inflammation		
CRP (per mg/L)	1.02 [1.00 - 1.04]	0.09
C3 (per 0.1 mg/mL)	0.96 [0.72 - 1.27]	0.08
Factor D (per µg/mL)	0.99 [0.79 - 1.25]	0.96
Properdin (per µg/mL)	0.97 [0.94 - 1.00]	0.10
Fibrin clot structure and function		
Fibrinogen (per g/L)	0.84 [0.36-1.93]	0.67
Lag time (per 10s)	0.97 [0.91-1.04]	0.40
Maximum absorbance (per 0.1 au)	0.75 [0.47-1.21]	0.24
$Lys50_{t0}$ (per 100s)	0.98 [0.91-1.07]	0.70

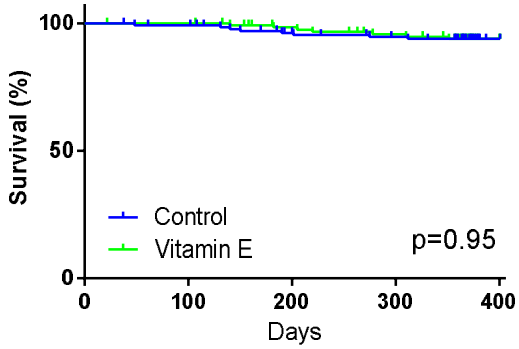


Figure 57 - Comparison of 12-month mortality between study groups. No mortality benefit of dialysis with the VE-bonded membrane was observed.

9.2 Baseline predictors of cardiovascular events

During the 12-month study period 33 patients experienced a total of 41 CV events; a breakdown of the CV events by vascular territory is shown Figure 58. The majority of events pertained to cardiac or peripheral vascular events. Of the 15 deaths during the study, 8 (53%) were CV in aetiology: 7 were the result of acute coronary syndromes and 1 was a haemorrhagic stroke.

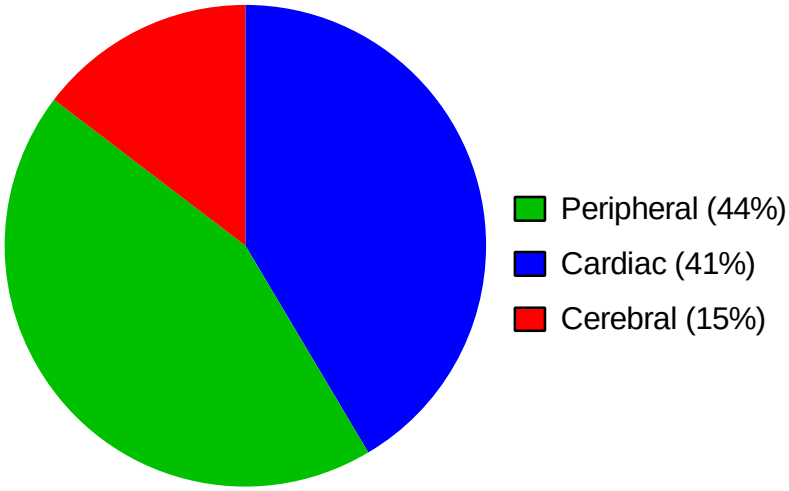


Figure 58 - Breakdown of cardiovascular events by vascular territory.

There were no significant associations between the patient sex (p=0.65), ethnicity (p=0.66), smoking habits (p=0.08), dialysis access (p=0.14) or the aetiology of renal failure (p=0.06) and the subsequent CV events rate. Kaplan-Meier analyses were performed after dividing the continuous variables age, weight, blood pressure, dialysis vintage and dialysis dose into tertiles at baseline and there were no evidence of non-linear associations. Univariate Cox-regression analyses modelling CV events with each of these variables were undertaken and no significant associations were observed (see Table 47).

Table 47 - Univariate Cox-regression hazard ratios for patient characteristics and cardiovascular events.

	Hazard ratio [95% CI]	p
Age (per 5 years)	1.11 [0.99 - 1.25]	0.08
Weight (per kg)	1.01 [0.99 - 1.02]	0.42
Systolic blood pressure (per mmHg)	1.00 [0.98 - 1.01]	0.55
Diastolic blood pressure (per mmHg)	0.98 [0.95 - 1.01]	0.18
Time on dialysis (years)	0.99 [0.93 - 1.06]	0.78
Urea reduction ratio (per 1%)	0.98 [0.94 - 1.02]	0.31

Considering the baseline co-morbidities, there were no associations between a history of diabetes (p=0.57), malignancy (p=0.23) or left ventricular dysfunction (p=0.96) and subsequent CV events. Patients with a history of IHD or PVD at baseline experienced a higher rate of CV events during the study (see Figure 59).

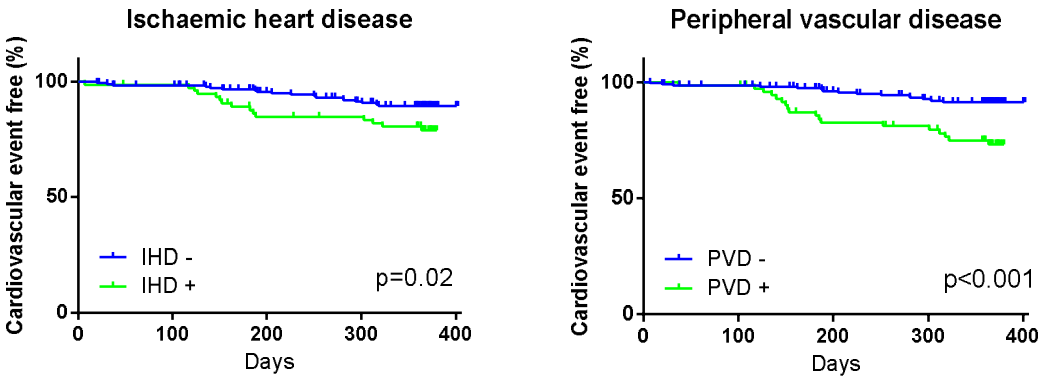


Figure 59 - Cardiovascular event rates compared on the bases of a history of ischaemic heart disease or peripheral vascular disease at baseline

Kaplan-Meier analyses found no evidence of non-linear associations between the anaemia, oxidative stress, inflammation or fibrin clot structure and function parameters and CV events with the exception of the SC5b-9 levels. Again, patients in the middle tertile of SC5b-9 levels at baseline fared best experiencing a significantly lower CV event rate (see Figure 60). The Cox-regression hazard ratios for the remainder of these variables are shown in Table 48. The statistically significant findings were that both higher CRP or C3 levels at baseline were positively associated with subsequent CV events. The incidence of CV events did not differ between study groups (see Figure 61).

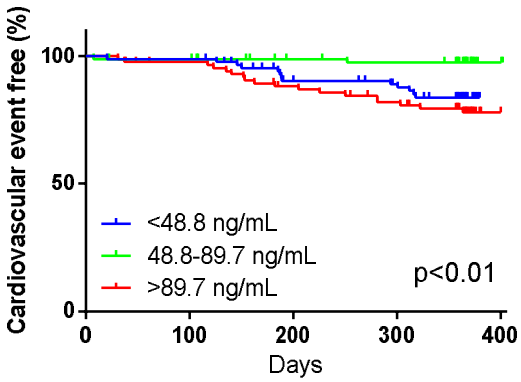


Figure 60 - Cardiovascular event free survival by baseline tertiles of SC5b-9. Patients with SC5b-9 levels in the middle tertile at baseline experienced significantly fewer cardiovascular events.

Table 48 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and cardiovascular events.

	Hazard ratio [95% CI]	p
Anaemia		
Haemoglobin (per g/dL)	1.11 [0.85-1.46]	0.44
ERI (per IU/wk/g/dL Hb)	1.01 [0.95-1.08]	0.71
Oxidative stress		
Ox-LDL (per ng/mL)	1.00 [1.00-1.00]	0.054
TBARS (per µM)	1.11 [0.71-1.72]	0.65
Inflammation		
CRP (per mg/L)	1.02 [1.01-1.03]	<0.01
C3 (per 0.1 mg/mL)	1.20 [1.01 - 1.42]	0.04
Factor D (per µg/mL)	1.04 [0.92-1.16]	0.55
Properdin (per µg/mL)	1.00 [0.99-1.01]	0.92
Fibrin clot structure and function		
Fibrinogen (per g/L)	0.99 [0.54-1.83]	0.99
Lag time (per 10s)	0.99 [0.96-1.03]	0.69
Maximum absorbance (per 0.1 au)	1.17 [0.90-1.52]	0.24
$Lys50_{t0}$ (per 100s)	0.97 [0.90-1.05]	0.44

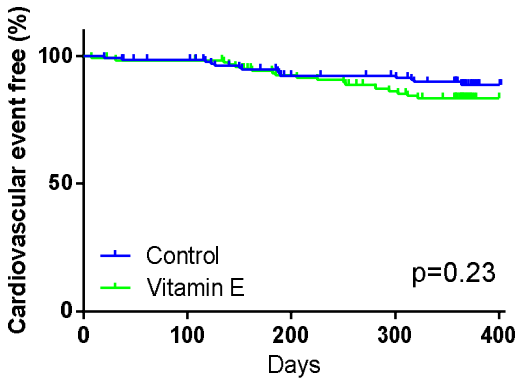


Figure 61 - Comparison of cardiovascular events between study groups. There was no difference in the CV event rates between study groups.

9.3 Baseline predictors of infective events

As detailed in section 3.7.6, infective events were defined as infections necessitating an admission to hospital. Fifty-two patients (20%) had at least one infective event during the study period and there were a total of 70 events. The commonest sites of primary infection were the respiratory tract, followed by bone and soft tissue and then dialysis access (see Figure 62).

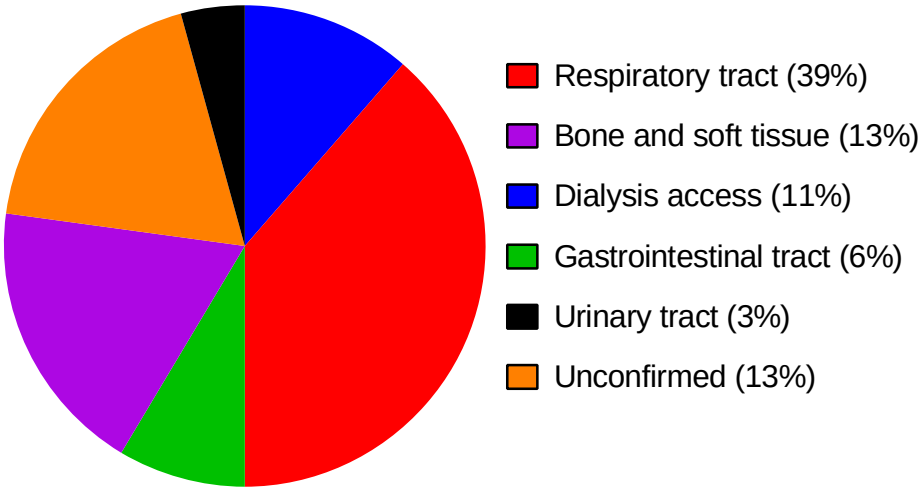


Figure 62 - Primary sites of infections for study patients.

The incidence of infective events did not differ when patients were compared on the bases of sex (p=0.17), ethnicity (p=0.92), smoking habits (p=0.30) or renal failure aetiology (p=0.18). Patients dialysing via CVCs experience significantly more infections (see Figure 63). Kaplan-Meier analyses were performed comparing infective events between tertiles of age, weight, dialysis vintage and dialysis dose at baseline and there were no evidence of non-linear associations. The univariate Cox-regression hazard ratios were therefore

calculated for these variables (see Table 49) and both younger age and lower dialysis dose were associated with an increased infection rate.

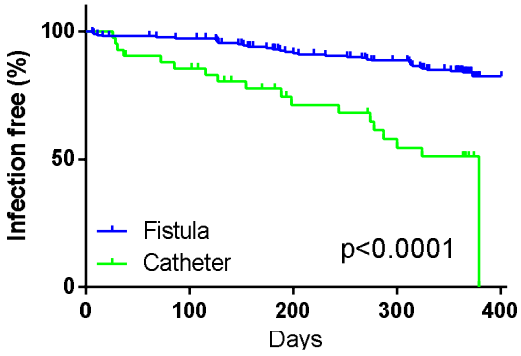


Figure 63 - Infective event rates compared by dialysis access at baseline. Patients dialysing via central venous catheters experienced a greater rate of infections.

Table 49 - Univariate Cox-regression hazard ratios for patient characteristics and infective events.

	Hazard ratio [95% CI]	p
Age (per 5 years)	0.92 [0.85 - 0.99]	0.04
Weight (per kg)	0.99 [0.92 - 1.07]	0.86
Time on dialysis (years)	1.00 [0.96 - 1.06]	0.85
Urea reduction ratio (per 1%)	0.97 [0.94 - 0.99]	0.03

Considering the comorbidities present at baseline, patients with malignancy were more likely to experience an infection during the study (p=0.04). There were no differences in the infection rates when patients were compared on the bases of diabetic status (p=0.07), or the presence of IHD (p=0.12) or PVD (p=0.87). Kaplan-Meier analyses were performed after categorising the continuous anaemia, oxidative stress, inflammation and fibrin clot structure and function variables into tertiles. There were no evidence of non-linear relationships with the exception of the association between CRP levels and infection rates, with patients in the middle tertile at baseline experiencing a higher infection rate (p=0.01). The Cox-regression hazard ratios for the remaining variables are shown in Table 50. The only statistically significant finding was the positive association between TBARS levels and the risk of infection. A comparison of the infection rates between the two study groups revealed no significant difference (see Figure 64) suggesting no effect of dialysis with the VE-bonded membranes.

Table 50 - Univariate Cox-regression hazard ratios for the anaemia, oxidative stress, inflammation and fibrin clot parameters and infective events.

	Hazard ratio [95% CI]	p
Anaemia		
Haemoglobin (per g/dL)	1.11 [0.89-1.38]	0.36
ERI (per IU/wk/g/dL Hb)	1.02 [0.97-1.07]	0.45
Oxidative stress		
Ox-LDL (per ng/mL)	1.00 [1.00-1.00]	0.97
TBARS (per µM)	1.49 [1.05-2.11]	0.03
Inflammation		
C3 (per 0.1 mg/mL)	0.94 [0.80-1.10]	0.44
Factor D (per µg/mL)	0.97 [0.85-1.12]	0.71
Properdin (per µg/mL)	1.00 [0.99-1.01]	0.70
Fibrin clot structure and function		
Fibrinogen (per g/L)	0.84 [0.54-1.32]	0.45
Lag time (per 10s)	1.02 [0.99-1.05]	0.26
Maximum absorbance (per 0.1 au)	1.06 [0.84-1.35]	0.62
$Lys50_{t0}$ (per 100s)	0.97 [0.91-1.04]	0.38

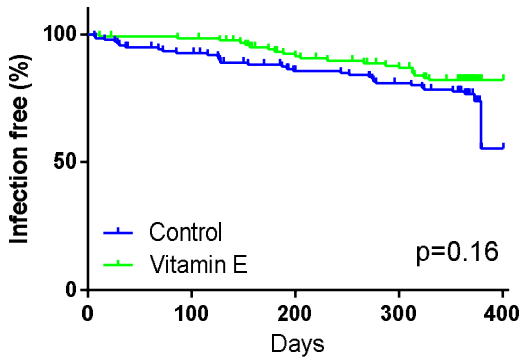


Figure 64 - Comparison of infective events between study groups. There was no difference in the incidence of CV events between study groups.

9.4 Baseline predictors of non-infective dialysis access events

During the 12-month study period 67 (26%) patients experienced a total of 114 dialysis access events. These, as detailed in section 3.7.6, were defined as any hospital admission or access procedure for non-infective dialysis access problems. Frequently encountered events included thrombosed AVFs, poor CVC blood flow necessitating a urokinase infusion or a line change, or poor AVF blood flow requiring a fistuloplasty. A breakdown of the non-infective dialysis access events encountered is provided in Figure 65.

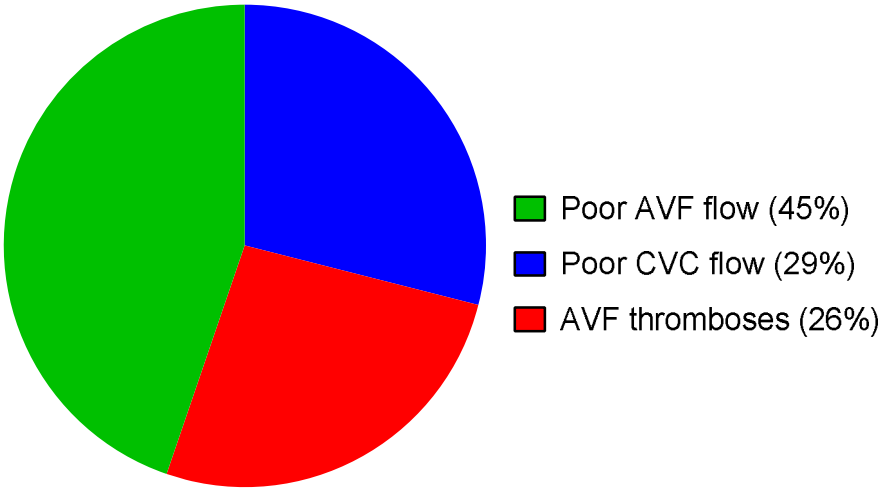


Figure 65 - Breakdown of non-infective dialysis access events. AVF: Arteriovenous fistula, CVC: Central venous catheter.

A focused evaluation of the associations between dialysis access events and some of the patient characteristics previously thought or known to be related to access problems was undertaken. Dialysis via a CVC [7] and the presence of diabetes [1148] have previously been shown by others to be associated with increased dialysis access complications, and both aspirin and warfarin have been prescribed to patients for the purposes of maintaining access patency (reviewed in [1149]); therefore the associations between these factors and non-infective dialysis access events in the present study were considered initially. Patients dialysing via CVCs experienced significantly more access related events compared to patients dialysing via AVFs (see Figure 66); there were no associations between the presence of diabetes (p=0.42), or therapy with aspirin (p=0.69) or warfarin (p=0.28) and the non-infective dialysis access events rates.

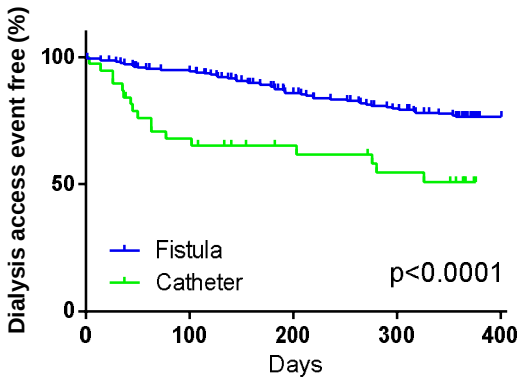


Figure 66 - Non-infective dialysis events compared by dialysis access at baseline. Patients dialysing with dialysis catheters experienced a higher rate of non-infective dialysis access events.

Comparison of the non-infective dialysis access events between study groups revealed a non-significant tendency towards more events in the control group (see Figure 67). This non-significant tendency was likely accounted for by the increased usage of CVCs in the control arm at the time of the study visits (16% vs 8%, p<0.01) and the greater rate of non-infective dialysis access complications for patients dialysing via CVCs (see Figure 66). To test this supposition, Cox-regression was performed by constructing a model which contained variables coding for dialysis access and study group. In this model, the regression coefficient for study group was not significant (p=0.16) and it indicated a higher incidence rate of non-infective dialysis access problems for patients dialysing with CVCs, as compared to AVFs (HR 2.77 [1.58 - 4.84]), independent of study group allocation.

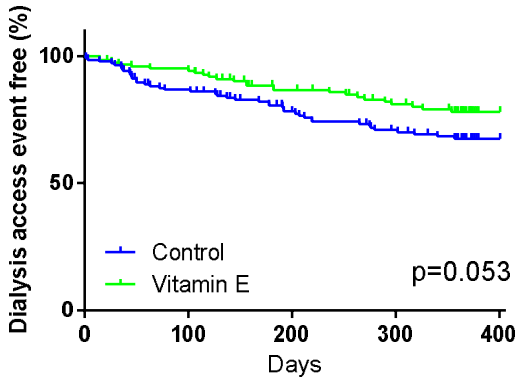


Figure 67 - Comparison of non-infective dialysis access events between study groups. There was no statistically significant difference in the non-infective dialysis access events between the study groups.

Given that a large number of the non-infective access events were related to thrombotic phenomena the relationships between fibrinogen levels, the fibrin clot characteristics and the incidence of access events were explored. The fibrin clot data obtained from patients dialysing via CVCs had been removed from the dataset owing to concerns regarding heparin contamination of the samples giving rise to spurious results (see section 8.1) therefore associations between fibrin clot parameters and CVC-related events could not be assessed. Of the 114 non-infective dialysis access events, 30 (26%) were for thrombosed AVFs (see Figure 65) and it was decided to analyse this subset of non-infective dialysis access events in more detail. Kaplan-Meier analyses found no evidence of non-linear associations therefore univariate Cox regression analyses were undertaken. This revealed shorter lag times to be associated with an increased rate of AVF thromboses, but no associations with fibrinogen levels, maximum absorbance or fibrinolysis times (see Table 51). The incidence of AVF thromboses did not differ when this subset of patients were compared on the bases of aspirin (p=0.93) or warfarin (p=0.59) therapy at baseline.

Table 51 - Univariate Cox-regression hazard ratios for the baseline fibrin clot parameters and the subsequent risk of arteriovenous fistula thrombosis.

	Hazard ratio [95% CI]	p
Fibrinogen (per g/L)	1.19 [0.60-2.37]	0.61
Lag time (per 10s)	0.95 [0.91-0.99]	0.04
Maximum absorbance (per 0.1 au)	1.06 [0.80-1.42]	0.68
$Lys50_{t0}$ (per 100s)	0.96 [0.88-1.05]	0.41

9.5 Discussion

A number of clinical endpoints were evaluated in this chapter: mortality, CV events, infections and non-infective dialysis access events. The associations between these outcomes and the baseline variables in the dataset were analysed before evaluating if the data collected on the study patients pertaining to anaemia, oxidative stress, inflammation and fibrin clot structure and function, added any additional prognostic information. Finally, the influence of dialysis with the VE-bonded membranes on each of the endpoints was determined.

9.5.1 Mortality and cardiovascular events

The 1 year mortality rate in the present study was 5.8% which is significantly lower than the UK national average annual mortality rate of approximately 11% in prevalent HD patients [4]. This apparent discrepancy is likely to have arisen, at least in part, because patients with significant inflammation were excluded from the study and HD patients with increased levels of inflammation are known to be at higher risk of adverse outcomes, particularly CV events and mortality [163, 429, 434-438, 1150]. In terms of the predictors of CV events and deaths, of which 53% in the present study were CV in aetiology, none of the so-called traditional CV risk factors, i.e. diabetes, blood pressure or smoking, were significantly associated with the outcomes. This is consistent with other studies which have shown that consideration of the traditional CV risk factors alone cannot account for the high observed rates of CV disease in renal populations [15, 41]. Part of the explanation for this, as outlined in section 1.2.1, is thought to be the increased prevalence of so-called non-traditional CV risk factors in renal patients, such as oxidative stress and complement activation with reference to the present thesis.

Increased TBARS, but not Ox-LDL, levels were associated with greater mortality although neither were significantly associated with CV events. On the face of it, this might suggest

that oxidative stress increases mortality which is unrelated to CV disease, perhaps by enhancing susceptibility to infection which is a leading causing of death in UK HD patients [4] and in the present study. In support of this theory there was a positive association between TBARS levels and infective events. The reason for this association may be impaired immune function in the presence of oxidative stress. This has been demonstrated in mice [1151] and increases in oxidative stress have been shown to precede infections in patients infected with the human immunodeficiency virus [1152], yet convincing data in humans to support an association between heightened oxidative stress and susceptibility to infection are lacking.

In terms of the complement components measured, patients in the higher and lower tertiles of SC5b-9 levels at baseline appeared to fare worse than patients with levels in the middle tertile with respect to mortality and CV events. As highlighted in Chapter 7, it is hard to know what the levels of SC5b-9 mean in terms of complement activity. High levels almost certainly reflect enhanced complement activity but whether low levels reflect reduced complement activity, and hence reduced SC5b-9 synthesis, or enhanced complement activity with consumption of the terminal complement components remains unclear. It is therefore possible that the apparent U-shaped association between SC5b-9 levels and both mortality and CV events in the present study reflects an association between complement activation and outcomes. From the small body of literature concerning complement activation and CV disease, there are evidence of enhanced complement activation following AMI [543-545] or ischaemic stroke [546] and, in patients with type 2 diabetes mellitus presenting with AMI, increased levels of SC5b-9 appear to be predictive of future CV events [541]. However, all of these data pertain to the acute situation rather than examining the predictive utility of SC5b-9 levels measured outside of these settings as in the present study. Indirect evidence for a potential role of complement activation in the pathogenesis of adverse outcomes for HD patients may come from studies reporting the impact of dialysis membrane composition on patient outcomes, such as CV events, infections and mortality, in which patients dialysing with less biocompatible membranes, which are more avid activators of the complement system, tend to fare worse [831, 832, 834, 835]. However other differences exist between the membranes other than simply the ability to activate complement, such as the potential to activate neutrophils, induce oxidative stress and the membrane permeability and solute clearance characteristics; although the ability to activate complement may form part of the explanation for the increased adverse event rates with less biocompatible membranes. Much of the data regarding outcomes with less biocompatible membranes are historical,

as many HD units now use more biocompatible membranes, and many other aspects of patient care besides simply dialyser technology have advanced in recent times making it hard to attribute improved survival in HD patients over time [4] to changes in dialyser composition.

There was a significant association between higher C3 levels at baseline and the subsequent risk of CV events. As highlighted in section 1.5.3, a number of observational series in non-renal patients have reported positive associations between C3 levels and both prevalent [532-536] and incident [537-540] CV disease, yet there are little or no published data exploring this association in chronic HD patients. A potential oversight given that dialysis is a powerful stimulus for complement activation [230-233] and CV disease is a leading cause of death for HD patients [4]. Much of the data attempting to elucidate the mechanism, or mechanisms, through which increased C3 levels might contribute to CV disease have focused on atherosclerosis. As discussed in section 1.2, atherosclerosis is not the sole process involved in the pathogenesis of CV disease in chronic HD patients, with vascular calcification playing an important role. The role of the complement system in vascular calcification has received far less attention. The complement component C3 is an acute phase reactant [428], and the positive association between another acute phase reactant, CRP levels, and adverse outcomes in HD patients has been well documented by others [163, 434, 435, 437, 1150] and indeed was evident in the present study; therefore the finding with respect to C3 levels is perhaps not surprising. It is interesting, however, that the associations between C3 and CRP levels and the subsequent risk of CV events persisted in the present study which only enrolled patients without significant inflammation. It is hard to separate the contributions of inflammation *per se*, and the aspects of inflammation which relate to complement activation - particularly given that markers of generalised inflammation, such as CRP levels measured in the present study, and a number of the complement components are increased as part of the acute phase response [428]. There is still debate as to whether the association between inflammation and CV disease arises as a consequence of inflammation driving processes which contribute to the development of CV disease, or whether the presence of CV disease is itself pro-inflammatory (see section 1.5.3). Unsurprisingly, a history of IHD or PVD at baseline was significantly associated with future risk of CV events. Patients with ESRF secondary to renovascular disease had a greater mortality, as has been reported by others [1153], which is likely a reflection of the high burden of vascular disease in these patients.

In terms of the anaemia parameters considered, there were no associations between the haemoglobin levels and either mortality or CV events. This finding is in accord with the literature that haemoglobin levels *per se* do not contribute to worse outcomes in HD patients [50, 291]. Greater ESA requirements have been shown to be an important predictor of worse outcomes for patients on HD [51, 52] and, indeed, increased ERI was predictive of mortality in the present study. This finding is particularly interesting given that patients with significant inflammation, and who were therefore likely to be most ESA resistant, were excluded from the study. The data from the present study would therefore tend to suggest that the association between ESA resistance and adverse patient outcomes persists in HD patients without overt inflammation. A univariate Cox-regression analysis modelling the mortality found a significant effect of higher baseline ESA doses (HR 1.02 [1.01 - 1.04] per µg/wk). It is therefore possible that the positive association between ERI and mortality reported here reflects harm associated with high ESA doses. As outlined in section 1.3.4, a number of mechanisms have been put forward to explain the potential for harm with ESAs including the large number of non-erythroid cells which express EPO [293] and the ability of ESAs to stimulate vascular smooth cell proliferation [295-297], cause vasoconstriction [300, 301] and increase the levels of pro-inflammatory cytokines [303, 304]. However, many of these factors are associated with the pathogenesis of CV disease yet no associations between CV events and the ERI or ESA dose were observed in the present study. Approaching 50% of the deaths were infective in aetiology and there is emerging evidence that EPO may interfere with immunity [1154], for example by impairing macrophage function [1155], yet there was no association between ESA requirements and infections suggesting that this mechanism was unlikely to explain the association between ERI and mortality in the present study.

With regard to the fibrin clot data, neither the fibrinogen levels nor the fibrin clot parameters with significantly associated with either the mortality or CV events. In contrast to this, several previous studies in HD patients have demonstrated a positive association between fibrinogen levels and mortality [45, 435, 646], akin to data from the general population [690, 696-700]. Whether this association was driven by patients with high levels of inflammation, and consequently fibrinogen levels, who were excluded from the present study to explain the discrepant results is not clear. Only two published studies to date have examined the prognostic utility of fibrin clot parameters in high risk groups: patients with PVD [715] and patients on HD [646]. In both of these studies [646, 715], published by the same group, the formation of less permeable, denser clots with shorter lag times and increased fibrinolytic resistance were associated with an increased risk of subsequent

adverse CV outcomes. Such findings were not evident in the present study. Of particular relevance to the present thesis was the study by Undas *et al*. [646] which followed 33 HD patients for 36 months during which time 10 patients experienced a fatal CV event. At baseline, the fibrin clots derived from these patients were less permeable and more resistant to fibrinolysis. There are a number of potential reasons why the apparent prognostic utility of the fibrin clot parameters reported by Undas *et al*. [646] was not found in the present study. Broadly similar exclusion criteria were used with respect to excluding patients with acute illness (as defined by high CRP in the present study) but the number of patients, study duration, methodology employed for fibrin clot analysis, statistical techniques used, event rates and the endpoint definitions differed between the studies preventing a direct comparison. Of the 15 deaths in the present study, 8 had a CV aetiology. Comparing the baseline fibrin clot parameters in these patients, i.e. adopting a similar endpoint definition to Undas *et al*. [646], with those who did not suffer a CV death over the following 12 months still found no significant differences ($p>0.45$ in all cases), suggesting the discrepancies could not be explained by different endpoint definitions. The mortality rate was higher in the Undas *et al*. [646] study, with 33% of patients dying within 3 years compared to the 5.8% 1 year mortality in the present study, and 10 out of the 11 deaths were CV in aetiology contrasting with only 53% in the present study. This suggests that there may be fundamental differences between the studies in terms of the patient cohorts or the treatments they received. The differences in clot characteristics between the survivors and the patients who subsequently died in the Undas *et al*. [646] study could potentially be explained by the significantly higher fibrinogen levels at baseline in the patients who went on to have a fatal CV event; no such finding was evident in the present study.

There was no difference in mortality or CV events between the two study groups suggesting no effect of dialysis with the VE-bonded dialysis membranes. No study to date has studied the effects of switching patients to HD with a VE-bonded membrane on these endpoints. The putative mechanisms through which VE-bonded membranes may reduce CV events in HD patients, i.e. reducing oxidative stress and inflammation, were not demonstrated in the present study therefore an absence of effect on these clinical endpoints may have been anticipated. It is important to recognise, however, that the present study was not adequately powered to detect a difference in mortality or CV events therefore the absence of benefit observed with the VE-bonded membrane here should be regarded as speculative.

9.5.2 Infective events

Patients on HD experience considerably higher rates of infection compared to aged match controls in the general population [1156] and infective episodes are linked to subsequent CV events [457, 458]. A number of contributory factors have been put forward to explain this including acquired immune dysfunction associated with uraemia [1157, 1158], multiple hospital attendances and the frequent use of intravascular devices [1159, 1160]. One-fifth of the study cohort experienced an infective event, defined here as an infection necessitating an admission to hospital, with the commonest site being the respiratory tract. Respiratory infections have been shown to be common among HD patients [1161] and associated with significant mortality [1162]. Dialysis access related infections were the third commonest site of infection, despite less than 15% of the study population dialysing via a CVC, and of these infections approaching 90% were related to CVCs. Several large series have similarly demonstrated higher infection rates for patients dialysing via CVCs, compared to grafts and AVFs [6, 457]. In the present study, a lower dialysis dose was associated with a greater infection rate. However, dialysis dose was lower in patients dialysing via CVCs compared to AVFs (urea reduction ratio 0.73 [±0.00] vs 0.76 [±0.01]; p=0.03), likely as a consequence of lower achievable blood pump speeds, although there were no data available to corroborate this. It is therefore likely that in these unadjusted analyses a reduced dialysis dose was a proxy for dialysis via a CVC.

Patients with diabetes in the present study did not appear to experience more infective events. The renal literature is mixed in this regard with two large series, based on the United States Renal Data Systems (USRDS) data, reporting differing conclusions on the influence of diabetes on the occurrence of infections [457, 458]. Interestingly none of the complement components measured were significantly associated with infective events. The complement component C3 forms an integral part of the innate immune system and C3 deficiency is associated with severe recurrent infections [1063]. However, no associations were observed between the baseline C3 levels and the risk of infections in the present study suggesting that the C3 levels were not an important contributor to infection risk in this study cohort. The incidence of infective events did not differ between the study groups suggesting no effect of the VE-membranes.

9.5.3 Non-infective dialysis access events

Unsurprisingly, dialysis access events were more common among patients dialysing via CVCs. This finding is consistent with the literature reporting higher rates of complication for

patients dialysing with CVCs compared to AVFs [1163, 1164]. At the time of the study it was LTHT policy to admit patients if they required a urokinase infusion for poor CVC blood flow rates during HD and this was a significant contributor (14%) to the access events recorded. (This practice contrasted with many other HD units and the unit policy has since been modified such that patients can receive intra-dialytic urokinase infusions without the need for hospital admission). Given that a significant number of non-infective dialysis access complications were related to thrombosis or presumed fibrin deposition within CVCs, the associations between the all-cause dialysis access events and concomitant aspirin or warfarin therapy, the fibrinogen levels and the fibrin clot characteristics measured were explored. No significant associations were found. Observational data such as these does not take account of the indications for aspirin or warfarin therapy and it is possible that a number of patients were taking these medications for vascular access patency. In other words, patients previously at high risk of thrombotic vascular access complications may have been receiving aspirin or warfarin for this indication to explain the apparent lack of observed benefit in the present study.

Unfortunately, no fibrin clot data were available for samples obtained via CVCs owing to concerns regarding heparin contamination (see section 8.1 and Figure 50), therefore it was not possible to examine the associations between the fibrin clot parameters and non-infective dialysis access events which pertained to CVCs, a significant contributor to the events. It was therefore decided to examine the subset of non-infective dialysis access events which related to AVF thrombosis and, in particular, how these were associated with the fibrin clot parameters. There were no significant associations between AVF thromboses and the fibrinogen levels, clot maximum absorbance or $Lys50_{t0}$ times but the lag times were significantly shorter in patients who went on to have an AVF thrombosis. There are no data examining the link between the fibrin clot parameters measured in the present study and the potential for thrombosis. Data from patients with idiopathic venous thromboembolism suggest no difference in the lag times between patients and controls [717], suggesting lag times may not be related to thrombotic potential. Conversely, studies examining fibrin clot formation in the setting of CV disease have reported shorter lag times in patient groups either with or at high risk of developing CV disease such as patients on HD [646], patients with heart failure [718], peripheral arterial disease [715] or acute coronary syndromes [648] and in the healthy first degree relatives of patients with premature coronary artery disease [709] suggesting shorter lag times may be associated with an increased thrombotic risk. It is conceivable that shorter lag times, which represents the time taken for sufficient protofibrils to form to enable lateral aggregation, may

predispose patients to thromboses particularly in the setting of AVF thrombosis where the rapid, turbulent blood is likely to disrupt many clots, and particularly those which are slower to form. Shorter lag times tend to be associated with the formation of denser clots (see Table 30 and Table 31) owing to the associations between clot assembly kinetics and clot structure [639]. It is important to realise, however, that the main drivers for AVF thrombosis are endothelial disruption at the site of needling or attenuated blood flow in the region of a vascular stenosis rather than pro-coagulant blood.

There was a non-significant tendency towards fewer non-infective dialysis access events among patients dialysing with the VE-bonded membrane. There were, however, more patients dialysing with CVCs in the control arm which was a significant contributor to the access events. To explore this further, Cox-regression modelling the non-infective dialysis access events incorporating the dialysis access and study group as covariates was undertaken. This confirmed no significant effect of the VE-bonded membrane on dialysis access events and the strong positive association with CVC dialysis access.

Despite the present study not being powered to detect differences in the endpoints considered in this chapter, there were a number of potentially interesting findings. Worse outcomes have previously been reported for patients with higher CRP levels or greater ESA-resistance and, in the present study, these associations persisted despite the exclusion of patients with significant inflammation. This suggests that it may not be a subset of patients with high levels of inflammation driving this association in the published studies, rather that increasing inflammation or ERI represents a continuum of risk. The finding that higher C3 levels were predictive of future CV events, as has been similarly reported in non-HD patients, is a novel finding of the present study. There was also an association between the SC5b-9 levels, both high and low, and the occurrence of mortality and CV events. Taking these findings together, particularly in light of the facts that HD is a potent stimulus for complement activation and HD patients have high rates of CV disease, would suggest that complement may play a role in the adverse outcomes for HD patients. The findings with respect to a decline in C3 levels across study visits (see Figure 42) and the negative correlation with length of time on dialysis (see Table 18, Chapter 7) are particularly interesting in this context. More work to understand the role of complement in the pathogenesis of CV disease in HD patients is warranted.

No effects of the VE-bonded membrane were observed on any of the clinical endpoints considered. This may, of course, represent a type II statistical error owing to the fact the

study was not powered to detect a difference after 12 months. The SPACE trial [825] was a secondary prevention trial examining the effects of oral VE on CV event reduction in HD patients and, according to the authors, with approximately 100 patients in each arm it had an 80% power to detect a relative risk of 0.6 in a composite CV endpoint over two years at a significance level of 5% and assuming a 30% event rate. To detect a relative risk of 0.6 in the present study, given the one year CV event rate of 14/137 in the control arm, would have required approximately 1600 patients (i.e. 800 in each arm) at a similar power and significance level. To detect a 10% difference in the fibrin clot maximum absorbance between survivors and patients who died, as reported by Undas *et al*. [646], at the same α- and β-level and based on the mean and standard deviation at baseline, would require 65 events in contrast to the 15 deaths or 8 CV deaths observed. These calculations serve to underscore that the present study was not powered to make inferences based on clinical endpoints such as mortality or CV events. However the putative mechanisms through which the VE-bonded membrane might be expected to improve these outcomes, i.e. reduce oxidative stress and / or inflammation, were not observed here as documented in the foregoing chapters. It therefore seems unlikely, even in an adequately powered study, that the wholesale switching of non-inflamed prevalent HD patients to dialysis with a VE-bonded membrane would result in better patient outcomes.

Chapter 10 : Conclusions and future perspectives

The study forming the basis for this thesis is the largest study of VE-bonded polysulfone membranes undertaken to date. Novel aspects of this work included the measurement of a number of the alternative complement pathway components and the *ex vivo* fibrin clot properties. The key findings of the study, with respect to the VE-bonded dialysis membranes, were that switching prevalent HD patients without significant inflammation to dialysis with these membranes had no beneficial effects on renal anaemia, oxidative stress, inflammation, *ex vivo* fibrin clot properties or patient outcomes when compared to identical uncoated membranes over 12 months. This may reflect a true absence of benefit from the VE-coating or, conversely, reflect the excellent biocompatible profile of the latest generation polysulfone membranes.

In terms of the anaemia data, *post hoc* analyses identified a reduction in the ERI for patients with greater levels of ESA-resistance at baseline dialysing with the VE-bonded, but not the control, membranes. The starting ERI for this subgroup of patients was comparable to the other studies reporting an ESA-sparing effect of VE-bonded polysulfone membranes [889, 893]. It is therefore possible that the VE-bonded membranes have ESA-sparing utility in patients who are ESA-resistant; further studies specifically designed and powered to answer this question are required. Approaches to this might include enrolling patient with evidence of ESA-resistance, for example with ERIs of ≥8 IU/wk/kg/g/dL Hb based on data from the present study and those detailed in Table 13, or stratifying patients *a priori* on the basis of their ERI. In the subset of patients with higher ERIs, who appeared to benefit from HD with the VE-bonded membrane, the reduction in ERI was not mirrored by changes in oxidative stress or inflammation. This may be because the improvements in ESA-resistance were not the result of reductions in oxidative stress or inflammation, or reflect the choice of oxidative stress and inflammation biomarkers measured.

Previous work with VE-bonded modified cellulose membranes has demonstrated improvements in RBC lifespan [878] by measuring the RBC creatine content [1165]. It would therefore be interesting to determine if coating polysulfone membranes with VE has any effect on RBC lifespan, particularly in ESA-resistant patients who appeared to benefit in the present study. Such studies should incorporate measures of inflammation and perhaps different or more extensive markers of oxidative stress than were measured here. If these studies demonstrated evidence of prolongation of the RBC lifespan with the VE-

bonded membranes, and no effects on oxidative stress or inflammation as were found in the present study, this again may reflect the choice of oxidative stress or inflammation biomarkers measured or point to the fact that other mechanisms, distinct from lowering of oxidative stress or inflammation, are responsible for the improvements in ESA-resistance or RBC lifespan. One such mechanism may relate to the haemostatic effects of VE outlined in section 1.7.1.3. Oral VE supplementation has been shown to reduce platelet activation, as measured by circulating P-selectin levels [754, 764], thereby reducing their thrombotic potential. Studies examining the haemostatic effects of VE-bonded dialysers, which have only incorporated very small numbers of patients, have reported beneficial effects in terms of reduced dialyser clotting in patients with a propensity to clot [877], reduced anticoagulation requirements in children [890] and less dialyser clotting when anticoagulation is avoided [1166]. It is therefore possible that the VE-bonded membranes reduce dialyser clotting, and hence the RBC mass sequestered in the dialysers and tubing at the end of dialysis, to explain improvements in ESA-resistance or increased RBC lifespan. This theory could be tested prospectively by incorporating measurements of dialyser clotting and intra-dialytic thrombotic events, such as clotted dialysis circuits, in future studies. Another potential extension in future studies would be to include data on residual renal function, which has been shown to be a determinant of ESA-requirements and patient outcomes [1167], but was not measured here.

The other key findings in the present study relate to the complement components measured. There was a progressive decline in C3 levels across study visits and the C3 levels at baseline were negatively correlated with the number of years on dialysis. The mechanisms underpinning these findings and the implications for patients are unclear and warrant further study; particularly in light of the associations between C3 and CV events. For example, do these findings reflect reduced C3 synthesis, increased C3 consumption or increased C3 clearance? Data from the present study would suggest that the C3 levels fell in the majority of patients (see Figure 43) rather than the findings reflecting large reductions in a minority of patients. It therefore seems likely that the fall in C3 levels is a feature of chronic HD rather than being restricted to a specific subset of patients. Polysulfone HD membranes have been shown to adsorb C3 [1045, 1046], although the impact of this clearance on the circulating pre-dialysis C3 levels measured in the present study is unclear. In terms of relating the decline in C3 levels to the degree of complement activation, making inferences as to the degree of complement activation from the levels of the complement components measured in the present study were not straightforward as highlighted in section 7.4.2. For example, low circulating levels of a particular complement

component may arise as a consequence of reduced synthesis in the setting of low complement activity or increased catabolism in the setting of high complement activity. Future work might be directed at measuring pre- and post- HD levels of the complement components, in addition to measuring other specific markers of complement activation such as C3a and C5a. This would provide insights into the overall degree of complement activation, how much of it is attributable to the HD process itself and permit a more meaningful interpretation of the levels of the other complement components. Contemporaneous measurement of the complement components in the afferent and efferent limbs of the HD circuit and in the dialysate would provide information about the contribution of dialyser clearance and adsorption to the circulating complement levels. The fall in C3 levels may also reflect a degree of protein-energy malnutrition which is common in HD patients and is linked to time on dialysis [1053]. To investigate this further, longitudinal studies measuring complement levels in conjunction with markers of nutritional status, such as anthropometry or bioimpedance body composition data, could be conducted.

As detailed in section 1.5.3, there is emerging evidence of a role for complement in the pathogenesis of CV disease. Given that CV disease is a leading cause of death for patients on HD [4], and HD is a powerful stimulus for complement activation [230-233], it is a potential oversight that in the modern treatment era this has received little attention in the renal literature. In the present study there was a positive association between C3 levels and future CV events. This is the first time that this association, which has previously been described in non-renal patients [538-540], has been similarly reported in HD patients. The findings with regards to C3, combined with the independent associations between the SC5b-9 levels and the subsequent risk of death or CV events, suggest that the complement system may have a role to play in adverse outcomes for HD patients. It remains unclear whether these associations reflect a functional role for complement, as has been postulated in the development of atherosclerotic lesions for example [511, 547-552], or represent an epiphenomenon such that the complement components measured may merely be reflective of more generalised inflammation which influences CV risk through a myriad of mechanisms not specifically related to complement (see section 1.5.3). The relationships between the complement components and CV disease observed here require validating in other prevalent HD populations. Designing studies to try to unpick whether complement has a functional role in the development of CV disease, or whether it is a marker for other processes which influence CV risk, is not straightforward. Such a distinction is an important one to make as if complement had a functional role in

the development of CV disease, manipulation of the complement system may be a potential therapeutic avenue for ameliorating the high CV risk in HD patients. Given that complement is an integral part of the innate immune system and a number of the complement components have pleiotropic actions, there is considerable potential for harm with such approaches. Larger scale observational studies in HD patients, incorporating the measurement of a number of different complement components (e.g. C3, C4, C3a, C5a, MASPs, factor H, properdin and SC5b-9), inflammatory markers (e.g. CRP, IL-6, ICAM-1, VCAM-1) and haemostatic factors (e.g. fibrinogen, P-selectin, PAI-1, fibrin clot structure) may provide a clearer picture of how the various complement activation pathways interact with other aspects of the inflammatory response and conspire to cause the pathophysiological changes associated with the development of CV disease, such as endothelial dysfunction, vascular calcification, atherosclerosis and altered haemostasis.

References

1. Stevens PE, O'Donoghue DJ, de Lusignan S*, et al.* Chronic kidney disease management in the United Kingdom: NEOERICA project results. *Kidney Int* 2007;72(1):92-9.

2. Coresh J, Selvin E, Stevens LA*, et al.* Prevalence of chronic kidney disease in the United States. *JAMA* 2007;298(17):2038-47.

3. Evans PD, Taal MW. Epidemiology and causes of chronic kidney disease. *Medicine* 2011;39(7):402-406.

4. The Renal Association. UK Renal Registry: The Fourteenth Annual Report. 2011.

5. NHS Kidney Care. 2010. Kidney Disease: Key Facts and Figures. *Available at: www.kidneycare.nhs.uk/document.php?o=476 [Accessed January 2013]*.

6. Stevenson KB, Hannah EL, Lowder CA*, et al.* Epidemiology of hemodialysis vascular access infections from longitudinal infection surveillance data: predicting the impact of NKF-DOQI clinical practice guidelines for vascular access. *Am J Kidney Dis* 2002;39(3):549-55.

7. Pisoni RL, Arrington CJ, Albert JM*, et al.* Facility hemodialysis vascular access use and mortality in countries participating in DOPPS: an instrumental variable analysis. *Am J Kidney Dis* 2009;53(3):475-91.

8. Zucchelli P, Santoro A, Zuccala A. Genesis and control of hypertension in hemodialysis patients. *Semin Nephrol* 1988;8(2):163-8.

9. Cunningham J, Locatelli F, Rodriguez M. Secondary hyperparathyroidism: pathogenesis, disease progression, and therapeutic options. *Clin J Am Soc Nephrol* 2011;6(4):913-21.

10. Office for National Statistics. Mortality Statistics for deaths registered in England and Wales. In. London; 2008.

11. The Renal Association. UK Renal Registry: The Nineth annual report. 2006.

12. Foley R, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis* 1998;32 (Suppl):S112-119.

13. Shoji T, Emoto M, Tabata T*, et al.* Advanced atherosclerosis in predialysis patients with chronic renal failure. *Kidney Int* 2002;61(6):2187-92.

14. Baigent C, Burbury K, Wheeler D. Premature cardiovascular disease in chronic renal failure. *Lancet* 2000;356:147-152.

15. Cheung AK, Sarnak MJ, Yan G*, et al.* Atherosclerotic cardiovascular disease risk in chronic hemodialysis patients. *Kidney Int* 2000;58:353-362.

16. Lewington S, Clarke R, Qizilbash N*, et al.* Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet* 2002;360(9349):1903-13.

17. Iseki K, Miyasato F, Tokuyama K*, et al.* Low diastolic blood pressure, hypoalbuminemia, and risk of death in a cohort of chronic hemodialysis patients. *Kidney Int* 1997;51(4):1212-7.

18. Port FK, Hulbert-Shearon TE, Wolfe RA*, et al.* Predialysis blood pressure and mortality risk in a national sample of maintenance hemodialysis patients. *Am J Kidney Dis* 1999;33(3):507-17.

19. Zager PG, Nikolic J, Brown RH*, et al.* "U" curve association of blood pressure and mortality in hemodialysis patients. Medical Directors of Dialysis Clinic, Inc. *Kidney Int* 1998;54(2):561-9.

20. Kalantar-Zadeh K, Block G, Humphreys MH*, et al.* Reverse epidemiology of cardiovascular risk factors in maintenance dialysis patients. *Kidney Int* 2003;63(3):793-808.

21. Tomson CR. Blood pressure and outcome in patients on dialysis. *Lancet* 2009;373(9668):981-2.

22. Lynn KL, McGregor DO, Moesbergen T*, et al.* Hypertension as a determinant of survival for patients treated with home dialysis. *Kidney Int* 2002;62(6):2281-7.

23. Mazzuchi N, Carbonell E, Fernandez-Cean J. Importance of blood pressure control in hemodialysis patient survival. *Kidney Int* 2000;58(5):2147-54.

24. Wilt TJ, Bloomfield HE, MacDonald R*, et al.* Effectiveness of statin therapy in adults with coronary heart disease. *Arch Intern Med* 2004;164(13):1427-36.

25. Marchioli R, Marfisi RM, Carinci F*, et al.* Meta-analysis, clinical trials, and transferability of research results into practice. The case of cholesterol-lowering interventions in the secondary prevention of coronary heart disease. *Arch Intern Med* 1996;156(11):1158-72.

26. Sever PS, Dahlof B, Poulter NR*, et al.* Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial--Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* 2003;361(9364):1149-58.

27. Downs JR, Clearfield M, Weis S*, et al.* Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA* 1998;279(20):1615-22.

28. Shepherd J, Cobbe SM, Ford I*, et al.* Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995;333(20):1301-7.

29. Fellstrom BC, Jardine AG, Schmieder RE*, et al.* Rosuvastatin and cardiovascular events in patients undergoing hemodialysis. *N Engl J Med* 2009;360(14):1395-407.

30. Wanner C, Krane V, Marz W*, et al.* Atorvastatin in patients with type 2 diabetes mellitus undergoing hemodialysis. *N Engl J Med* 2005;353(3):238-48.

31. Baigent C, Landray MJ, Reith C*, et al.* The effects of lowering LDL cholesterol with simvastatin plus ezetimibe in patients with chronic kidney disease (Study of Heart and Renal Protection): a randomised placebo-controlled trial. *Lancet* 2011;377(9784):2181-92.

32. Iseki K, Yamazato M, Tozawa M*, et al.* Hypocholesterolemia is a significant predictor of death in a cohort of chronic hemodialysis patients. *Kidney Int* 2002;61(5):1887-93.

33. Liu Y, Coresh J, Eustace JA*, et al.* Association between cholesterol level and mortality in dialysis patients: role of inflammation and malnutrition. *JAMA* 2004;291(4):451-9.

34. Lew EA, Garfinkel L. Variations in mortality by weight among 750,000 men and women. *J Chronic Dis* 1979;32(8):563-76.

35. Manson JE, Willett WC, Stampfer MJ*, et al.* Body weight and mortality among women. *N Engl J Med* 1995;333(11):677-85.

36. Byers T. Body weight and mortality. *N Engl J Med* 1995;333(11):723-4.

37. Wong JS, Port FK, Hulbert-Shearon TE*, et al.* Survival advantage in Asian American end-stage renal disease patients. *Kidney Int* 1999;55(6):2515-23.

38. Fleischmann E, Teal N, Dudley J*, et al.* Influence of excess weight on mortality and hospital stay in 1346 hemodialysis patients. *Kidney Int* 1999;55(4):1560-7.

39. Leavey SF, Strawderman RL, Jones CA*, et al.* Simple nutritional indicators as independent predictors of mortality in hemodialysis patients. *Am J Kidney Dis* 1998;31(6):997-1006.

40. Leavey SF, McCullough K, Hecking E*, et al.* Body mass index and mortality in 'healthier' as compared with 'sicker' haemodialysis patients: results from the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephrol Dial Transplant* 2001;16(12):2386-94.

41. Shah DS, Polkinghorne KR, Pellicano R*, et al.* Are traditional risk factors valid for assessing cardiovascular risk in end-stage renal failure patients? *Nephrology* 2008;13(8):667-71.

42. Danesh J, Wheeler JG, Hirschfield GM*, et al.* C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med* 2004;350(14):1387-97.

43. Himmelfarb J, Stenvinkel P, Ikizler TA*, et al.* The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int* 2002;62(5):1524-38.

44. Ernst E, Resch KL. Fibrinogen as a cardiovascular risk factor: a meta-analysis and review of the literature. *Ann Intern Med* 1993;118(12):956-63.

45. Zoccali C, Mallamaci F, Tripepi G*, et al.* Fibrinogen, mortality and incident cardiovascular complications in end-stage renal failure. *J Intern Med* 2003;254(2):132-9.

46. Foley RN, Parfrey PS, Harnett JD*, et al.* Hypoalbuminemia, cardiac morbidity, and mortality in end-stage renal disease. *J Am Soc Nephrol* 1996;7(5):728-36.

47. Lowrie EG, Lew NL. Death risk in hemodialysis patients: the predictive value of commonly measured variables and an evaluation of death rate differences between facilities. *Am J Kidney Dis* 1990;15(5):458-82.

48. Stenvinkel P, Heimburger O, Paultre F*, et al.* Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. *Kidney Int* 1999;55(5):1899-911.

49. Nygard O, Nordrehaug JE, Refsum H*, et al.* Plasma homocysteine levels and mortality in patients with coronary artery disease. *N Engl J Med* 1997;337(4):230-6.

50. Szczech LA, Barnhart HX, Inrig JK*, et al.* Secondary analysis of the CHOIR trial epoetin-alpha dose and achieved hemoglobin outcomes. *Kidney Int* 2008;74(6):791-8.

51. Zhang Y, Thamer M, Stefanik K*, et al.* Epoetin requirements predict mortality in hemodialysis patients. *Am J Kidney Dis* 2004;44(5):866-76.

52. Regidor DL, Kopple JD, Kovesdy CP*, et al.* Associations between changes in hemoglobin and administered erythropoiesis-stimulating agent and survival in hemodialysis patients. *J Am Soc Nephrol* 2006;17(4):1181-91.

53. Ross R. Atherosclerosis - an inflammatory disease. *N Engl J Med* 1999;340(2):115-26.

54. Lindner A, Charra B, Sherrard DJ*, et al.* Accelerated atherosclerosis in prolonged maintenance hemodialysis. *N Engl J Med* 1974;290(13):697-701.

55. Suzuki C, Nakamura S, Ishibashi-Ueda H*, et al.* Evidence for severe atherosclerotic changes in chronic hemodialysis patients: comparative autopsy study against cardiovascular disease patients without chronic kidney disease. *Ther Apher Dial* 2011;15(1):51-7.

56. Gross ML, Meyer HP, Ziebart H*, et al.* Calcification of coronary intima and media: immunohistochemistry, backscatter imaging, and x-ray analysis in renal and nonrenal patients. *Clin J Am Soc Nephrol* 2007;2(1):121-34.

57. Nakamura S, Ishibashi-Ueda H, Niizuma S*, et al.* Coronary calcification in patients with chronic kidney disease and coronary artery disease. *Clin J Am Soc Nephrol* 2009;4(12):1892-900.

58. Schwarz U, Buzello M, Ritz E*, et al.* Morphology of coronary atherosclerotic lesions in patients with end-stage renal failure. *Nephrol Dial Transplant* 2000;15(2):218-23.

59. Amann K. Media calcification and intima calcification are distinct entities in chronic kidney disease. *Clin J Am Soc Nephrol* 2008;3(6):1599-605.

60. Zieman SJ, Melenovsky V, Kass DA. Mechanisms, pathophysiology, and therapy of arterial stiffness. *Arterioscler Thromb Vasc Biol* 2005;25(5):932-43.

61. London GM, Drueke TB. Atherosclerosis and arteriosclerosis in chronic renal failure. *Kidney Int* 1997;51(6):1678-95.

62. Guerin AP, Marchais SJ, Metivier F*, et al.* Arterial structural and functional alterations in uraemia. *Eur J Clin Invest* 2005;35 Suppl 3:85-8.

63. O'Rourke MF, Kelly RP. Wave reflection in the systemic circulation and its implications in ventricular function. *J Hypertens* 1993;11(4):327-37.

64. London G, Guerin A, Pannier B*, et al.* Increased systolic pressure in chronic uremia. Role of arterial wave reflections. *Hypertension* 1992;20(1):10-9.

65. Gusbeth-Tatomir P, Covic A. Causes and consequences of increased arterial stiffness in chronic kidney disease patients. *Kidney Blood Press Res* 2007;30(2):97-107.

66. Chang KC, Tseng YZ, Kuo TS*, et al.* Impaired left ventricular relaxation and arterial stiffness in patients with essential hypertension. *Clin Sci* 1994;87(6):641-7.

67. Watanabe H, Ohtsuka S, Kakihana M*, et al.* Coronary circulation in dogs with an experimental decrease in aortic compliance. *J Am Coll Cardiol* 1993;21(6):1497-506.

68. London GM, Marchais SJ, Guerin AP. Arterial stiffness and function in end-stage renal disease. *Adv Chronic Kidney Dis* 2004;11(2):202-9.

69. KDIGO Clinical Practice Guideline for the Diagnosis, Evaluation, Prevention and Treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int* 2009;76(Suppl 113).

70. Braun J, Oldendorf M, Moshage W*, et al.* Electron beam computed tomography in the evaluation of cardiac calcification in chronic dialysis patients. *Am J Kidney Dis* 1996;27(3):394-401.

71. Goodman WG, Goldin J, Kuizon BD*, et al.* Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. *N Engl J Med* 2000;342(20):1478-83.

72. Blacher J, Guerin AP, Pannier B*, et al.* Arterial calcifications, arterial stiffness, and cardiovascular risk in end-stage renal disease. *Hypertension* 2001;38(4):938-42.

73. London GM, Guerin AP, Marchais SJ*, et al.* Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. *Nephrol Dial Transplant* 2003;18(9):1731-40.

74. Mathiesen EB, Bonaa KH, Joakimsen O. Echolucent plaques are associated with high risk of ischemic cerebrovascular events in carotid stenosis: the tromso study. *Circulation* 2001;103(17):2171-5.

75. Johnson JM, Kennelly MM, Decesare D*, et al.* Natural history of asymptomatic carotid plaque. *Arch Surg* 1985;120(9):1010-2.

76. Gronholdt ML, Nordestgaard BG, Schroeder TV*, et al.* Ultrasonic echolucent carotid plaques predict future strokes. *Circulation* 2001;104(1):68-73.

77. Hunt JL, Fairman R, Mitchell ME*, et al.* Bone formation in carotid plaques: a clinicopathological study. *Stroke* 2002;33(5):1214-9.

78. Beckman JA, Ganz J, Creager MA*, et al.* Relationship of clinical presentation and calcification of culprit coronary artery stenoses. *Arterioscler Thromb Vasc Biol* 2001;21(10):1618-22.

79. Haydar AA, Hujairi NM, Covic AA*, et al.* Coronary artery calcification is related to coronary atherosclerosis in chronic renal disease patients: a study comparing EBCT-

generated coronary artery calcium scores and coronary angiography. *Nephrol Dial Transplant* 2004;19(9):2307-12.

80. Sharples EJ, Pereira D, Summers S*, et al.* Coronary artery calcification measured with electron-beam computerized tomography correlates poorly with coronary artery angiography in dialysis patients. *Am J Kidney Dis* 2004;43(2):313-9.

81. Bonifacio DL, Malineni K, Kadakia RA*, et al.* Coronary calcification and cardiac events after percutaneous intervention in dialysis patients. *J Cardiovasc Risk* 2001;8(3):133-7.

82. Everhart JE, Pettitt DJ, Knowler WC*, et al.* Medial arterial calcification and its association with mortality and complications of diabetes. *Diabetologia* 1988;31(1):16-23.

83. Jono S, McKee MD, Murry CE*, et al.* Phosphate regulation of vascular smooth muscle cell calcification. *Circ Res* 2000;87(7):E10-7.

84. Lopez I, Mendoza FJ, Aguilera-Tejero E*, et al.* The effect of calcitriol, paricalcitol, and a calcimimetic on extraosseous calcifications in uremic rats. *Kidney Int* 2008;73(3):300-7.

85. Mallick NP, Berlyne GM. Arterial calcification after vitamin-D therapy in hyperphosphatemic renal failure. *Lancet* 1968;2(7582):1316-20.

86. Goldsmith DJ, Covic A, Sambrook PA*, et al.* Vascular calcification in long-term haemodialysis patients in a single unit: a retrospective analysis. *Nephron* 1997;77(1):37-43.

87. Chertow GM, Burke SK, Raggi P. Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. *Kidney Int* 2002;62(1):245-52.

88. Block GA, Spiegel DM, Ehrlich J*, et al.* Effects of sevelamer and calcium on coronary artery calcification in patients new to hemodialysis. *Kidney Int* 2005;68(4):1815-24.

89. Block GA, Raggi P, Bellasi A*, et al.* Mortality effect of coronary calcification and phosphate binder choice in incident hemodialysis patients. *Kidney Int* 2007;71(5):438-41.

90. Herzog CA, Littrell K, Arko C*, et al.* Clinical characteristics of dialysis patients with acute myocardial infarction in the United States: a collaborative project of the United States Renal Data System and the National Registry of Myocardial Infarction. *Circulation* 2007;116(13):1465-72.

91. Sosnov J, Lessard D, Goldberg RJ*, et al.* Differential symptoms of acute myocardial infarction in patients with kidney disease: a community-wide perspective. *Am J Kidney Dis* 2006;47(3):378-84.

92. Cardiovascular disease in patients with end-stage renal disease. In. *USRDS 2011 Annual Data Report*. Bethesda, MD: National Institute of Health, National Institutes of Diabetes and Digestive and Kidney Diseases; 2011, 217-24.

93. Jadoul M, Thumma J, Fuller DS*, et al.* Modifiable practices associated with sudden death among hemodialysis patients in the Dialysis Outcomes and Practice Patterns Study. *Clin J Am Soc Nephrol* 2012;7(5):765-74.

94. Parekh RS, Plantinga LC, Kao WH*, et al.* The association of sudden cardiac death with inflammation and other traditional risk factors. *Kidney Int* 2008;74(10):1335-42.

95. Priori SG, Aliot E, Blomstrom-Lundqvist C*, et al.* Task Force on Sudden Cardiac Death of the European Society of Cardiology. *Eur Heart J* 2001;22(16):1374-450.

96. Chugh SS, Jui J, Gunson K*, et al.* Current burden of sudden cardiac death: multiple source surveillance versus retrospective death certificate-based review in a large U.S. community. *J Am Coll Cardiol* 2004;44(6):1268-75.

97. Thomas AC, Knapman PA, Krikler DM*, et al.* Community study of the causes of "natural" sudden death. *BMJ* 1988;297(6661):1453-6.

98. Leach IH, Blundell JW, Rowley JM*, et al.* Acute ischaemic lesions in death due to ischaemic heart disease. An autopsy study of 333 cases of out-of-hospital death. *Eur Heart J* 1995;16(9):1181-5.

99. Takeda K, Harada A, Okuda S*, et al.* Sudden death in chronic dialysis patients. *Nephrol Dial Transplant* 1997;12(5):952-5.

100. Kannel WB, Wilson PW, D'Agostino RB*, et al.* Sudden coronary death in women. *Am Heart J* 1998;136(2):205-12.

101. Genovesi S, Valsecchi MG, Rossi E*, et al.* Sudden death and associated factors in a historical cohort of chronic haemodialysis patients. *Nephrol Dial Transplant* 2009;24(8):2529-36.

102. Haider AW, Larson MG, Benjamin EJ*, et al.* Increased left ventricular mass and hypertrophy are associated with increased risk for sudden death. *J Am Coll Cardiol* 1998;32(5):1454-9.

103. Westenbrink BD, Hovinga TK, Kloppenburg WD*, et al.* B-type natriuretic peptide and interdialytic fluid retention are independent and incremental predictors of mortality in hemodialysis patients. *Clin Nephrol* 2011;76(5):373-9.

104. Kovesdy CP, Regidor DL, Mehrotra R*, et al.* Serum and dialysate potassium concentrations and survival in hemodialysis patients. *Clin J Am Soc Nephrol* 2007;2(5):999-1007.

105. Di Iorio BR, Bortone S, Piscopo C*, et al.* Cardiac vascular calcification and QT interval in ESRD patients: is there a link? *Blood Purif* 2006;24(5-6):451-9.

106. Astor BC, Muntner P, Levin A*, et al.* Association of kidney function with anemia: the Third National Health and Nutrition Examination Survey (1988-1994). *Arch Intern Med* 2002;162(12):1401-8.

107. Hsu CY, McCulloch CE, Curhan GC. Epidemiology of anemia associated with chronic renal insufficiency among adults in the United States: results from the Third National Health and Nutrition Examination Survey. *J Am Soc Nephrol* 2002;13(2):504-10.

108. Moreno F, Lopez Gomez JM, Sanz-Guajardo D*, et al.* Quality of life in dialysis patients. A spanish multicentre study. Spanish Cooperative Renal Patients Quality of Life Study Group. *Nephrol Dial Transplant* 1996;11 Suppl 2:125-9.

109. Mayer G, Thum J, Graf H. Anaemia and reduced exercise capacity in patients on chronic haemodialysis. *Clin Sci* 1989;76(3):265-8.

110. Benz RL, Pressman MR, Hovick ET*, et al.* A preliminary study of the effects of correction of anemia with recombinant human erythropoietin therapy on sleep, sleep disorders, and daytime sleepiness in hemodialysis patients (The SLEEPO study). *Am J Kidney Dis* 1999;34(6):1089-95.

111. Levin NW, Lazarus JM, Nissenson AR. National Cooperative rHu Erythropoietin Study in patients with chronic renal failure--an interim report. The National Cooperative rHu Erythropoietin Study Group. *Am J Kidney Dis* 1993;22(2 Suppl 1):3-12.

112. Beusterien KM, Nissenson AR, Port FK*, et al.* The effects of recombinant human erythropoietin on functional health and well-being in chronic dialysis patients. *J Am Soc Nephrol* 1996;7(5):763-73.

113. Ma JZ, Ebben J, Xia H*, et al.* Hematocrit level and associated mortality in hemodialysis patients. *J Am Soc Nephrol* 1999;10(3):610-9.

114. Locatelli F, Pisoni RL, Combe C*, et al.* Anaemia in haemodialysis patients of five European countries: association with morbidity and mortality in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephrol Dial Transplant* 2004;19(1):121-32.

115. Locatelli F, Conte F, Marcelli D. The impact of haematocrit levels and erythropoietin treatment on overall and cardiovascular mortality and morbidity--the experience of the Lombardy Dialysis Registry. *Nephrol Dial Transplant* 1998;13(7):1642-4.

116. Collins AJ, Li S, St Peter W*, et al.* Death, hospitalization, and economic associations among incident hemodialysis patients with hematocrit values of 36 to 39%. *J Am Soc Nephrol* 2001;12(11):2465-73.

117. Parfrey PS, Foley RN, Harnett JD*, et al.* Outcome and risk factors for left ventricular disorders in chronic uraemia. *Nephrol Dial Transplant* 1996;11(7):1277-85.

118. Foley RN, Parfrey PS, Harnett JD*, et al.* The impact of anemia on cardiomyopathy, morbidity, and and mortality in end-stage renal disease. *Am J Kidney Dis* 1996;28(1):53-61.

119. Collins AJ, Ma JZ, Xia A*, et al.* Trends in anemia treatment with erythropoietin usage and patient outcomes. *Am J Kidney Dis* 1998;32(6 Suppl 4):S133-41.

120. Duke M, Abelmann WH. The hemodynamic response to chronic anemia. *Circulation* 1969;39(4):503-15.

121. Mann JF. What are the short-term and long-term consequences of anaemia in CRF patients? *Nephrol Dial Transplant* 1999;14 Suppl 2:29-36.

122. Silberberg JS, Rahal DP, Patton DR*, et al.* Role of anemia in the pathogenesis of left ventricular hypertrophy in end-stage renal disease. *Am J Cardiol* 1989;64(3):222-4.

123. Zoccali C, Benedetto FA, Mallamaci F*, et al.* Left ventricular mass monitoring in the follow-up of dialysis patients: prognostic value of left ventricular hypertrophy progression. *Kidney Int* 2004;65(4):1492-8.

124. Foley RN, Parfrey PS, Harnett JD*, et al.* The prognostic importance of left ventricular geometry in uremic cardiomyopathy. *J Am Soc Nephrol* 1995;5(12):2024-31.

125. Jones M, Schenkel B, Just J. Epoetin alfa's effect on left ventricular hypertrophy and subsequent mortality. *Int J Cardiol* 2005;100(2):253-65.

126. Evans RW, Rader B, Manninen DL. The quality of life of hemodialysis recipients treated with recombinant human erythropoietin. Cooperative Multicenter EPO Clinical Trial Group. *JAMA* 1990;263(6):825-30.

127. Marsh JT, Brown WS, Wolcott D*, et al.* rHuEPO treatment improves brain and cognitive function of anemic dialysis patients. *Kidney Int* 1991;39(1):155-63.

128. Pickett JL, Theberge DC, Brown WS*, et al.* Normalizing hematocrit in dialysis patients improves brain function. *Am J Kidney Dis* 1999;33(6):1122-30.

129. Sennesael JJ, Van der Niepen P, Verbeelen DL. Treatment with recombinant human erythropoietin increases antibody titers after hepatitis B vaccination in dialysis patients. *Kidney Int* 1991;40(1):121-8.

130. Veys N, Vanholder R, Ringoir S. Correction of deficient phagocytosis during erythropoietin treatment in maintenance hemodialysis patients. *Am J Kidney Dis* 1992;19(4):358-63.

131. Gordge MP, Leaker B, Patel A*, et al.* Recombinant human erythropoietin shortens the uraemic bleeding time without causing intravascular haemostatic activation. *Thromb Res* 1990;57(2):171-82.

132. Macdougall IC, Davies ME, Hallett I*, et al.* Coagulation studies and fistula blood flow during erythropoietin therapy in haemodialysis patients. *Nephrol Dial Transplant* 1991;6(11):862-7.

133. Moia M, Mannucci PM, Vizzotto L*, et al.* Improvement in the haemostatic defect of uraemia after treatment with recombinant human erythropoietin. *Lancet* 1987;2(8570):1227-9.

134. Jelkmann W. Erythropoietin: structure, control of production, and function. *Physiol Rev* 1992;72(2):449-89.

135. Jelkmann W. Molecular biology of erythropoietin. *Intern Med* 2004;43(8):649-59.

136. Wojchowski DM, Sathyanarayana P, Dev A. Erythropoietin receptor response circuits. *Curr Opin Hematol* 2010;17(3):169-76.

137. Maxwell P. HIF-1: an oxygen response system with special relevance to the kidney. *J Am Soc Nephrol* 2003;14(11):2712-22.

138. Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 2004;5(5):343-54.

139. Macdougall IC, Eckardt K. Anemia in chronic kidney disease. In: Feehally J, Floege J, Johnson RJ, (eds). *Comprehensive clinical nephrology*. St. Louis: Mosby; 2007, p853-860.

140. Semenza GL. Involvement of oxygen-sensing pathways in physiologic and pathologic erythropoiesis. *Blood* 2009;114(10):2015-9.

141. Eckardt KU. Erythropoietin: oxygen-dependent control of erythropoiesis and its failure in renal disease. *Nephron* 1994;67(1):7-23.

142. Tan CC, Eckardt KU, Ratcliffe PJ. Organ distribution of erythropoietin messenger RNA in normal and uremic rats. *Kidney Int* 1991;40(1):69-76.

143. Naets JP, Garcia JF, Tousaaint C*, et al.* Radioimmunoassay of erythropoietin in chronic uraemia or anephric patients. *Scand J Haematol* 1986;37(5):390-4.

144. Eschbach JW. The anemia of chronic renal failure: pathophysiology and the effects of recombinant erythropoietin. *Kidney Int* 1989;35(1):134-48.

145. Caro J, Brown S, Miller O*, et al.* Erythropoietin levels in uremic nephric and anephric patients. *J Lab Clin Med* 1979;93(3):449-58.

146. Macdougall IC. Monitoring of iron status and iron supplementation in patients treated with erythropoietin. *Curr Opin Nephrol Hypertens* 1994;3(6):620-5.

147. Kooistra MP, Niemantsverdriet EC, van Es A*, et al.* Iron absorption in erythropoietin-treated haemodialysis patients: effects of iron availability, inflammation and aluminium. *Nephrol Dial Transplant* 1998;13(1):82-8.

148. Tessitore N, Solero GP, Lippi G*, et al.* The role of iron status markers in predicting response to intravenous iron in haemodialysis patients on maintenance erythropoietin. *Nephrol Dial Transplant* 2001;16(7):1416-23.

149. Schaefer RM, Schaefer L. Iron monitoring and supplementation: how do we achieve the best results? *Nephrol Dial Transplant* 1998;13 Suppl 2:9-12.

150. KDOQI. Clinical practice guidelines and clinical practice recommendations for anemia in chronic kidney disease in adults. *Am J Kidney Dis* 2006;47(Suppl 3).

151. European Renal Association. Revised European best practice guidelines for the management of anaemia in patients with chronic renal failure. *Nephrol Dial Transplant* 2004;29(Suppl 2).

152. Nemeth E, Valore EV, Territo M*, et al.* Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* 2003;101(7):2461-3.

153. Malyszko J, Malyszko JS, Pawlak K*, et al.* Hepcidin, iron status, and renal function in chronic renal failure, kidney transplantation, and hemodialysis. *Am J Hematol* 2006;81(11):832-7.

154. Tomosugi N, Kawabata H, Wakatabe R*, et al.* Detection of serum hepcidin in renal failure and inflammation by using ProteinChip System. *Blood* 2006;108(4):1381-7.

155. Taes YE, Wuyts B, Boelaert JR*, et al.* Prohepcidin accumulates in renal insufficiency. *Clin Chem Lab Med* 2004;42(4):387-9.

156. Jurado RL. Iron, infections, and anemia of inflammation. *Clin Infect Dis* 1997;25(4):888-95.

157. Trey JE, Kushner I. The acute phase response and the hematopoietic system: the role of cytokines. *Crit Rev Oncol Hematol* 1995;21(1-3):1-18.

158. Schooley JC, Kullgren B, Allison AC. Inhibition by interleukin-1 of the action of erythropoietin on erythroid precursors and its possible role in the pathogenesis of hypoplastic anaemias. *Br J Haematol* 1987;67(1):11-7.

159. Allen DA, Breen C, Yaqoob MM*, et al.* Inhibition of CFU-E colony formation in uremic patients with inflammatory disease: role of IFN-gamma and TNF-alpha. *J Investig Med* 1999;47(5):204-11.

160. Jelkmann W. Proinflammatory cytokines lowering erythropoietin production. *J Interferon Cytokine Res* 1998;18(8):555-9.

161. Locatelli F, Andrulli S, Memoli B*, et al.* Nutritional-inflammation status and resistance to erythropoietin therapy in haemodialysis patients. *Nephrol Dial Transplant* 2006;21(4):991-8.

162. Lopez-Gomez JM, Portoles JM, Aljama P. Factors that condition the response to erythropoietin in patients on hemodialysis and their relation to mortality. *Kidney Int Suppl* 2008(111):S75-81.

163. Yeun JY, Levine RA, Mantadilok V*, et al.* C-Reactive protein predicts all-cause and cardiovascular mortality in hemodialysis patients. *Am J Kidney Dis* 2000;35(3):469-76.

164. Kalantar-Zadeh K, Ikizler TA, Block G*, et al.* Malnutrition-inflammation complex syndrome in dialysis patients: causes and consequences. *Am J Kidney Dis* 2003;42(5):864-81.

165. Boxer M, Ellman L, Geller R*, et al.* Anemia in primary hyperparathyroidism. *Arch Intern Med* 1977;137(5):588-93.

166. Falko JM, Guy JT, Smith RE*, et al.* Primary hyperparathyroidism and anemia. *Arch Intern Med* 1976;136(8):887-9.

167. Zingraff J, Drueke T, Marie P*, et al.* Anemia and secondary hyperparathyroidism. *Arch Intern Med* 1978;138(11):1650-2.

168. Barbour GL. Effect of parathyroidectomy on anemia in chronic renal failure. *Arch Intern Med* 1979;139(8):889-91.

169. Rao DS, Shih MS, Mohini R. Effect of serum parathyroid hormone and bone marrow fibrosis on the response to erythropoietin in uremia. *N Engl J Med* 1993;328(3):171-5.

170. Meytes D, Bogin E, Ma A*, et al.* Effect of parathyroid hormone on erythropoiesis. *J Clin Invest* 1981;67(5):1263-9.

171. Komatsuda A, Hirokawa M, Haseyama T*, et al.* Human parathyroid hormone does not influence human erythropoiesis in vitro. *Nephrol Dial Transplant* 1998;13(8):2088-91.

172. Delwiche F, Garrity MJ, Powell JS*, et al.* High levels of the circulating form of parathyroid hormone do not inhibit in vitro erythropoiesis. *J Lab Clin Med* 1983;102(4):613-20.

173. Drueke TB, Eckardt KU. Role of secondary hyperparathyroidism in erythropoietin resistance of chronic renal failure patients. *Nephrol Dial Transplant* 2002;17 Suppl 5:28-31.

174. Radtke HW, Frei U, Erbes PM*, et al.* Improving anemia by hemodialysis: effect of serum erythropoietin. *Kidney Int* 1980;17(3):382-7.

175. De Paepe MB, Schelstraete KH, Ringoir SM*, et al.* Influence of continuous ambulatory peritoneal dialysis on the anemia of endstage renal disease. *Kidney Int* 1983;23(5):744-8.

176. Zappacosta AR, Caro J, Erslev A. Normalization of hematocrit in patients with end-stage renal disease on continuous ambulatory peritoneal dialysis: the role of erythropoietin. *Am J Med* 1982;72(1):53-7.

177. Movilli E, Cancarini GC, Zani R*, et al.* Adequacy of dialysis reduces the doses of recombinant erythropoietin independently from the use of biocompatible membranes in haemodialysis patients. *Nephrol Dial Transplant* 2001;16(1):111-4.

178. Coladonato JA, Frankenfield DL, Reddan DN*, et al.* Trends in anemia management among US hemodialysis patients. *J Am Soc Nephrol* 2002;13(5):1288-95.

179. Madore F, Lowrie EG, Brugnara C*, et al.* Anemia in hemodialysis patients: variables affecting this outcome predictor. *J Am Soc Nephrol* 1997;8(12):1921-9.

180. Ifudu O, Feldman J, Friedman EA. The intensity of hemodialysis and the response to erythropoietin in patients with end-stage renal disease. *N Engl J Med* 1996;334(7):420-5.

181. McGonigle RJ, Wallin JD, Shadduck RK*, et al.* Erythropoietin deficiency and inhibition of erythropoiesis in renal insufficiency. *Kidney Int* 1984;25(2):437-44.

182. Wallner SF, Vautrin RM. Evidence that inhibition of erythropoiesis is important in the anemia of chronic renal failure. *J Lab Clin Med* 1981;97(2):170-8.

183. Delwiche F, Segal GM, Eschbach JW*, et al.* Hematopoietic inhibitors in chronic renal failure: lack of in vitro specificity. *Kidney Int* 1986;29(3):641-8.

184. Segal GM, Eschbach JW, Egrie JC*, et al.* The anemia of end-stage renal disease: hematopoietic progenitor cell response. *Kidney Int* 1988;33(5):983-8.

185. Weidmann P, Maxwell MH, Lupu AN*, et al.* Plasma renin activity and blood pressure in terminal renal failure. *N Engl J Med* 1971;285(14):757-62.

186. Brown JJ, Curtis JR, Lever AF*, et al.* Plasma renin concentration and the control of blood pressure in patients on maintenance haemodialysis. *Nephron* 1969;6(3):329-49.

187. Vertes V, Cangiano JL, Berman LB*, et al.* Hypertension in end-stage renal disease. *N Engl J Med* 1969;280(18):978-81.

188. Kimura G, Takahashi N, Kawano Y*, et al.* Plasma renin activity in hemodialyzed patients during long-term follow-up. *Am J Kidney Dis* 1995;25(4):589-92.

189. K/DOQI clinical practice guidelines for cardiovascular disease in dialysis patients. *Am J Kidney Dis* 2005;45(4 Suppl 3):S1-153.

190. Tarazi RC, Frohlich ED, Dustan HP*, et al.* Hypertension and high hematocrit. Another clue to renal arterial disease. *Am J Cardiol* 1966;18(6):855-8.

191. Bacon BR, Rothman SA, Ricanati ES*, et al.* Renal artery stenosis with erythrocytosis after renal transplantation. *Arch Intern Med* 1980;140(9):1206-11.

192. Shand BI, Bailey RR, Lynn KL*, et al.* Effect of enalapril on erythrocytosis in hypertensive patients with renal disease. *Blood Press* 1995;4(4):238-40.

193. Kamper AL, Nielsen OJ. Effect of enalapril on haemoglobin and serum erythropoietin in patients with chronic nephropathy. *Scand J Clin Lab Invest* 1990;50(6):611-8.

194. Olsen MH, Wachtell K, Beevers G*, et al.* Prognostic importance of hemoglobin in hypertensive patients with electrocardiographic left ventricular hypertrophy: the Losartan Intervention For End point reduction in hypertension (LIFE) study. *Am Heart J* 2009;157(1):177-84.

195. Griffing GT, Melby JC. Enalapril (MK-421) and the white cell count and haematocrit. *Lancet* 1982;1(8285):1361.

196. Odabas AR, Cetinkaya R, Selcuk Y*, et al.* The effect of high dose losartan on erythropoietin resistance in patients undergoing haemodialysis. *Panminerva Med* 2003;45(1):59-62.

197. Hirakata H, Onoyama K, Iseki K*, et al.* Worsening of anemia induced by long-term use of captopril in hemodialysis patients. *Am J Nephrol* 1984;4(6):355-60.

198. Erturk S, Nergizoglu G, Ates K*, et al.* The impact of withdrawing ACE inhibitors on erythropoietin responsiveness and left ventricular hypertrophy in haemodialysis patients. *Nephrol Dial Transplant* 1999;14(8):1912-6.

199. Mrug M, Stopka T, Julian BA*, et al.* Angiotensin II stimulates proliferation of normal early erythroid progenitors. *J Clin Invest* 1997;100(9):2310-4.

200. Gupta M, Miller BA, Ahsan N*, et al.* Expression of angiotensin II type I receptor on erythroid progenitors of patients with post transplant erythrocytosis. *Transplantation* 2000;70(8):1188-94.

201. Kato H, Ishida J, Imagawa S*, et al.* Enhanced erythropoiesis mediated by activation of the renin-angiotensin system via angiotensin II type 1a receptor. *FASEB J* 2005;19(14):2023-5.

202. Gould AB, Goodman SA. Effect of an angiotensin-converting enzyme inhibitor on blood pressure and erythropoiesis in rats. *Eur J Pharmacol* 1990;181(3):225-34.

203. Vlahakos DV, Balodimos C, Papachristopoulos V*, et al.* Renin-angiotensin system stimulates erythropoietin secretion in chronic hemodialysis patients. *Clin Nephrol* 1995;43(1):53-9.

204. Jensen JD, Eiskjaer H, Bagger JP*, et al.* Elevated level of erythropoietin in congestive heart failure relationship to renal perfusion and plasma renin. *J Intern Med* 1993;233(2):125-30.

205. Le Meur Y, Lorgeot V, Comte L*, et al.* Plasma levels and metabolism of AcSDKP in patients with chronic renal failure: relationship with erythropoietin requirements. *Am J Kidney Dis* 2001;38(3):510-7.

206. van der Meer P, Lipsic E, Westenbrink BD*, et al.* Levels of hematopoiesis inhibitor N-acetyl-seryl-aspartyl-lysyl-proline partially explain the occurrence of anemia in heart failure. *Circulation* 2005;112(12):1743-7.

207. Kurtides ES, Rambach WA, Alt HL*, et al.* Effect of Hemodialysis on Erythrokinetics in Anemia of Uremia. *J Lab Clin Med* 1964;63:469-79.

208. Adamson JW, Eschbach J, Finch CA. The kidney and erythropoiesis. *Am J Med* 1968;44(5):725-33.

209. Desforges JF, Dawson JP. The anemia of renal failure. *Arch Intern Med* 1958;101(2):326-32.

210. Joske RA, McAlister JM, Prankerd TA. Isotope investigations of red cell production and destruction in chronic renal disease. *Clin Sci* 1956;15(4):511-22.

211. Koch KM, Patyna WD, Shaldon S*, et al.* Anemia of the regular hemodialysis patient and its treatment. *Nephron* 1974;12(5):405-19.

212. Vos FE, Schollum JB, Coulter CV*, et al.* Red blood cell survival in long-term dialysis patients. *Am J Kidney Dis* 2011;58(4):591-8.

213. Decamps A, Zandecki M, Ribiere M*, et al.* Red cell filterability and chronic renal failure. *Scand J Clin Lab Invest Suppl* 1981;156:177-9.

214. Kikuchi Y, Koyama T, Koyama Y*, et al.* Red blood cell deformability in renal failure. *Nephron* 1982;30(1):8-14.

215. Ota K, Aida K, Era K*, et al.* Subhemolytic damage of red blood cells during hemodialysis examined by cyclic path centrifuge. *Trans Am Soc Artif Intern Organs* 1976;22:682-6.

216. Forman S, Bischel M, Hochstein P. Erythrocyte deformability in uremic hemodialyzed patients. *Ann Intern Med* 1973;79(6):841-3.

217. Viljoen M, de Oliveira AA, Milne FJ. Physical properties of the red blood cells in chronic renal failure. *Nephron* 1991;59(2):271-8.

218. Rosenmund A, Binswanger U, Straub PW. Oxidative injury to erythrocytes, cell rigidity, and splenic hemolysis in hemodialyzed uremic patients. *Ann Intern Med* 1975;82(4):460-5.

219. Hartley LC, Morgan TO, Innis MD*, et al.* Splenectomy for anaemia in patients on regular haemodialysis. *Lancet* 1971;2(7738):1343-5.

220. Baskurt OK, Meiselman HJ. Activated polymorphonuclear leukocytes affect red blood cell aggregability. *J Leukoc Biol* 1998;63(1):89-93.

221. Siems W, Carluccio F, Radenkovic S*, et al.* Oxidative stress in renal anemia of hemodialysis patients is mitigated by epoetin treatment. *Kidney Blood Press Res* 2005;28(5-6):295-301.

222. Sommerburg O, Grune T, Hampl H*, et al.* Does long-term treatment of renal anaemia with recombinant erythropoietin influence oxidative stress in haemodialysed patients? *Nephrol Dial Transplant* 1998;13(10):2583-7.

223. Wiswedel I, Peter D, Gardemann A*, et al.* Serum Concentrations of F2-Isoprostanes and 4-Hydroxynonenal in Hemodialysis Patients in Relation to Inflammation and Renal Anemia. *Biomark Insights* 2008;3:419-428.

224. Siems W, Carluccio F, Grune T*, et al.* Elevated serum concentration of cardiotoxic lipid peroxidation products in chronic renal failure in relation to severity of renal anemia. *Clin Nephrol* 2002;58 Suppl 1:S20-5.

225. Gallucci MT, Lubrano R, Meloni C*, et al.* Red blood cell membrane lipid peroxidation and resistance to erythropoietin therapy in hemodialysis patients. *Clin Nephrol* 1999;52(4):239-45.

226. Kato A, Odamaki M, Hishida A. Blood 8-hydroxy-2'-deoxyguanosine is associated with erythropoietin resistance in haemodialysis patients. *Nephrol Dial Transplant* 2003;18(5):931-6.

227. Himmelfarb J, McMonagle E, Holbrook D*, et al.* Increased susceptibility to erythrocyte C5b-9 deposition and complement-mediated lysis in chronic renal failure. *Kidney Int* 1999;55(2):659-66.

228. Volanakis JE, Barnum SR, Giddens M*, et al.* Renal filtration and catabolism of complement protein D. *N Engl J Med* 1985;312(7):395-9.

229. Pascual M, Steiger G, Estreicher J*, et al.* Metabolism of complement factor D in renal failure. *Kidney Int* 1988;34(4):529-36.

230. Chanard J, Lavaud S, Randoux C*, et al.* New insights in dialysis membrane biocompatibility: relevance of adsorption properties and heparin binding. *Nephrol Dial Transplant* 2003;18(2):252-7.

231. Deppisch R, Gohl H, Smeby L. Microdomain structure of polymeric surfaces--potential for improving blood treatment procedures. *Nephrol Dial Transplant* 1998;13(6):1354-9.

232. Hauser AC, Derfler K, Stockenhuber F*, et al.* Generation of the membrane attack complex during haemodialysis: impact of classical and alternative pathway components. *Clin Sci* 1990;79(5):471-6.

233. Cheung AK. Biocompatibility of hemodialysis membranes. *J Am Soc Nephrol* 1990;1(2):150-61.

234. Andersson J, Ekdahl KN, Larsson R*, et al.* C3 adsorbed to a polymer surface can form an initiating alternative pathway convertase. *J Immunol* 2002;168(11):5786-91.

235. Richardson D, Bartlett C, Goutcher E*, et al.* Erythropoietin resistance due to dialysate chloramine: the two-way traffic of solutes in haemodialysis. *Nephrol Dial Transplant* 1999;14(11):2625-7.

236. Weinstein T, Chagnac A, Korzets A*, et al.* Haemolysis in haemodialysis patients: evidence for impaired defence mechanisms against oxidative stress. *Nephrol Dial Transplant* 2000;15(6):883-7.

237. Manzler AD, Schreiner AW. Copper-induced acute hemolytic anemia. A new complication of hemodialysis. *Ann Intern Med* 1970;73(3):409-12.

238. Petrie JJ, Row PG. Dialysis anaemia caused by subacute zinc toxicity. *Lancet* 1977;1(8023):1178-80.

239. Cheung J, Yu A, LaBossiere J*, et al.* Peptic ulcer bleeding outcomes adversely affected by end-stage renal disease. *Gastrointest Endosc* 2010;71(1):44-9.

240. Saeed F, Agrawal N, Greenberg E*, et al.* Lower gastrointestinal bleeding in chronic hemodialysis patients. *Int J Nephrol* 2011;2011:272535.

241. Toke AB. GI bleeding risk in patients undergoing dialysis. *Gastrointest Endosc* 2010;71(1):50-2.

242. Linton AL, Clark WF, Driedger AA*, et al.* Correctable factors contributing to the anemia of dialysis patients. *Nephron* 1977;19(2):95-8.

243. Kaw D, Malhotra D. Platelet dysfunction and end-stage renal disease. *Semin Dial* 2006;19(4):317-22.

244. Remuzzi G. Bleeding in renal failure. *Lancet* 1988;1(8596):1205-8.

245. Kasanen A, Forsstrom J. The effect of simultaneous intramuscular ascorbic acid and cobalt therapy of renal anemia. *Ann Med Exp Biol Fenn* 1963;41:103-5.

246. Shahidi NT. Androgens and erythropoiesis. *N Engl J Med* 1973;289(2):72-80.

247. Eschbach JW, Mladenovic J, Garcia JF*, et al.* The anemia of chronic renal failure in sheep. Response to erythropoietin-rich plasma in vivo. *J Clin Invest* 1984;74(2):434-41.

248. Miyake T, Kung CK, Goldwasser E. Purification of human erythropoietin. *J Biol Chem* 1977;252(15):5558-64.

249. Jacobs K, Shoemaker C, Rudersdorf R*, et al.* Isolation and characterization of genomic and cDNA clones of human erythropoietin. *Nature* 1985;313(6005):806-10.

250. Lin FK, Suggs S, Lin CH*, et al.* Cloning and expression of the human erythropoietin gene. *Proc Natl Acad Sci U S A* 1985;82(22):7580-4.

251. Eschbach JW, Egrie JC, Downing MR*, et al.* Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. *N Engl J Med* 1987;316(2):73-8.

252. Winearls CG, Oliver DO, Pippard MJ*, et al.* Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* 1986;2(8517):1175-8.

253. Jelkmann W. Erythropoietin after a century of research: younger than ever. *Eur J Haematol* 2007;78(3):183-205.

254. Macdougall IC, Padhi D, Jang G. Pharmacology of darbepoetin alfa. *Nephrol Dial Transplant* 2007;22 Suppl 4:iv2-iv9.

255. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Nephrol Dial Transplant* 2001;16 Suppl 3:3-13.

256. Trachsler J, Gluck Z, Dickenmann M*, et al.* Parameters for successful monthly extended dosing of darbepoetin-alpha in patients undergoing hemodialysis. *Clin Nephrol* 2009;71(6):697-702.

257. Johnson DW, Pollock CA, Macdougall IC. Erythropoiesis-stimulating agent hyporesponsiveness. *Nephrology* 2007;12(4):321-30.

258. Besarab A, Coyne DW. Iron supplementation to treat anemia in patients with chronic kidney disease. *Nat Rev Nephrol* 2010;6(12):703-10.

259. Markowitz GS, Kahn GA, Feingold RE*, et al.* An evaluation of the effectiveness of oral iron therapy in hemodialysis patients receiving recombinant human erythropoietin. *Clin Nephrol* 1997;48(1):34-40.

260. Wingard RL, Parker RA, Ismail N*, et al.* Efficacy of oral iron therapy in patients receiving recombinant human erythropoietin. *Am J Kidney Dis* 1995;25(3):433-9.

261. Macdougall IC. Strategies for iron supplementation: oral versus intravenous. *Kidney Int Suppl* 1999;69:S61-6.

262. Rozen-Zvi B, Gafter-Gvili A, Paul M*, et al.* Intravenous versus oral iron supplementation for the treatment of anemia in CKD: systematic review and meta-analysis. *Am J Kidney Dis* 2008;52(5):897-906.

263. Fudin R, Jaichenko J, Shostak A*, et al.* Correction of uremic iron deficiency anemia in hemodialyzed patients: a prospective study. *Nephron* 1998;79(3):299-305.

264. Kapoian T, O'Mara NB, Singh AK*, et al.* Ferric gluconate reduces epoetin requirements in hemodialysis patients with elevated ferritin. *J Am Soc Nephrol* 2008;19(2):372-9.

265. Besarab A, Amin N, Ahsan M*, et al.* Optimization of epoetin therapy with intravenous iron therapy in hemodialysis patients. *J Am Soc Nephrol* 2000;11(3):530-8.

266. Macdougall IC, Tucker B, Thompson J*, et al.* A randomized controlled study of iron supplementation in patients treated with erythropoietin. *Kidney Int* 1996;50(5):1694-9.

267. Fishbane S, Frei GL, Maesaka J. Reduction in recombinant human erythropoietin doses by the use of chronic intravenous iron supplementation. *Am J Kidney Dis* 1995;26(1):41-6.

268. Richardson D, Bartlett C, Will EJ. Optimizing erythropoietin therapy in hemodialysis patients. *Am J Kidney Dis* 2001;38(1):109-17.

269. Coyne DW, Kapoian T, Suki W*, et al.* Ferric gluconate is highly efficacious in anemic hemodialysis patients with high serum ferritin and low transferrin saturation: results of the Dialysis Patients' Response to IV Iron with Elevated Ferritin (DRIVE) Study. *J Am Soc Nephrol* 2007;18(3):975-84.

270. Schiller B, Doss S, E DEC*, et al.* Costs of managing anemia with erythropoiesis-stimulating agents during hemodialysis: a time and motion study. *Hemodial Int* 2008;12(4):441-9.

271. Bommer J, Alexiou C, Muller-Buhl U*, et al.* Recombinant human erythropoietin therapy in haemodialysis patients--dose determination and clinical experience. *Nephrol Dial Transplant* 1987;2(4):238-42.

272. Bahlmann J, Schoter KH, Scigalla P*, et al.* Morbidity and mortality in hemodialysis patients with and without erythropoietin treatment: a controlled study. *Contrib Nephrol* 1991;88:90-106.

273. Canadian Erythropoietin Study Group. Association between recombinant human erythropoietin and quality of life and exercise capacity of patients receiving haemodialysis. Canadian Erythropoietin Study Group. *BMJ* 1990;300(6724):573-8.

274. Locatelli F, Pisoni RL, Akizawa T*, et al.* Anemia management for hemodialysis patients: Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines and Dialysis Outcomes and Practice Patterns Study (DOPPS) findings. *Am J Kidney Dis* 2004;44(5 Suppl 2):27-33.

275. Pisoni RL, Bragg-Gresham JL, Young EW*, et al.* Anemia management and outcomes from 12 countries in the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Am J Kidney Dis* 2004;44(1):94-111.

276. Lu WX, Jones-Burton C, Zhan M*, et al.* Survival benefit of recombinant human erythropoietin administration prior to onset of end-stage renal disease: variations across surrogates for quality of care and time. *Nephron Clin Pract* 2005;101(2):c79-86.

277. Wolfe RA, Hulbert-Shearon TE, Ashby VB*, et al.* Improvements in dialysis patient mortality are associated with improvements in urea reduction ratio and hematocrit, 1999 to 2002. *Am J Kidney Dis* 2005;45(1):127-35.

278. Messana JM, Chuang CC, Turenne M*, et al.* Association of quarterly average achieved hematocrit with mortality in dialysis patients: a time-dependent comorbidity-adjusted model. *Am J Kidney Dis* 2009;53(3):503-12.

279. Robinson BM, Joffe MM, Berns JS*, et al.* Anemia and mortality in hemodialysis patients: accounting for morbidity and treatment variables updated over time. *Kidney Int* 2005;68(5):2323-30.

280. Ofsthun N, Labrecque J, Lacson E*, et al.* The effects of higher hemoglobin levels on mortality and hospitalization in hemodialysis patients. *Kidney Int* 2003;63(5):1908-14.

281. Li S, Collins AJ. Association of hematocrit value with cardiovascular morbidity and mortality in incident hemodialysis patients. *Kidney Int* 2004;65(2):626-33.

282. Xia H, Ebben J, Ma JZ*, et al.* Hematocrit levels and hospitalization risks in hemodialysis patients. *J Am Soc Nephrol* 1999;10(6):1309-16.

283. Gilbertson DT, Ebben JP, Foley RN*, et al.* Hemoglobin level variability: associations with mortality. *Clin J Am Soc Nephrol* 2008;3(1):133-8.

284. Besarab A, Bolton WK, Browne JK*, et al.* The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. *N Engl J Med* 1998;339(9):584-90.

285. Clement FM, Klarenbach S, Tonelli M*, et al.* The impact of selecting a high hemoglobin target level on health-related quality of life for patients with chronic kidney disease: a systematic review and meta-analysis. *Arch Intern Med* 2009;169(12):1104-12.

286. Phrommintikul A, Haas SJ, Elsik M*, et al.* Mortality and target haemoglobin concentrations in anaemic patients with chronic kidney disease treated with erythropoietin: a meta-analysis. *Lancet* 2007;369(9559):381-8.

287. Strippoli GF, Craig JC, Manno C*, et al.* Hemoglobin targets for the anemia of chronic kidney disease: a meta-analysis of randomized, controlled trials. *J Am Soc Nephrol* 2004;15(12):3154-65.

288. Palmer SC, Navaneethan SD, Craig JC*, et al.* Meta-analysis: erythropoiesis-stimulating agents in patients with chronic kidney disease. *Ann Intern Med* 2010;153(1):23-33.

289. Volkova N, Arab L. Evidence-based systematic literature review of hemoglobin/hematocrit and all-cause mortality in dialysis patients. *Am J Kidney Dis* 2006;47(1):24-36.

290. Raine AE. Hypertension, blood viscosity, and cardiovascular morbidity in renal failure: implications of erythropoietin therapy. *Lancet* 1988;1(8577):97-100.

291. Goodkin DA, Fuller DS, Robinson BM*, et al.* Naturally occurring higher hemoglobin concentration does not increase mortality among hemodialysis patients. *J Am Soc Nephrol* 2011;22(2):358-65.

292. Erslev AJ. Erythropoietin. *N Engl J Med* 1991;324(19):1339-44.

293. Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. *Br J Haematol* 2008;141(1):14-31.

294. Fishbane S, Besarab A. Mechanism of increased mortality risk with erythropoietin treatment to higher hemoglobin targets. *Clin J Am Soc Nephrol* 2007;2(6):1274-82.

295. Haller H, Christel C, Dannenberg L*, et al.* Signal transduction of erythropoietin in endothelial cells. *Kidney Int* 1996;50(2):481-8.

296. Ammarguellat F, Llovera M, Kelly PA*, et al.* Low doses of EPO activate MAP kinases but not JAK2-STAT5 in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 2001;284(4):1031-8.

297. Desai A, Zhao Y, Lankford HA*, et al.* Nitric oxide suppresses EPO-induced monocyte chemoattractant protein-1 in endothelial cells: implications for atherogenesis in chronic renal disease. *Lab Invest* 2006;86(4):369-79.

298. De Marchi S, Cecchin E, Falleti E*, et al.* Long-term effects of erythropoietin therapy on fistula stenosis and plasma concentrations of PDGF and MCP-1 in hemodialysis patients. *J Am Soc Nephrol* 1997;8(7):1147-56.

299. Aguilera A, Selgas R, Ruiz-Caravaca ML*, et al.* Effects of recombinant human erythropoietin on functional and injury endothelial markers in peritoneal dialysis patients. *Perit Dial Int* 1999;19 Suppl 2:S161-6.

300. Rodrigue ME, Moreau C, Lariviere R*, et al.* Relationship between eicosanoids and endothelin-1 in the pathogenesis of erythropoietin-induced hypertension in uremic rats. *J Cardiovasc Pharmacol* 2003;41(3):388-95.

301. Takahashi K, Totsune K, Imai Y*, et al.* Plasma concentrations of immunoreactive-endothelin in patients with chronic renal failure treated with recombinant human erythropoietin. *Clin Sci* 1993;84(1):47-50.

302. Barrett JD, Zhang Z, Zhu JH*, et al.* Erythropoietin upregulates angiotensin receptors in cultured rat vascular smooth muscle cells. *J Hypertens* 1998;16(12 Pt 1):1749-57.

303. Ferrucci L, Guralnik JM, Woodman RC*, et al.* Proinflammatory state and circulating erythropoietin in persons with and without anemia. *Am J Med* 2005;118(11):1288.

304. Keithi-Reddy SR, Addabbo F, Patel TV*, et al.* Association of anemia and erythropoiesis stimulating agents with inflammatory biomarkers in chronic kidney disease. *Kidney Int* 2008;74(6):782-90.

305. Baune BT, Rothermundt M, Ladwig KH*, et al.* Systemic inflammation (Interleukin 6) predicts all-cause mortality in men: results from a 9-year follow-up of the MEMO Study. *Age* 2011;33(2):209-17.

306. Haddy N, Sass C, Droesch S*, et al.* IL-6, TNF-alpha and atherosclerosis risk indicators in a healthy family population: the STANISLAS cohort. *Atherosclerosis* 2003;170(2):277-83.

307. Fort J, Cuevas X, Garcia F*, et al.* Mortality in incident haemodialysis patients: time-dependent haemoglobin levels and erythropoiesis-stimulating agent dose are independent predictive factors in the ANSWER study. *Nephrol Dial Transplant* 2010;25(8):2702-10.

308. McMahon LP, Cai MX, Baweja S*, et al.* Mortality in dialysis patients may not be associated with ESA dose: a 2-year prospective observational study. *BMC Nephrol* 2012;13(1):40.

309. Cotter D, Zhang Y, Thamer M*, et al.* The effect of epoetin dose on hematocrit. *Kidney Int* 2008;73(3):347-53.

310. Bradbury BD, Wang O, Critchlow CW*, et al.* Exploring relative mortality and epoetin alfa dose among hemodialysis patients. *Am J Kidney Dis* 2008;51(1):62-70.

311. Cotter DJ, Stefanik K, Zhang Y*, et al.* Hematocrit was not validated as a surrogate end point for survival among epoetin-treated hemodialysis patients. *J Clin Epidemiol* 2004;57(10):1086-95.

312. Kilpatrick RD, Critchlow CW, Fishbane S*, et al.* Greater epoetin alfa responsiveness is associated with improved survival in hemodialysis patients. *Clin J Am Soc Nephrol* 2008;3(4):1077-83.

313. Strippoli GF. Effects of the dose of erythropoiesis stimulating agents on cardiovascular events, quality of life, and health-related costs in hemodialysis patients: the clinical evaluation of the dose of erythropoietins (C.E. DOSE) trial protocol. *Trials* 2010;11:70.

314. Stryer L. *Biochemistry*. 4th ed: W.H. Freeman; 1995.

315. Papa S, Skulachev VP. Reactive oxygen species, mitochondria, apoptosis and aging. *Mol Cell Biochem* 1997;174(1-2):305-19.

316. Portaluppi F, Boari B, Manfredini R. Oxidative stress in essential hypertension. *Curr Pharm Des* 2004;10(14):1695-8.

317. Andrew PJ, Mayer B. Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 1999;43(3):521-31.

318. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann Bot* 2003;91 Spec No:179-94.

319. Abuja PM, Albertini R. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. *Clinica Chimica Acta* 2001;306(1-2):1-17.

320. Cutler RG. Oxidative stress profiling: part I. Its potential importance in the optimization of human health. *Ann N Y Acad Sci* 2005;1055:93-135.

321. Cutler RG, Plummer J, Chowdhury K*, et al.* Oxidative stress profiling: part II. Theory, technology, and practice. *Ann N Y Acad Sci* 2005;1055:136-58.

322. Pryor WA. Oxidative stress status-the second set. *Free Radic Biol Med* 2000;28(4):503-504.

323. Pryor WA. Oxidative stress status: OSS, BOSS, and "Wild Bill" Donovan. *Free Radic Biol Med* 1999;27(11-12):1135-6.

324. Locatelli F, Canaud B, Eckardt K-U*, et al.* Oxidative stress in end-stage renal disease: an emerging threat to patient outcome. *Nephrol Dial Transplant* 2003;18(7):1272-80.

325. Malecka-Massalska T, Wladysiuk M, Ksiazek A. *A Systematic Review of the Effect of Vitamin C Infusion and Vitamin E-Coated Membrane on Hemodialysis-Induced Oxidative Stress*. Hemodialysis - Different Aspects, Maria Goretti Penido (Ed.). Available from: http://www.intechopen.com/books/hemodialysis-different-aspects/a-systematic-review-of-the-effect-of-vitamin-c-infusion-and-vitamin-e-coated-membrane-on-hemodialysi (2011-11-14; date last accessed).

326. Coskun C, Kural A, Doventas Y*, et al.* Hemodialysis and protein oxidation products. *Ann N Y Acad Sci* 2007;1100:404-8.

327. Handelman GJ, Walter MF, Adhikarla R*, et al.* Elevated plasma F2-isoprostanes in patients on long-term hemodialysis. *Kidney Int* 2001;59(5):1960-6.

328. Himmelfarb J, McMonagle E, McMenamin E. Plasma protein thiol oxidation and carbonyl formation in chronic renal failure. *Kidney Int* 2000;58(6):2571-8.

329. Himmelfarb J, McMenamin E, McMonagle E. Plasma aminothiol oxidation in chronic hemodialysis patients. *Kidney Int* 2002;61(2):705-16.

330. Chen MF, Chang CL, Liou SY. Increase in resting levels of superoxide anion in the whole blood of uremic patients on chronic hemodialysis. *Blood Purif* 1998;16(5):290-300.

331. Odetti P, Garibaldi S, Gurreri G*, et al.* Protein oxidation in hemodialysis and kidney transplantation. *Metabolism* 1996;45(11):1319-22.

332. Nguyen-Khoa T, Massy ZA, De Bandt JP*, et al.* Oxidative stress and haemodialysis: role of inflammation and duration of dialysis treatment. *Nephrol Dial Transplant* 2001;16(2):335-40.

333. Himmelfarb J, McMenamin ME, Loseto G*, et al.* Myeloperoxidase-catalyzed 3-chlorotyrosine formation in dialysis patients. *Free Radic Biol Med* 2001;31(10):1163-9.

334. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C*, et al.* Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int* 1996;49(5):1304-13.

335. Loughrey CM, Young IS, Lightbody JH*, et al.* Oxidative stress in haemodialysis. *QJM* 1994;87(11):679-83.

336. Kose K, Dogan P, Gunduz Z*, et al.* Oxidative stress in hemodialyzed patients and the long-term effects of dialyzer reuse practice. *Clin Biochem* 1997;30(8):601-6.

337. Castoldi G, Antolini L, Bombardi C*, et al.* Oxidative stress biomarkers and chromogranin A in uremic patients: effects of dialytic treatment. *Clin Biochem* 2010;43(18):1387-92.

338. Karamouzis I, Sarafidis PA, Karamouzis M*, et al.* Increase in oxidative stress but not in antioxidant capacity with advancing stages of chronic kidney disease. *Am J Nephrol* 2008;28(3):397-404.

339. Dounousi E, Papavasiliou E, Makedou A*, et al.* Oxidative stress is progressively enhanced with advancing stages of CKD. *Am J Kidney Dis* 2006;48(5):752-60.

340. Haffner SM, Agil A, Mykkanen L*, et al.* Plasma oxidizability in subjects with normal glucose tolerance, impaired glucose tolerance, and NIDDM. *Diabetes Care* 1995;18(5):646-53.

341. Nourooz-Zadeh J, Rahimi A, Tajaddini-Sarmadi J*, et al.* Relationships between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologia* 1997;40(6):647-53.

342. Pandey KB, Rizvi SI. Markers of oxidative stress in erythrocytes and plasma during aging in humans. *Oxid Med Cell Longev* 2010;3(1):2-12.

343. Romano AD, Serviddio G, de Matthaeis A*, et al.* Oxidative stress and aging. *J Nephrol* 2010;23 Suppl 15:S29-36.

344. Peuchant E, Carbonneau MA, Dubourg L*, et al.* Lipoperoxidation in plasma and red blood cells of patients undergoing haemodialysis: vitamins A, E, and iron status. *Free Radic Biol Med* 1994;16(3):339-46.

345. Wu CC, Chen JS, Wu WM*, et al.* Myeloperoxidase serves as a marker of oxidative stress during single haemodialysis session using two different biocompatible dialysis membranes. *Nephrol Dial Transplant* 2005;20(6):1134-9.

346. Jackson P, Loughrey CM, Lightbody JH*, et al.* Effect of hemodialysis on total antioxidant capacity and serum antioxidants in patients with chronic renal failure. *Clin Chem* 1995;41(8 Pt 1):1135-8.

347. Klein JB, McLeish KR, Ward RA. Transplantation, not dialysis, corrects azotemia-dependent priming of the neutrophil oxidative burst. *Am J Kidney Dis* 1999;33(3):483-91.

348. Paul JL, Roch-Arveiller M, Man NK*, et al.* Influence of uremia on polymorphonuclear leukocytes oxidative metabolism in end-stage renal disease and dialyzed patients. *Nephron* 1991;57(4):428-32.

349. Ward RA, McLeish KR. Polymorphonuclear leukocyte oxidative burst is enhanced in patients with chronic renal insufficiency. *J Am Soc Nephrol* 1995;5(9):1697-702.

350. Rhee MS, McGoldrick MD, Meuwissen HJ. Serum factor from patients with chronic renal failure enhances polymorphonuclear leukocyte oxidative metabolism. *Nephron* 1986;42(1):6-13.

351. Ward RA, Ouseph R, McLeish KR. Effects of high-flux hemodialysis on oxidant stress. *Kidney Int* 2003;63(1):353-9.

352. Ward RA, McLeish KR. Oxidant stress in hemodialysis patients: what are the determining factors? *Artif Organs* 2003;27(3):230-6.

353. Cristol JP, Canaud B, Rabesandratana H*, et al.* Enhancement of reactive oxygen species production and cell surface markers expression due to haemodialysis. *Nephrol Dial Transplant* 1994;9(4):389-94.

354. Nguyen AT, Lethias C, Zingraff J*, et al.* Hemodialysis membrane-induced activation of phagocyte oxidative metabolism detected in vivo and in vitro within microamounts of whole blood. *Kidney Int* 1985;28(2):158-67.

355. Wanner C, Bahner U, Mattern R*, et al.* Effect of dialysis flux and membrane material on dyslipidaemia and inflammation in haemodialysis patients. *Nephrol Dial Transplant* 2004;19(10):2570-5.

356. Van Epps DE, Garcia ML. Enhancement of neutrophils function as a result of prior exposure to chemotactic factor. *J Clin Invest* 1980;66(2):167-75.

357. Webster RO, Hong SR, Johnston RB, Jr.*, et al.* Biologial effects of the human complement fragments C5a and C5ades Arg on neutrophil function. *Immunopharmacology* 1980;2(3):201-19.

358. Morena M, Cristol JP, Senecal L*, et al.* Oxidative stress in hemodialysis patients: is NADPH oxidase complex the culprit? *Kidney Int Suppl* 2002(80):109-14.

359. DeLeo FR, Renee J, McCormick S*, et al.* Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J Clin Invest* 1998;101(2):455-63.

360. Kumano K, Yokota S, Nanbu M*, et al.* Do cytokine-inducing substances penetrate through dialysis membranes and stimulate monocytes? *Kidney Int Suppl* 1993;41:S205-8.

361. Nockher WA, Scherberich JE. Monocyte cell-surface CD14 expression and soluble CD14 antigen in hemodialysis: evidence for chronic exposure to LPS. *Kidney Int* 1995;48(5):1469-76.

362. McIntyre CW, Harrison LE, Eldehni MT*, et al.* Circulating endotoxemia: a novel factor in systemic inflammation and cardiovascular disease in chronic kidney disease. *Clin J Am Soc Nephrol* 2011;6(1):133-41.

363. Mehmetoglu I, Yerlikaya FH, Kurban S*, et al.* Oxidative stress markers in hemodialysis and peritoneal dialysis patients, including coenzyme Q10 and ischemia-modified albumin. *Int J Artif Organs* 2012;35(3):226-32.

364. Salahudeen AK, Oliver B, Bower JD*, et al.* Increase in plasma esterified F2-isoprostanes following intravenous iron infusion in patients on hemodialysis. *Kidney Int* 2001;60(4):1525-31.

365. Mimic-Oka J, Savic-Radojevic A, Pljesa-Ercegovac M*, et al.* Evaluation of oxidative stress after repeated intravenous iron supplementation. *Ren Fail* 2005;27(3):345-51.

366. Lim PS, Wei YH, Yu YL*, et al.* Enhanced oxidative stress in haemodialysis patients receiving intravenous iron therapy. *Nephrol Dial Transplant* 1999;14(11):2680-7.

367. Zanen AL, Adriaansen HJ, van Bommel EF*, et al.* 'Oversaturation' of transferrin after intravenous ferric gluconate (Ferrlecit(R)) in haemodialysis patients. *Nephrol Dial Transplant* 1996;11(5):820-4.

368. Roob JM, Khoschsorur G, Tiran A*, et al.* Vitamin E attenuates oxidative stress induced by intravenous iron in patients on hemodialysis. *J Am Soc Nephrol* 2000;11(3):539-49.

369. Lahera V, Goicoechea M, de Vinuesa SG*, et al.* Oxidative stress in uremia: the role of anemia correction. *J Am Soc Nephrol* 2006;17(12 Suppl 3):S174-7.

370. Sutton HC. Efficiency of chelated iron compounds as catalysts for the Haber-Weiss reaction. *J Free Radic Biol Med* 1985;1(3):195-202.

371. Koppenol WH. The Haber-Weiss cycle--70 years later. *Redox Rep* 2001;6(4):229-34.

372. Aronoff GR. Safety of intravenous iron in clinical practice: implications for anemia management protocols. *J Am Soc Nephrol* 2004;15 Suppl 2:S99-106.

373. Auerbach M, Ballard H. Clinical use of intravenous iron: administration, efficacy, and safety. *Hematology Am Soc Hematol Educ Program* 2010;2010:338-47.

374. Kumbasar A, Gursu M, Kaya C*, et al.* The effect of different doses and types of intravenous iron on oxidative stress and inflammation in hemodialysis patients. *J Nephrol* 2012;25(5):825-32.

375. Chen HC, Tsai JC, Tsai JH*, et al.* Recombinant human erythropoietin enhances superoxide production by FMLP-stimulated polymorphonuclear leukocytes in hemodialysis patients. *Kidney Int* 1997;52(5):1390-4.

376. Turi S, Nemeth I, Varga I*, et al.* The effect of erythropoietin on the cellular defence mechanism of red blood cells in children with chronic renal failure. *Pediatr Nephrol* 1992;6(6):536-41.

377. Delmas-Beauvieux MC, Combe C, Peuchant E*, et al.* Evaluation of red blood cell lipoperoxidation in hemodialysed patients during erythropoietin therapy supplemented or not with iron. *Nephron* 1995;69(4):404-10.

378. Mimic-Oka J, Simic T, Ekmescic V*, et al.* Erythrocyte glutathione peroxidase and superoxide dismutase activities in different stages of chronic renal failure. *Clin Nephrol* 1995;44(1):44-8.

379. Ceballos-Picot I, Witko-Sarsat V, Merad-Boudia M*, et al.* Glutathione antioxidant system as a marker of oxidative stress in chronic renal failure. *Free Radic Biol Med* 1996;21(6):845-53.

380. Klemm A, Voigt C, Friedrich M*, et al.* Determination of erythrocyte antioxidant capacity in haemodialysis patients using electron paramagnetic resonance. *Nephrol Dial Transplant* 2001;16(11):2166-71.

381. Yawata Y, Jacob HS. Abnormal red cell metabolism in patients with chronic uremia: Nature of the defect and its persistence despite adequate hemodialysis. *Blood* 1975;45(2):231-9.

382. Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. *Arch Biochem Biophys* 1990;280(1):1-8.

383. Suliman ME, Anderstam B, Lindholm B*, et al.* Total, free, and protein-bound sulphur amino acids in uraemic patients. *Nephrol Dial Transplant* 1997;12(11):2332-8.

384. Hultberg B, Andersson A, Arnadottir M. Reduced, free and total fractions of homocysteine and other thiol compounds in plasma from patients with renal failure. *Nephron* 1995;70(1):62-7.

385. Chauveau P, Chadefaux B, Coude M*, et al.* Hyperhomocysteinemia, a risk factor for atherosclerosis in chronic uremic patients. *Kidney Int Suppl* 1993;41:S72-7.

386. Himmelfarb J, McMonagle E. Albumin is the major plasma protein target of oxidant stress in uremia. *Kidney Int* 2001;60(1):358-63.

387. Soriani M, Pietraforte D, Minetti M. Antioxidant potential of anaerobic human plasma: role of serum albumin and thiols as scavengers of carbon radicals. *Arch Biochem Biophys* 1994;312(1):180-8.

388. Soejima A, Matsuzawa N, Miyake N*, et al.* Hypoalbuminemia accelerates erythrocyte membrane lipid peroxidation in chronic hemodialysis patients. *Clin Nephrol* 1999;51(2):92-7.

389. Himmelfarb J, Kane J, McMonagle E*, et al.* Alpha and gamma tocopherol metabolism in healthy subjects and patients with end-stage renal disease. *Kidney Int* 2003;64(3):978-91.

390. Hultqvist M, Hegbrant J, Nilsson-Thorell C*, et al.* Plasma concentrations of vitamin C, vitamin E and/or malondialdehyde as markers of oxygen free radical production during hemodialysis. *Clin Nephrol* 1997;47(1):37-46.

391. Montazerifar F, Hashemi M, Karajibani M*, et al.* Hemodialysis alters lipid profiles, total antioxidant capacity, and vitamins A, E, and C concentrations in humans. *J Med Food* 2010;13(6):1490-3.

392. Maggi E, Bellazzi R, Falaschi F*, et al.* Enhanced LDL oxidation in uremic patients: an additional mechanism for accelerated atherosclerosis? *Kidney Int* 1994;45(3):876-83.

393. Nakayama H, Akiyama S, Inagaki M*, et al.* Dehydroascorbic acid and oxidative stress in haemodialysis patients. *Nephrol Dial Transplant* 2001;16(3):574-9.

394. Morena M, Cristol JP, Bosc JY*, et al.* Convective and diffusive losses of vitamin C during haemodiafiltration session: a contributive factor to oxidative stress in haemodialysis patients. *Nephrol Dial Transplant* 2002;17(3):422-7.

395. Cavdar C, Camsari T, Semin I*, et al.* Lipid peroxidation and antioxidant activity in chronic haemodialysis patients treated with recombinant human erythropoietin. *Scand J Urol Nephrol* 1997;31(4):371-5.

396. Boaz M, Matas Z, Biro A*, et al.* Serum malondialdehyde and prevalent cardiovascular disease in hemodialysis. *Kidney Int* 1999;56(3):1078-83.

397. Boaz M, Matas Z, Biro A*, et al.* Comparison of hemostatic factors and serum malondialdehyde as predictive factors for cardiovascular disease in hemodialysis patients. *Am J Kidney Dis* 1999;34(3):438-44.

398. Shoji T, Fukumoto M, Kimoto E*, et al.* Antibody to oxidized low-density lipoprotein and cardiovascular mortality in end-stage renal disease. *Kidney Int* 2002;62(6):2230-7.

399. Bayes B, Pastor MC, Bonal J*, et al.* Oxidative stress, inflammation and cardiovascular mortality in haemodialysis--role of seniority and intravenous ferrotherapy: analysis at 4 years of follow-up. *Nephrol Dial Transplant* 2006;21(4):984-90.

400. Zhang R, Brennan ML, Fu X*, et al.* Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 2001;286(17):2136-42.

401. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 1993;91(6):2546-51.

402. Burke A, Fitzgerald GA. Oxidative stress and smoking-induced vascular injury. *Prog Cardiovasc Dis* 2003;46(1):79-90.

403. Raij L, DeMaster EG, Jaimes EA. Cigarette smoke-induced endothelium dysfunction: role of superoxide anion. *J Hypertens* 2001;19(5):891-7.

404. Dursun B, Dursun E, Suleymanlar G*, et al.* Carotid artery intima-media thickness correlates with oxidative stress in chronic haemodialysis patients with accelerated atherosclerosis. *Nephrol Dial Transplant* 2008;23(5):1697-703.

405. Dursun B, Dursun E, Suleymanlar G*, et al.* The effect of hemodialysis on accelerated atherosclerosis in diabetic patients: correlation of carotid artery intima-media thickness with oxidative stress. *J Diabetes Complications* 2009;23(4):257-64.

406. Honda H, Ueda M, Kojima S*, et al.* Assessment of myeloperoxidase and oxidative alpha1-antitrypsin in patients on hemodialysis. *Clin J Am Soc Nephrol* 2009;4(1):142-51.

407. Suwaidi JA, Hamasaki S, Higano ST*, et al.* Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 2000;101(9):948-54.

408. Annuk M, Zilmer M, Lind L*, et al.* Oxidative stress and endothelial function in chronic renal failure. *J Am Soc Nephrol* 2001;12(12):2747-52.

409. Munzel T, Sinning C, Post F*, et al.* Pathophysiology, diagnosis and prognostic implications of endothelial dysfunction. *Ann Med* 2008;40(3):180-96.

410. Moro MA, Darley-Usmar VM, Goodwin DA*, et al.* Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc Natl Acad Sci U S A* 1994;91(14):6702-6.

411. Beckman JS, Beckman TW, Chen J*, et al.* Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990;87(4):1620-4.

412. Steinberg D, Parthasarathy S, Carew TE*, et al.* Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 1989;320(14):915-24.

413. Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet* 1994;344(8925):793-5.

414. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 1996;20(5):707-27.

415. Westhuyzen J. The oxidation hypothesis of atherosclerosis: an update. *Ann Clin Lab Sci* 1997;27(1):1-10.

416. Dasgupta A, Hussain S, Ahmad S. Increased lipid peroxidation in patients on maintenance hemodialysis. *Nephron* 1992;60(1):56-9.

417. Samouilidou E, Karpouza A, Grapsa E*, et al.* Serum oxidized LDL is inversely associated with HDL2-cholesterol subclass in renal failure patients on hemodialysis. *Nephron Clin Pract* 2010;115(4):c289-94.

418. Samouilidou EC, Karpouza AP, Kostopoulos V*, et al.* Lipid abnormalities and oxidized LDL in chronic kidney disease patients on hemodialysis and peritoneal dialysis. *Ren Fail* 2012;34(2):160-4.

419. Ando M, Lundkvist I, Bergstrom J*, et al.* Enhanced scavenger receptor expression in monocyte-macrophages in dialysis patients. *Kidney Int* 1996;49(3):773-80.

420. Vogiatzi G, Tousoulis D, Stefanadis C. The role of oxidative stress in atherosclerosis. *Hellenic J Cardiol* 2009;50(5):402-9.

421. Dalfino G, Simone S, Porreca S*, et al.* Bone morphogenetic protein-2 may represent the molecular link between oxidative stress and vascular stiffness in chronic kidney disease. *Atherosclerosis* 2010;211(2):418-23.

422. Byon CH, Javed A, Dai Q*, et al.* Oxidative stress induces vascular calcification through modulation of the osteogenic transcription factor Runx2 by AKT signaling. *J Biol Chem* 2008;283(22):15319-27.

423. Danielski M, Ikizler TA, McMonagle E*, et al.* Linkage of hypoalbuminemia, inflammation, and oxidative stress in patients receiving maintenance hemodialysis therapy. *Am J Kidney Dis* 2003;42(2):286-94.

424. Ikizler TA, Morrow JD, Roberts LJ*, et al.* Plasma F2-isoprostane levels are elevated in chronic hemodialysis patients. *Clin Nephrol* 2002;58(3):190-7.

425. Mezzano D, Pais EO, Aranda E*, et al.* Inflammation, not hyperhomocysteinemia, is related to oxidative stress and hemostatic and endothelial dysfunction in uremia. *Kidney Int* 2001;60(5):1844-50.

426. Podrez EA, Abu-Soud HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med* 2000;28(12):1717-25.

427. Hu ML, Louie S, Cross CE*, et al.* Antioxidant protection against hypochlorous acid in human plasma. *J Lab Clin Med* 1993;121(2):257-62.

428. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999;340(6):448-54.

429. Bologa RM, Levine DM, Parker TS*, et al.* Interleukin-6 predicts hypoalbuminemia, hypocholesterolemia, and mortality in hemodialysis patients. *Am J Kidney Dis* 1998;32(1):107-14.

430. Veneskoski M, Turunen SP, Kummu O*, et al.* Specific recognition of malondialdehyde and malondialdehyde acetaldehyde adducts on oxidized LDL and apoptotic cells by complement anaphylatoxin C3a. *Free Radic Biol Med* 2011;51(4):834-43.

431. Vogt W, Damerau B, von Zabern I*, et al.* Non-enzymic activation of the fifth component of human complement, by oxygen radicals. Some properties of the activation product, C5b-like C5. *Mol Immunol* 1989;26(12):1133-42.

432. Collard CD, Agah A, Stahl GL. Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF-kappaB and new protein synthesis. *Immunopharmacology* 1998;39(1):39-50.

433. Kilgore KS, Homeister JW, Satoh PS*, et al.* Sulfhydryl compounds, captopril, and MPG inhibit complement-mediated myocardial injury. *Am J Physiol* 1994;266(1 Pt 2):H28-35.

434. Iseki K, Tozawa M, Yoshi S*, et al.* Serum C-reactive protein (CRP) and risk of death in chronic dialysis patients. *Nephrol Dial Transplant* 1999;14(8):1956-60.

435. Zimmermann J, Herrlinger S, Pruy A*, et al.* Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. *Kidney Int* 1999;55(2):648-58.

436. Kimmel PL, Phillips TM, Simmens SJ*, et al.* Immunologic function and survival in hemodialysis patients. *Kidney Int* 1998;54(1):236-44.

437. Stenvinkel P, Wanner C, Metzger T*, et al.* Inflammation and outcome in end-stage renal failure: does female gender constitute a survival advantage? *Kidney Int* 2002;62(5):1791-8.

438. Reddan DN, Klassen PS, Szczech LA*, et al.* White blood cells as a novel mortality predictor in haemodialysis patients. *Nephrol Dial Transplant* 2003;18(6):1167-73.

439. Panichi V, Maggiore U, Taccola D*, et al.* Interleukin-6 is a stronger predictor of total and cardiovascular mortality than C-reactive protein in haemodialysis patients. *Nephrol Dial Transplant* 2004;19(5):1154-60.

440. Bazeley J, Bieber B, Li Y*, et al.* C-reactive protein and prediction of 1-year mortality in prevalent hemodialysis patients. *Clin J Am Soc Nephrol* 2011;6(10):2452-61.

441. Qureshi AR, Alvestrand A, Divino-Filho JC*, et al.* Inflammation, malnutrition, and cardiac disease as predictors of mortality in hemodialysis patients. *J Am Soc Nephrol* 2002;13 Suppl 1:S28-36.

442. Kawaguchi T, Tong L, Robinson BM*, et al.* C-reactive protein and mortality in hemodialysis patients: the Dialysis Outcomes and Practice Patterns Study (DOPPS). *Nephron Clin Pract* 2011;117(2):c167-78.

443. Xue JL, Dahl D, Ebben JP*, et al.* The association of initial hemodialysis access type with mortality outcomes in elderly Medicare ESRD patients. *Am J Kidney Dis* 2003;42(5):1013-9.

444. Ayus JC, Sheikh-Hamad D. Silent infection in clotted hemodialysis access grafts. *J Am Soc Nephrol* 1998;9(7):1314-7.

445. Stenvinkel P, Heimburger O, Jogestrand T*, et al.* Does persistent infection with Chlamydia pneumoniae increase the risk of atherosclerosis in chronic renal failure? *Kidney Int* 1999;55(6):2531-2.

446. Haubitz M, Brunkhorst R. C-reactive protein and chronic Chlamydia pneumoniae infection--long-term predictors for cardiovascular disease and survival in patients on peritoneal dialysis. *Nephrol Dial Transplant* 2001;16(4):809-15.

447. Zoccali C, Benedetto FA, Mallamaci F*, et al.* Inflammation is associated with carotid atherosclerosis in dialysis patients. Creed Investigators. Cardiovascular Risk Extended Evaluation in Dialysis Patients. *J Hypertens* 2000;18(9):1207-13.

448. Rahmati MA, Craig RG, Homel P*, et al.* Serum markers of periodontal disease status and inflammation in hemodialysis patients. *Am J Kidney Dis* 2002;40(5):983-9.

449. Craig RG, Spittle MA, Levin NW. Importance of periodontal disease in the kidney patient. *Blood Purif* 2002;20(1):113-9.

450. Lopez-Gomez JM, Perez-Flores I, Jofre R*, et al.* Presence of a failed kidney transplant in patients who are on hemodialysis is associated with chronic inflammatory state and erythropoietin resistance. *J Am Soc Nephrol* 2004;15(9):2494-501.

451. Niebauer J, Volk HD, Kemp M*, et al.* Endotoxin and immune activation in chronic heart failure: a prospective cohort study. *Lancet* 1999;353(9167):1838-42.

452. Perl J, Wald R, McFarlane P*, et al.* Hemodialysis vascular access modifies the association between dialysis modality and survival. *J Am Soc Nephrol* 2011;22(6):1113-21.

453. Leavey SF, Strawderman RL, Young EW*, et al.* Cross-sectional and longitudinal predictors of serum albumin in hemodialysis patients. *Kidney Int* 2000;58(5):2119-28.

454. Kaysen GA. Biochemistry and biomarkers of inflamed patients: why look, what to assess. *Clin J Am Soc Nephrol* 2009;4 Suppl 1:S56-63.

455. Allon M, Daugirdas J, Depner TA*, et al.* Effect of change in vascular access on patient mortality in hemodialysis patients. *Am J Kidney Dis* 2006;47(3):469-77.

456. Hung A, Pupim L, Yu C*, et al.* Determinants of C-reactive protein in chronic hemodialysis patients: relevance of dialysis catheter utilization. *Hemodial Int* 2008;12(2):236-43.

457. Ishani A, Collins AJ, Herzog CA*, et al.* Septicemia, access and cardiovascular disease in dialysis patients: the USRDS Wave 2 study. *Kidney Int* 2005;68(1):311-8.

458. Foley RN, Guo H, Snyder JJ*, et al.* Septicemia in the United States dialysis population, 1991 to 1999. *J Am Soc Nephrol* 2004;15(4):1038-45.

459. Allon M. Dialysis catheter-related bacteremia: treatment and prophylaxis. *Am J Kidney Dis* 2004;44(5):779-91.

460. Percival SL, Kite P. Intravascular catheters and biofilm control. *J Vasc Access* 2007;8(2):69-80.

461. Kanaa M, Wright MJ, Sandoe JA. Examination of tunnelled haemodialysis catheters using scanning electron microscopy. *Clin Microbiol Infect* 2010;16(6):780-6.

462. van Riemsdijk-van Overbeeke IC, Baan CC, Hesse CJ*, et al.* TNF-alpha: mRNA, plasma protein levels and soluble receptors in patients on chronic hemodialysis, on CAPD and with end-stage renal failure. *Clin Nephrol* 2000;53(2):115-23.

463. Descamps-Latscha B, Herbelin A, Nguyen AT*, et al.* Balance between IL-1 beta, TNF-alpha, and their specific inhibitors in chronic renal failure and maintenance dialysis. Relationships with activation markers of T cells, B cells, and monocytes. *J Immunol* 1995;154(2):882-92.

464. Brockhaus M, Bar-Khayim Y, Gurwicz S*, et al.* Plasma tumor necrosis factor soluble receptors in chronic renal failure. *Kidney Int* 1992;42(3):663-7.

465. Oppermann M, Kurts C, Zierz R*, et al.* Elevated plasma levels of the immunosuppressive complement fragment Ba in renal failure. *Kidney Int* 1991;40(5):939-47.

466. Schwedler S, Schinzel R, Vaith P*, et al.* Inflammation and advanced glycation end products in uremia: simple coexistence, potentiation or causal relationship? *Kidney Int Suppl* 2001;78:S32-6.

467. Meerwaldt R, Zeebregts CJ, Navis G*, et al.* Accumulation of advanced glycation end products and chronic complications in ESRD treated by dialysis. *Am J Kidney Dis* 2009;53(1):138-50.

468. Noordzij MJ, Lefrandt JD, Smit AJ. Advanced glycation end products in renal failure: an overview. *J Ren Care* 2008;34(4):207-12.

469. Vlassara H, Brownlee M, Manogue KR*, et al.* Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science* 1988;240(4858):1546-8.

470. Morohoshi M, Fujisawa K, Uchimura I*, et al.* Glucose-dependent interleukin 6 and tumor necrosis factor production by human peripheral blood monocytes in vitro. *Diabetes* 1996;45(7):954-9.

471. Sarnak MJ, Poindexter A, Wang SR*, et al.* Serum C-reactive protein and leptin as predictors of kidney disease progression in the Modification of Diet in Renal Disease Study. *Kidney Int* 2002;62(6):2208-15.

472. Oberg BP, McMenamin E, Lucas FL*, et al.* Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004;65(3):1009-16.

473. Haubitz M, Schulze M, Koch KM. Increase of C-reactive protein serum values following haemodialysis. *Nephrol Dial Transplant* 1990;5(7):500-3.

474. Zaoui P, Hakim RM. The effects of the dialysis membrane on cytokine release. *J Am Soc Nephrol* 1994;4(9):1711-8.

475. Panichi V, Migliori M, De Pietro S*, et al.* Plasma C-reactive protein in hemodialysis patients: a cross-sectional, longitudinal clinical survey. *Blood Purif* 2000;18(1):30-6.

476. Tielemans C, Husson C, Schurmans T*, et al.* Effects of ultrapure and non-sterile dialysate on the inflammatory response during in vitro hemodialysis. *Kidney Int* 1996;49(1):236-43.

477. Schindler R, Boenisch O, Fischer C*, et al.* Effect of the hemodialysis membrane on the inflammatory reaction in vivo. *Clin Nephrol* 2000;53(6):452-9.

478. Stenvinkel P. Inflammation in end-stage renal failure: could it be treated? *Nephrol Dial Transplant* 2002;17 Suppl 8:33-8; discussion 40.

479. Chenoweth DE. Complement activation in extracorporeal circuits. *Ann N Y Acad Sci* 1987;516:306-13.

480. Deppisch RM, Beck W, Goehl H*, et al.* Complement components as uremic toxins and their potential role as mediators of microinflammation. *Kidney Int Suppl* 2001;78:S271-7.

481. Volanakis JE. Transcriptional regulation of complement genes. *Annu Rev Immunol* 1995;13:277-305.

482. McGrath FD, Brouwer MC, Arlaud GJ*, et al.* Evidence that complement protein C1q interacts with C-reactive protein through its globular head region. *J Immunol* 2006;176(5):2950-7.

483. Volanakis JE. Complement activation by C-reactive protein complexes. *Ann N Y Acad Sci* 1982;389:235-50.

484. Jarva H, Jokiranta TS, Hellwage J*, et al.* Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8-11. *J Immunol* 1999;163(7):3957-62.

485. Ricklin D, Hajishengallis G, Yang K*, et al.* Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010;11(9):785-97.

486. Markiewski MM, Lambris JD. The role of complement in inflammatory diseases from behind the scenes into the spotlight. *Am J Pathol* 2007;171(3):715-27.

487. Muller-Eberhard HJ. The killer molecule of complement. *J Invest Dermatol* 1985;85(1 Suppl):47s-52s.

488. Pangburn MK, Schreiber RD, Muller-Eberhard HJ. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med* 1981;154(3):856-67.

489. Bexborn F, Andersson PO, Chen H*, et al.* The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb). *Mol Immunol* 2008;45(8):2370-9.

490. Hourcade DE. Properdin and complement activation: a fresh perspective. *Curr Drug Targets* 2008;9(2):158-64.

491. Kemper C, Atkinson JP, Hourcade DE. Properdin: emerging roles of a pattern-recognition molecule. *Annu Rev Immunol* 2010;28:131-55.

492. Spitzer D, Mitchell LM, Atkinson JP*, et al.* Properdin can initiate complement activation by binding specific target surfaces and providing a platform for de novo convertase assembly. *J Immunol* 2007;179(4):2600-8.

493. Harboe M, Ulvund G, Vien L*, et al.* The quantitative role of alternative pathway amplification in classical pathway induced terminal complement activation. *Clin Exp Immunol* 2004;138(3):439-46.

494. Klos A, Tenner AJ, Johswich KO*, et al.* The role of the anaphylatoxins in health and disease. *Mol Immunol* 2009;46(14):2753-66.

495. Janeway CA, Travers P, Walport M*, et al. Immunobiology 5*. 5th ed. New York: Garland Science; 2001.

496. Cicardi M, Zingale L, Zanichelli A*, et al.* C1 inhibitor: molecular and clinical aspects. *Springer Semin Immunopathol* 2005;27(3):286-98.

497. Jozsi M, Zipfel PF. Factor H family proteins and human diseases. *Trends Immunol* 2008;29(8):380-7.

498. Podack ER, Muller-Eberhard HJ. SC5b-9 complex of complement: formation of the dimeric membrane attack complex by removal of S-protein. *J Immunol* 1980;124(4):1779-83.

499. Tschopp J, French LE. Clusterin: modulation of complement function. *Clin Exp Immunol* 1994;97 Suppl 2:11-4.

500. Kolb WP, Muller-Eberhard HJ. The membrane attack mechanism of complement. Isolation and subunit composition of the C5b-9 complex. *J Exp Med* 1975;141(4):724-35.

501. Liszewski MK, Farries TC, Lublin DM*, et al.* Control of the complement system. *Adv Immunol* 1996;61:201-83.

502. Ninomiya H, Sims PJ. The human complement regulatory protein CD59 binds to the alpha-chain of C8 and to the "b"domain of C9. *J Biol Chem* 1992;267(19):13675-80.

503. Bellomo G, Lippi G, Saronio P*, et al.* Inflammation, infection and cardiovascular events in chronic hemodialysis patients: a prospective study. *J Nephrol* 2003;16(2):245-51.

504. Desai AA, Nissenson A, Chertow GM*, et al.* The relationship between laboratory-based outcome measures and mortality in end-stage renal disease: a systematic review. *Hemodial Int* 2009;13(3):347-59.

505. Honda H, Qureshi AR, Heimburger O*, et al.* Serum albumin, C-reactive protein, interleukin 6, and fetuin a as predictors of malnutrition, cardiovascular disease, and mortality in patients with ESRD. *Am J Kidney Dis* 2006;47(1):139-48.

506. deFilippi C, Wasserman S, Rosanio S*, et al.* Cardiac troponin T and C-reactive protein for predicting prognosis, coronary atherosclerosis, and cardiomyopathy in patients undergoing long-term hemodialysis. *JAMA* 2003;290(3):353-9.

507. Kaptoge S, Di Angelantonio E, Lowe G*, et al.* C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* 2010;375(9709):132-40.

508. Reynolds GD, Vance RP. C-reactive protein immunohistochemical localization in normal and atherosclerotic human aortas. *Arch Pathol Lab Med* 1987;111(3):265-9.

509. Bhakdi S, Torzewski M, Klouche M*, et al.* Complement and atherogenesis: binding of CRP to degraded, nonoxidized LDL enhances complement activation. *Arterioscler Thromb Vasc Biol* 1999;19(10):2348-54.

510. Lagrand WK, Niessen HW, Wolbink GJ*, et al.* C-reactive protein colocalizes with complement in human hearts during acute myocardial infarction. *Circulation* 1997;95(1):97-103.

511. Torzewski J, Torzewski M, Bowyer DE*, et al.* C-reactive protein frequently colocalizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. *Arterioscler Thromb Vasc Biol* 1998;18(9):1386-92.

512. Zwaka TP, Hombach V, Torzewski J. C-reactive protein-mediated low density lipoprotein uptake by macrophages: implications for atherosclerosis. *Circulation* 2001;103(9):1194-7.

513. Torzewski M, Rist C, Mortensen RF*, et al.* C-reactive protein in the arterial intima: role of C-reactive protein receptor-dependent monocyte recruitment in atherogenesis. *Arterioscler Thromb Vasc Biol* 2000;20(9):2094-9.

514. Verma S, Wang CH, Li SH*, et al.* A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. *Circulation* 2002;106(8):913-9.

515. Pasceri V, Willerson JT, Yeh ET. Direct proinflammatory effect of C-reactive protein on human endothelial cells. *Circulation* 2000;102(18):2165-8.

516. Pasceri V, Cheng JS, Willerson JT*, et al.* Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation* 2001;103(21):2531-4.

517. Schwedler SB, Amann K, Wernicke K*, et al.* Native C-reactive protein increases whereas modified C-reactive protein reduces atherosclerosis in apolipoprotein E-knockout mice. *Circulation* 2005;112(7):1016-23.

518. Gill R, Kemp JA, Sabin C*, et al.* Human C-reactive protein increases cerebral infarct size after middle cerebral artery occlusion in adult rats. *J Cereb Blood Flow Metab* 2004;24(11):1214-8.

519. Griselli M, Herbert J, Hutchinson WL*, et al.* C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction. *J Exp Med* 1999;190(12):1733-40.

520. Pepys MB. C-reactive protein is neither a marker nor a mediator of atherosclerosis. *Nat Clin Pract Nephrol* 2008;4(5):234-5.

521. Elliott P, Chambers JC, Zhang W*, et al.* Genetic Loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA* 2009;302(1):37-48.

522. Lawlor DA, Harbord RM, Timpson NJ*, et al.* The association of C-reactive protein and CRP genotype with coronary heart disease: findings from five studies with 4,610 cases amongst 18,637 participants. *PLoS One* 2008;3(8):e3011.

523. Pai JK, Mukamal KJ, Rexrode KM*, et al.* C-reactive protein (CRP) gene polymorphisms, CRP levels, and risk of incident coronary heart disease in two nested case-control studies. *PLoS One* 2008;3(1):e1395.

524. Zacho J, Tybjaerg-Hansen A, Jensen JS*, et al.* Genetically elevated C-reactive protein and ischemic vascular disease. *N Engl J Med* 2008;359(18):1897-908.

525. Taylor KE, Giddings JC, van den Berg CW. C-reactive protein-induced in vitro endothelial cell activation is an artefact caused by azide and lipopolysaccharide. *Arterioscler Thromb Vasc Biol* 2005;25(6):1225-30.

526. Pepys MB, Hawkins PN, Kahan MC*, et al.* Proinflammatory effects of bacterial recombinant human C-reactive protein are caused by contamination with bacterial products, not by C-reactive protein itself. *Circ Res* 2005;97(11):e97-103.

527. Bhagat K, Vallance P. Inflammatory cytokines impair endothelium-dependent dilatation in human veins in vivo. *Circulation* 1997;96(9):3042-7.

528. Tintut Y, Patel J, Parhami F*, et al.* Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway. *Circulation* 2000;102(21):2636-42.

529. Huber SA, Sakkinen P, Conze D*, et al.* Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 1999;19(10):2364-7.

530. Ridker PM, Rifai N, Stampfer MJ*, et al.* Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. *Circulation* 2000;101(15):1767-72.

531. Harris TB, Ferrucci L, Tracy RP*, et al.* Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. *Am J Med* 1999;106(5):506-12.

532. van Greevenbroek MM, Jacobs M, van der Kallen CJ*, et al.* Human plasma complement C3 is independently associated with coronary heart disease, but only in heavy smokers (the CODAM study). *Int J Cardiol* 2012;154(2):158-62.

533. Carter AM, Prasad UK, Grant PJ. Complement C3 and C-reactive protein in male survivors of myocardial infarction. *Atherosclerosis* 2009;203(2):538-43.

534. Ajjan R, Grant PJ, Futers TS*, et al.* Complement C3 and C-reactive protein levels in patients with stable coronary artery disease. *Thromb Haemost* 2005;94(5):1048-53.

535. Muscari A, Massarelli G, Bastagli L*, et al.* Relationship of serum C3 to fasting insulin, risk factors and previous ischaemic events in middle-aged men. *Eur Heart J* 2000;21(13):1081-90.

536. Onat A, Uzunlar B, Hergenc G*, et al.* Cross-sectional study of complement C3 as a coronary risk factor among men and women. *Clin Sci* 2005;108(2):129-35.

537. Engstrom G, Hedblad B, Berglund G*, et al.* Plasma levels of complement C3 is associated with development of hypertension: a longitudinal cohort study. *J Hum Hypertens* 2007;21(4):276-82.

538. Engstrom G, Hedblad B, Janzon L*, et al.* Complement C3 and C4 in plasma and incidence of myocardial infarction and stroke: a population-based cohort study. *Eur J Cardiovasc Prev Rehabil* 2007;14(3):392-7.

539. Szeplaki G, Prohaszka Z, Duba J*, et al.* Association of high serum concentration of the third component of complement (C3) with pre-existing severe coronary artery disease and new vascular events in women. *Atherosclerosis* 2004;177(2):383-9.

540. Muscari A, Bozzoli C, Puddu GM*, et al.* Association of serum C3 levels with the risk of myocardial infarction. *Am J Med* 1995;98(4):357-64.

541. Mellbin LG, Bjerre M, Thiel S*, et al.* Complement activation and prognosis in patients with type 2 diabetes and myocardial infarction: a report from the DIGAMI 2 trial. *Diabetes Care* 2012;35(4):911-7.

542. Speidl WS, Exner M, Amighi J*, et al.* Complement component C5a predicts future cardiovascular events in patients with advanced atherosclerosis. *Eur Heart J* 2005;26(21):2294-9.

543. Yasuda M, Takeuchi K, Hiruma M*, et al.* The complement system in ischemic heart disease. *Circulation* 1990;81(1):156-63.

544. Langlois PF, Gawryl MS. Detection of the terminal complement complex in patient plasma following acute myocardial infarction. *Atherosclerosis* 1988;70(1-2):95-105.

545. Imai T, Takase S, Arai M*, et al.* Serial changes of complement titers in the acute phase of myocardial infarction. *Jpn Circ J* 1983;47(3):289-93.

546. Pedersen ED, Waje-Andreassen U, Vedeler CA*, et al.* Systemic complement activation following human acute ischaemic stroke. *Clin Exp Immunol* 2004;137(1):117-22.

547. Laine P, Pentikainen MO, Wurzner R*, et al.* Evidence for complement activation in ruptured coronary plaques in acute myocardial infarction. *Am J Cardiol* 2002;90(4):404-8.

548. Meuwissen M, van der Wal AC, Niessen HW*, et al.* Colocalisation of intraplaque C reactive protein, complement, oxidised low density lipoprotein, and macrophages in stable and unstable angina and acute myocardial infarction. *J Clin Pathol* 2006;59(2):196-201.

549. Vlaicu R, Niculescu F, Rus HG*, et al.* Immunohistochemical localization of the terminal C5b-9 complement complex in human aortic fibrous plaque. *Atherosclerosis* 1985;57(2-3):163-77.

550. Distelmaier K, Adlbrecht C, Jakowitsch J*, et al.* Local complement activation triggers neutrophil recruitment to the site of thrombus formation in acute myocardial infarction. *Thromb Haemost* 2009;102(3):564-72.

551. Doran JP, Howie AJ, Townend JN*, et al.* Detection of myocardial infarction by immunohistological staining for C9 on formalin fixed, paraffin wax embedded sections. *J Clin Pathol* 1996;49(1):34-7.

552. Niculescu F, Rus HG, Vlaicu R. Activation of the human terminal complement pathway in atherosclerosis. *Clin Immunol Immunopathol* 1987;45(2):147-55.

553. Seifert PS, Hugo F, Tranum-Jensen J*, et al.* Isolation and characterization of a complement-activating lipid extracted from human atherosclerotic lesions. *J Exp Med* 1990;172(2):547-57.

554. Schmiedt W, Kinscherf R, Deigner HP*, et al.* Complement C6 deficiency protects against diet-induced atherosclerosis in rabbits. *Arterioscler Thromb Vasc Biol* 1998;18(11):1790-5.

555. Buerke M, Murohara T, Lefer AM. Cardioprotective effects of a C1 esterase inhibitor in myocardial ischemia and reperfusion. *Circulation* 1995;91(2):393-402.

556. Amsterdam EA, Stahl GL, Pan HL*, et al.* Limitation of reperfusion injury by a monoclonal antibody to C5a during myocardial infarction in pigs. *Am J Physiol* 1995;268(1 Pt 2):H448-57.

557. Riley RD, Sato H, Zhao ZQ*, et al.* Recombinant human complement C5a receptor antagonist reduces infarct size after surgical revascularization. *J Thorac Cardiovasc Surg* 2000;120(2):350-8.

558. Nilsson B, Ekdahl KN, Mollnes TE*, et al.* The role of complement in biomaterial-induced inflammation. *Mol Immunol* 2007;44(1-3):82-94.

559. Strom A, Ahlqvist E, Franzen A*, et al.* Extracellular matrix components in atherosclerotic arteries of Apo E/LDL receptor deficient mice: an immunohistochemical study. *Histol Histopathol* 2004;19(2):337-47.

560. Okamoto Y, Arita Y, Nishida M*, et al.* An adipocyte-derived plasma protein, adiponectin, adheres to injured vascular walls. *Horm Metab Res* 2000;32(2):47-50.

561. Sjoberg A, Onnerfjord P, Morgelin M*, et al.* The extracellular matrix and inflammation: fibromodulin activates the classical pathway of complement by directly binding C1q. *J Biol Chem* 2005;280(37):32301-8.

562. Peake PW, Shen Y, Walther A*, et al.* Adiponectin binds C1q and activates the classical pathway of complement. *Biochem Biophys Res Commun* 2008;367(3):560-5.

563. Shields KJ, Stolz D, Watkins SC*, et al.* Complement proteins C3 and C4 bind to collagen and elastin in the vascular wall: a potential role in vascular stiffness and atherosclerosis. *Clin Transl Sci* 2011;4(3):146-52.

564. Knoll F, Zitt E, Intemann D*, et al.* Juvenile elastic arteries after 28 years of renal replacement therapy in a patient with complete complement C4 deficiency. *BMC Nephrol* 2012;13:161.

565. Pavlov VI, La Bonte LR, Baldwin WM*, et al.* Absence of mannose-binding lectin prevents hyperglycemic cardiovascular complications. *Am J Pathol* 2012;180(1):104-12.

566. Iyer A, Woodruff TM, Wu MC*, et al.* Inhibition of inflammation and fibrosis by a complement C5a receptor antagonist in DOCA-salt hypertensive rats. *J Cardiovasc Pharmacol* 2011;58(5):479-86.

567. Ando B, Wiedmer T, Hamilton KK*, et al.* Complement proteins C5b-9 initiate secretion of platelet storage granules without increased binding of fibrinogen or von Willebrand factor to newly expressed cell surface GPIIb-IIIa. *J Biol Chem* 1988;263(24):11907-14.

568. Tedesco F, Fischetti F, Pausa M*, et al.* Complement-endothelial cell interactions: pathophysiological implications. *Mol Immunol* 1999;36(4-5):261-8.

569. Polley MJ, Nachman RL. Human platelet activation by C3a and C3a des-arg. *J Exp Med* 1983;158(2):603-15.

570. Krarup A, Wallis R, Presanis JS*, et al.* Simultaneous activation of complement and coagulation by MBL-associated serine protease 2. *PLoS One* 2007;2(7):e623.

571. Huber-Lang M, Sarma JV, Zetoune FS*, et al.* Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 2006;12(6):682-7.

572. Lidington EA, Haskard DO, Mason JC. Induction of decay-accelerating factor by thrombin through a protease-activated receptor 1 and protein kinase C-dependent pathway protects vascular endothelial cells from complement-mediated injury. *Blood* 2000;96(8):2784-92.

573. Ohtsuka H, Imamura T, Matsushita M*, et al.* Thrombin generates monocyte chemotactic activity from complement factor H. *Immunology* 1993;80(1):140-5.

574. Amara U, Rittirsch D, Flierl M*, et al.* Interaction between the coagulation and complement system. *Adv Exp Med Biol* 2008;632:71-9.

575. Kirschfink M, Borsos T. Binding and activation of C4 and C3 on the red cell surface by non-complement enzymes. *Mol Immunol* 1988;25(5):505-12.

576. Ghebrehiwet B, Randazzo BP, Dunn JT*, et al.* Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. *J Clin Invest* 1983;71(5):1450-6.

577. Ozmen L, Ekdahl KN, Elgue G*, et al.* Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 2002;51(6):1779-84.

578. Wuillemin WA, Minnema M, Meijers JC*, et al.* Inactivation of factor XIa in human plasma assessed by measuring factor XIa-protease inhibitor complexes: major role for C1-inhibitor. *Blood* 1995;85(6):1517-26.

579. Davis AE, 3rd. Biological effects of C1 inhibitor. *Drug News Perspect* 2004;17(7):439-46.

580. Myles T, Nishimura T, Yun TH*, et al.* Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. *J Biol Chem* 2003;278(51):51059-67.

581. Ruggeri ZM. The role of von Willebrand factor in thrombus formation. *Thromb Res* 2007;120 Suppl 1:S5-9.

582. Yago T, Lou J, Wu T*, et al.* Platelet glycoprotein Ibalpha forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *J Clin Invest* 2008;118(9):3195-207.

583. Kehrel B, Wierwille S, Clemetson KJ*, et al.* Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood* 1998;91(2):491-9.

584. Varga-Szabo D, Pleines I, Nieswandt B. Cell adhesion mechanisms in platelets. *Arterioscler Thromb Vasc Biol* 2008;28(3):403-12.

585. Sonnenberg A, Modderman PW, Hogervorst F. Laminin receptor on platelets is the integrin VLA-6. *Nature* 1988;336(6198):487-9.

586. Lam SC, Plow EF, D'Souza SE*, et al.* Isolation and characterization of a platelet membrane protein related to the vitronectin receptor. *J Biol Chem* 1989;264(7):3742-9.

587. Emsley J, Knight CG, Farndale RW*, et al.* Structural basis of collagen recognition by integrin alpha2beta1. *Cell* 2000;101(1):47-56.

588. Mills DC. ADP receptors on platelets. *Thromb Haemost* 1996;76(6):835-56.

589. Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. *Circ J* 2010;74(4):597-607.

590. Vu TK, Hung DT, Wheaton VI*, et al.* Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991;64(6):1057-68.

591. Kahn ML, Zheng YW, Huang W*, et al.* A dual thrombin receptor system for platelet activation. *Nature* 1998;394(6694):690-4.

592. Macfarlane RG. An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier. *Nature* 1964;202:498-9.

593. Davie EW, Ratnoff OD. Waterfall Sequence for Intrinsic Blood Clotting. *Science* 1964;145:1310-2.

594. Dahlback B. Blood coagulation. *Lancet* 2000;355(9215):1627-32.

595. Adams RL, Bird RJ. Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. *Nephrology* 2009;14(5):462-70.

596. Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am J Pathol* 1989;134(5):1087-97.

597. Mandal SK, Pendurthi UR, Rao LV. Cellular localization and trafficking of tissue factor. *Blood* 2006;107(12):4746-53.

598. Monroe DM, Hoffman M. What does it take to make the perfect clot? *Arterioscler Thromb Vasc Biol* 2006;26(1):41-8.

599. Mann KG, Brummel-Ziedins K, Orfeo T*, et al.* Models of blood coagulation. *Blood Cells Mol Dis* 2006;36(2):108-17.

600. Butenas S, Mann KG. Blood coagulation. *Biochemistry* 2002;67(1):3-12.

601. Bungay SD. Modelling the effect of amplification pathway factors on thrombin generation: A comparison of hemophilias. *Transfus Apher Sci* 2008;38(1):41-7.

602. Roberts HR, White GC. The biology of haemostasis and thrombosis. In: Weatherall DA, Cox TM, Firth JD, et al., (eds). *Oxford Textbook of Medicine*. 4th ed. Oxford: Oxford University Press; 2003.

603. Ramakrishnan V, DeGuzman F, Bao M*, et al.* A thrombin receptor function for platelet glycoprotein Ib-IX unmasked by cleavage of glycoprotein V. *Proc Natl Acad Sci U S A* 2001;98(4):1823-8.

604. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 2005;3(8):1800-14.

605. Bilodeau ML, Hamm HE. Regulation of protease-activated receptor (PAR) 1 and PAR4 signaling in human platelets by compartmentalized cyclic nucleotide actions. *J Pharmacol Exp Ther* 2007;322(2):778-88.

606. Binnie CG, Lord ST. The fibrinogen sequences that interact with thrombin. *Blood* 1993;81(12):3186-92.

607. Muszbek L, Yee VC, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res* 1999;94(5):271-305.

608. Muszbek L, Bagoly Z, Bereczky Z*, et al.* The involvement of blood coagulation factor XIII in fibrinolysis and thrombosis. *Cardiovasc Hematol Agents Med Chem* 2008;6(3):190-205.

609. Rau JC, Beaulieu LM, Huntington JA*, et al.* Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost* 2007;5 Suppl 1:102-15.

610. Quinsey NS, Greedy AL, Bottomley SP*, et al.* Antithrombin: in control of coagulation. *Int J Biochem Cell Biol* 2004;36(3):386-9.

611. Baugh RJ, Broze GJ, Jr., Krishnaswamy S. Regulation of extrinsic pathway factor Xa formation by tissue factor pathway inhibitor. *J Biol Chem* 1998;273(8):4378-86.

612. Broze GJ, Jr. The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Semin Hematol* 1992;29(3):159-69.

613. Rezaie AR. Regulation of the protein C anticoagulant and antiinflammatory pathways. *Curr Med Chem* 2010;17(19):2059-69.

614. Guinto ER, Esmon CT. Loss of prothrombin and of factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. *J Biol Chem* 1984;259(22):13986-92.

615. Fulcher CA, Gardiner JE, Griffin JH*, et al.* Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. *Blood* 1984;63(2):486-9.

616. Hackeng TM, Rosing J. Protein S as cofactor for TFPI. *Arterioscler Thromb Vasc Biol* 2009;29(12):2015-20.

617. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *Br J Haematol* 2005;129(3):307-21.

618. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood* 1997;90(10):3819-43.

619. Lijnen HR, Collen D. Endothelium in hemostasis and thrombosis. *Prog Cardiovasc Dis* 1997;39(4):343-50.

620. Lijnen HR, Bachmann F, Collen D*, et al.* Mechanisms of plasminogen activation. *J Intern Med* 1994;236(4):415-24.

621. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol* 2002;3(12):932-43.

622. Vaughan DE. PAI-1 and atherothrombosis. *J Thromb Haemost* 2005;3(8):1879-83.

623. Sprengers ED, Kluft C. Plasminogen activator inhibitors. *Blood* 1987;69(2):381-7.

624. Samad F, Loskutoff DJ. Tissue distribution and regulation of plasminogen activator inhibitor-1 in obese mice. *Mol Med* 1996;2(5):568-82.

625. Loskutoff DJ, Ny T, Sawdey M*, et al.* Fibrinolytic system of cultured endothelial cells: regulation by plasminogen activator inhibitor. *J Cell Biochem* 1986;32(4):273-80.

626. Chomiki N, Henry M, Alessi MC*, et al.* Plasminogen activator inhibitor-1 expression in human liver and healthy or atherosclerotic vessel walls. *Thromb Haemost* 1994;72(1):44-53.

627. Declerck PJ, Alessi MC, Verstreken M*, et al.* Measurement of plasminogen activator inhibitor 1 in biologic fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood* 1988;71(1):220-5.

628. Lawrence DA, Palaniappan S, Stefansson S*, et al.* Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J Biol Chem* 1997;272(12):7676-80.

629. Zhou A, Huntington JA, Pannu NS*, et al.* How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nat Struct Biol* 2003;10(7):541-4.

630. Keijer J, Ehrlich HJ, Linders M*, et al.* Vitronectin governs the interaction between plasminogen activator inhibitor 1 and tissue-type plasminogen activator. *J Biol Chem* 1991;266(16):10700-7.

631. Rezaie AR. Vitronectin functions as a cofactor for rapid inhibition of activated protein C by plasminogen activator inhibitor-1. Implications for the mechanism of profibrinolytic action of activated protein C. *J Biol Chem* 2001;276(19):15567-70.

632. van Meijer M, Smilde A, Tans G*, et al.* The suicide substrate reaction between plasminogen activator inhibitor 1 and thrombin is regulated by the cofactors vitronectin and heparin. *Blood* 1997;90(5):1874-82.

633. Rezaie AR. Role of exosites 1 and 2 in thrombin reaction with plasminogen activator inhibitor-1 in the absence and presence of cofactors. *Biochemistry* 1999;38(44):14592-9.

634. Nesheim M. Thrombin and fibrinolysis. *Chest* 2003;124(3 Suppl):33S-9S.

635. Wang W, Boffa MB, Bajzar L*, et al.* A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *J Biol Chem* 1998;273(42):27176-81.

636. Collen D. The plasminogen (fibrinolytic) system. *Thromb Haemost* 1999;82(2):259-70.

637. Kimura S, Aoki N. Cross-linking site in fibrinogen for alpha 2-plasmin inhibitor. *J Biol Chem* 1986;261(33):15591-5.

638. Dunn EJ, Ariens RA, de Lange M*, et al.* Genetics of fibrin clot structure: a twin study. *Blood* 2004;103(5):1735-40.

639. Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys J* 1992;63(1):111-28.

640. Blomback B. Fibrinogen structure, activation, polymerization and fibrin gel structure. *Thromb Res* 1994;75(3):327-8.

641. Okada M, Blomback B. Factors influencing fibrin gel structure studied by flow measurement. *Ann N Y Acad Sci* 1983;408:233-53.

642. Blomback B, Carlsson K, Hessel B*, et al.* Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. *Biochimica et Biophysica Acta* 1989;997(1-2):96-110.

643. Ryan EA, Mockros LF, Weisel JW*, et al.* Structural origins of fibrin clot rheology. *Biophys J* 1999;77(5):2813-26.

644. Fatah K, Silveira A, Tornvall P*, et al.* Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age. *Thromb Haemost* 1996;76(4):535-40.

645. Sjoland JA, Sidelmann JJ, Brabrand M*, et al.* Fibrin clot structure in patients with end-stage renal disease. *Thromb Haemost* 2007;98(2):339-45.

646. Undas A, Kolarz M, Kopec G*, et al.* Altered fibrin clot properties in patients on long-term haemodialysis: relation to cardiovascular mortality. *Nephrol Dial Transplant* 2008;23(6):2010-2015.

647. Undas A, Nycz K, Pastuszczak M*, et al.* The effect of chronic kidney disease on fibrin clot properties in patients with acute coronary syndrome. *Blood Coagul Fibrinolysis* 2010;21(6):522-7.

648. Undas A, Szuldrzynski K, Stepien E*, et al.* Reduced clot permeability and susceptibility to lysis in patients with acute coronary syndrome: effects of inflammation and oxidative stress. *Atherosclerosis* 2008;196(2):551-7.

649. Fatah K, Hamsten A, Blomback B*, et al.* Fibrin gel network characteristics and coronary heart disease: relations to plasma fibrinogen concentration, acute phase protein, serum lipoproteins and coronary atherosclerosis. *Thromb Haemost* 1992;68(2):130-5.

650. Brownlee M, Vlassara H, Cerami A. Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. *Diabetes* 1983;32(7):680-4.

651. Nair CH, Azhar A, Wilson JD*, et al.* Studies on fibrin network structure in human plasma. Part II--Clinical application: diabetes and antidiabetic drugs. *Thromb Res* 1991;64(4):477-85.

652. Hess K, Alzahrani SH, Mathai M*, et al.* A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. *Diabetologia* 2011.

653. Jorneskog G, Egberg N, Fagrell B*, et al.* Altered properties of the fibrin gel structure in patients with IDDM. *Diabetologia* 1996;39(12):1519-23.

654. Dunn EJ, Ariens RA, Grant PJ. The influence of type 2 diabetes on fibrin structure and function. *Diabetologia* 2005;48(6):1198-206.

655. Bochenek M, Zalewski J, Sadowski J*, et al.* Type 2 diabetes as a modifier of fibrin clot properties in patients with coronary artery disease. *J Thromb Thrombolysis* 2013;35(2):264-70.

656. Antovic A, Perneby C, Ekman GJ*, et al.* Marked increase of fibrin gel permeability with very low dose ASA treatment. *Thromb Res* 2005;116(6):509-17.

657. Fatah K, Beving H, Albage A*, et al.* Acetylsalicylic acid may protect the patient by increasing fibrin gel porosity. Is withdrawing of treatment harmful to the patient? *Eur Heart J* 1996;17(9):1362-6.

658. Williams S, Fatah K, Hjemdahl P*, et al.* Better increase in fibrin gel porosity by low dose than intermediate dose acetylsalicylic acid. *Eur Heart J* 1998;19(11):1666-72.

659. Ajjan RA, Standeven KF, Khanbhai M*, et al.* Effects of aspirin on clot structure and fibrinolysis using a novel in vitro cellular system. *Arterioscler Thromb Vasc Biol* 2009;29(5):712-7.

660. Bjornsson TD, Schneider DE, Berger H, Jr. Aspirin acetylates fibrinogen and enhances fibrinolysis. Fibrinolytic effect is independent of changes in plasminogen activator levels. *J Pharmacol Exp Ther* 1989;250(1):154-61.

661. Hirsh J, Fuster V, Ansell J*, et al.* American Heart Association/American College of Cardiology Foundation guide to warfarin therapy. *Circulation* 2003;107(12):1692-711.

662. Nelsestuen GL, Zytkovicz TH, Howard JB. The mode of action of vitamin K. Identification of gamma-carboxyglutamic acid as a component of prothrombin. *J Biol Chem* 1974;249(19):6347-50.

663. Stenflo J, Fernlund P, Egan W*, et al.* Vitamin K dependent modifications of glutamic acid residues in prothrombin. *Proc Natl Acad Sci U S A* 1974;71(7):2730-3.

664. Choonara IA, Malia RG, Haynes BP*, et al.* The relationship between inhibition of vitamin K1 2,3-epoxide reductase and reduction of clotting factor activity with warfarin. *Br J Clin Pharmacol* 1988;25(1):1-7.

665. Whitlon DS, Sadowski JA, Suttie JW. Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. *Biochemistry* 1978;17(8):1371-7.

666. Cain D, Hutson SM, Wallin R. Warfarin resistance is associated with a protein component of the vitamin K 2,3-epoxide reductase enzyme complex in rat liver. *Thromb Haemost* 1998;80(1):128-33.

667. Furie B, Furie BC. The molecular basis of blood coagulation. *Cell* 1988;53(4):505-18.

668. Wallin R, Hutson SM. Warfarin and the vitamin K-dependent gamma-carboxylation system. *Trends Mol Med* 2004;10(7):299-302.

669. Blomback M, He S, Bark N*, et al.* Effects on fibrin network porosity of anticoagulants with different modes of action and reversal by activated coagulation factor concentrate. *Br J Haematol* 2011;152(6):758-65.

670. Azizova OA, Piryazev AP, Aseychev AV*, et al.* Oxidative modification of fibrinogen inhibits its transformation into fibrin under the effect of thrombin. *Bull Exp Biol Med* 2009;147(2):201-3.

671. Piryazev AP, Aseichev AV, Azizova OA. Effect of oxidation-modified fibrinogen on the formation and lysis of fibrin clot in the plasma. *Bull Exp Biol Med* 2009;148(6):881-5.

672. Vadseth C, Souza JM, Thomson L*, et al.* Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J Biol Chem* 2004;279(10):8820-6.

673. Inada Y, Hessel B, Blomback B. Photooxidation of fibrinogen in the presence of methylene blue and its effect on polymerization. *Biochim Biophys Acta* 1978;532(1):161-70.

674. Yoshimoto T, Saito Y, Inada Y. Non-clottable fibrinogen by photooxidation in blood plasma. *Photochem Photobiol* 1987;45(5):675-6.

675. Shacter E, Williams JA, Levine RL. Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. *Free Radic Biol Med* 1995;18(4):815-21.

676. Karpel R, Marx G, Chevion M. Free radical-induced fibrinogen coagulation: modulation of neofibe formation by concentration, pH and temperature. *Isr J Med Sci* 1991;27(2):61-6.

677. Galanakis DK, Laurent P, Janoff A. Cigarette smoke contains anticoagulants against fibrin aggregation and factor XIIIa in plasma. *Science* 1982;217(4560):642-5.

678. Morrow JD, Hill KE, Burk RF*, et al.* A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci U S A* 1990;87(23):9383-7.

679. Undas A, Topor-Madry R, Tracz W*, et al.* Effect of cigarette smoking on plasma fibrin clot permeability and susceptibility to lysis. *Thromb Haemost* 2009;102(6):1289-91.

680. Kwasny-Krochin B, Gluszko P, Undas A. Unfavorably altered fibrin clot properties in patients with active rheumatoid arthritis. *Thromb Res* 2010;126(1):e11-6.

681. Undas A, Plicner D, Stepien E*, et al.* Altered fibrin clot structure in patients with advanced coronary artery disease: a role of C-reactive protein, lipoprotein(a) and homocysteine. *J Thromb Haemost* 2007;5(9):1988-90.

682. Carter AM, Schroeder V, Polkinghorne VR*, et al.* The inflammatory protein complement C3 is a novel substrate for factor XIII and impairs fibrin clot lysis [Abstract]. *Circulation* 2009;120:S1099.

683. Rajzer M, Wojciechowska W, Kawecka-Jaszcz K*, et al.* Plasma fibrin clot properties in arterial hypertension and their modification by antihypertensive medication. *Thromb Res* 2012;130(1):99-103.

684. Salonen EM, Vartio T, Hedman K*, et al.* Binding of fibronectin by the acute phase reactant C-reactive protein. *J Biol Chem* 1984;259(3):1496-501.

685. Nikolajsen CL, Scavenius C, Enghild JJ. Human complement C3 is a substrate for transglutaminases. A functional link between non-protease-based members of the coagulation and complement cascades. *Biochemistry* 2012;51(23):4735-42.

686. Howes JM, Richardson VR, Smith KA*, et al.* Complement C3 is a novel plasma clot component with anti-fibrinolytic properties. *Diab Vasc Dis Res* 2012;9(3):216-25.

687. Shats-Tseytlina EA, Nair CH, Dhall DP. Complement activation: a new participant in the modulation of fibrin gel characteristics and the progression of atherosclerosis? *Blood Coagul Fibrinolysis* 1994;5(4):529-35.

688. Schroeder V, Carter AM, Dunne J*, et al.* Proinflammatory and hypofibrinolytic phenotype in healthy first-degree relatives of patients with Type 2 diabetes. *J Thromb Haemost* 2010;8(9):2080-2.

689. Kaptoge S, Di Angelantonio E, Pennells L*, et al.* C-reactive protein, fibrinogen, and cardiovascular disease prediction. *N Engl J Med* 2012;367(14):1310-20.

690. Danesh J, Lewington S, Thompson SG*, et al.* Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *JAMA* 2005;294(14):1799-809.

691. Levenson J, Giral P, Razavian M*, et al.* Fibrinogen and silent atherosclerosis in subjects with cardiovascular risk factors. *Arterioscler Thromb Vasc Biol* 1995;15(9):1263-8.

692. Handa K, Kono S, Saku K*, et al.* Plasma fibrinogen levels as an independent indicator of severity of coronary atherosclerosis. *Atherosclerosis* 1989;77(2-3):209-13.

693. Broadhurst P, Kelleher C, Hughes L*, et al.* Fibrinogen, factor VII clotting activity and coronary artery disease severity. *Atherosclerosis* 1990;85(2-3):169-73.

694. De Luca G, Verdoia M, Cassetti E*, et al.* High fibrinogen level is an independent predictor of presence and extent of coronary artery disease among Italian population. *J Thromb Thrombolysis* 2011;31(4):458-63.

695. Danielsen R, Onundarson PT, Thors H*, et al.* Activated and total coagulation factor VII, and fibrinogen in coronary artery disease. *Scand Cardiovasc J* 1998;32(2):87-95.

696. Resch KL, Ernst E, Matrai A*, et al.* Fibrinogen and viscosity as risk factors for subsequent cardiovascular events in stroke survivors. *Ann Intern Med* 1992;117(5):371-5.

697. Lindahl B, Toss H, Siegbahn A*, et al.* Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease. *N Engl J Med* 2000;343(16):1139-47.

698. Benderly M, Graff E, Reicher-Reiss H*, et al.* Fibrinogen is a predictor of mortality in coronary heart disease patients. The Bezafibrate Infarction Prevention (BIP) Study Group. *Arterioscler Thromb Vasc Biol* 1996;16(3):351-6.

699. Becker RC, Cannon CP, Bovill EG*, et al.* Prognostic value of plasma fibrinogen concentration in patients with unstable angina and non-Q-wave myocardial infarction (TIMI IIIB Trial). *Am J Cardiol* 1996;78(2):142-7.

700. Toss H, Lindahl B, Siegbahn A*, et al.* Prognostic influence of increased fibrinogen and C-reactive protein levels in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease. *Circulation* 1997;96(12):4204-10.

701. Koch M, Kutkuhn B, Grabensee B*, et al.* Apolipoprotein A, fibrinogen, age, and history of stroke are predictors of death in dialysed diabetic patients: a prospective study in 412 subjects. *Nephrol Dial Transplant* 1997;12(12):2603-11.

702. Folsom AR. Epidemiology of fibrinogen. *Eur Heart J* 1995;16 Suppl A:21-3; discussion 23-4.

703. Scarabin PY, Aillaud MF, Amouyel P*, et al.* Associations of fibrinogen, factor VII and PAI-1 with baseline findings among 10,500 male participants in a prospective study of myocardial infarction--the PRIME Study. Prospective Epidemiological Study of Myocardial Infarction. *Thromb Haemost* 1998;80(5):749-56.

704. Ernst E, Koenig W. Fibrinogen and cardiovascular risk. *Vasc Med* 1997;2(2):115-25.

705. Folsom AR. Fibrinogen and cardiovascular risk markers. *Blood Coagul Fibrinolysis* 1999;10 Suppl 1:S13-6.

706. Tapson VF. The role of smoking in coagulation and thromboembolism in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2(1):71-7.

707. Kaptoge S, White IR, Thompson SG*, et al.* Associations of plasma fibrinogen levels with established cardiovascular disease risk factors, inflammatory markers, and other characteristics: individual participant meta-analysis of 154,211 adults in 31 prospective studies: the fibrinogen studies collaboration. *Am J Epidemiol* 2007;166(8):867-79.

708. Collet JP, Allali Y, Lesty C*, et al.* Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb* 2006;26(11):2567-73.

709. Mills JD, Ariens RAS, Mansfield MW*, et al.* Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. *Circulation* 2002;106(15):1938-42.

710. Undas A, Zalewski J, Krochin M*, et al.* Altered plasma fibrin clot properties are associated with in-stent thrombosis. *Arterioscler Thromb Vasc Biol* 2010;30(2):276-82.

711. Undas A, Podolec P, Zawilska K*, et al.* Altered fibrin clot structure/function in patients with cryptogenic ischemic stroke. *Stroke* 2009;40(4):1499-501.

712. Undas A, Slowik A, Wolkow P*, et al.* Fibrin clot properties in acute ischemic stroke: relation to neurological deficit. *Thromb Res* 2010;125(4):357-61.

713. Scott DJ, Prasad P, Philippou H*, et al.* Clot architecture is altered in abdominal aortic aneurysms and correlates with aneurysm size. *Arterioscler Thromb Vasc Biol* 2011;31(12):3004-10.

714. Bhasin N, Parry DJ, Scott DJ*, et al.* Regarding "Altered fibrin clot structure and function in individuals with intermittent claudication". *J Vasc Surg* 2009;49(4):1088-9.

715. Undas A, Nowakowski T, Ciesla-Dul M*, et al.* Abnormal plasma fibrin clot characteristics are associated with worse clinical outcome in patients with peripheral arterial disease and thromboangiitis obliterans. *Atherosclerosis* 2011;215(2):481-6.

716. Bhasin N, Ariens RA, West RM*, et al.* Altered fibrin clot structure and function in the healthy first-degree relatives of subjects with intermittent claudication. *J Vasc Surg* 2008;48(6):1497-503, 1503 e1.

717. Undas A, Zawilska K, Ciesla-Dul M*, et al.* Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood* 2009;114(19):4272-8.

718. Palka I, Nessler J, Nessler B*, et al.* Altered fibrin clot properties in patients with chronic heart failure and sinus rhythm: a novel prothrombotic mechanism. *Heart* 2010;96(14):1114-8.

719. Pieters M, Covic N, Loots du T*, et al.* The effect of glycaemic control on fibrin network structure of type 2 diabetic subjects. *Thromb Haemost* 2006;96(5):623-9.

720. Pieters M, Covic N, van der Westhuizen FH*, et al.* Glycaemic control improves fibrin network characteristics in type 2 diabetes - a purified fibrinogen model. *Thromb Haemost* 2008;99(4):691-700.

721. Carter AM, Cymbalista CM, Spector TD*, et al.* Heritability of clot formation, morphology, and lysis: the EuroCLOT study. *Arterioscler Thromb Vasc Biol* 2007;27(12):2783-9.

722. Barua RS, Sy F, Srikanth S*, et al.* Effects of cigarette smoke exposure on clot dynamics and fibrin structure: an ex vivo investigation. *Arterioscler Thromb Vasc Biol* 2010;30(1):75-9.

723. Stepien E, Miszalski-Jamka T, Kapusta P*, et al.* Beneficial effect of cigarette smoking cessation on fibrin clot properties. *J Thromb Thrombolysis* 2011;32(2):177-82.

724. Sidelmann J, Jespersen J, Kluft C*, et al.* The effect of chemical anti-inhibitors on fibrinolytic enzymes and inhibitors. *Clin Chim Acta* 1997;261(1):43-56.

725. Kluft C. Elimination of inhibition in euglobulin fibrinolysis by use of flufenamate: involvement of C1-inactivator. *Haemostasis* 1977;6(6):351-69.

726. Collet JP, Park D, Lesty C*, et al.* Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. *Arterioscler Thromb Vasc Biol* 2000;20(5):1354-61.

727. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of tocopherols and related compounds. Recommendations 1981. *Mol Cell Biochem* 1982;49(3):183-5.

728. Dutta A, Dutta SK. Vitamin E and its role in the prevention of atherosclerosis and carcinogenesis: a review. *J Am Coll Nutr* 2003;22(4):258-68.

729. Wolf G. How an increased intake of alpha-tocopherol can suppress the bioavailability of gamma-tocopherol. *Nutr Rev* 2006;64(6):295-9.

730. Kornbrust DJ, Mavis RD. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content. *Lipids* 1980;15(5):315-22.

731. Burton GW, Joyce A, Ingold KU. First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. *Lancet* 1982;2(8293):327.

732. Burton GW, Joyce A, Ingold KU. Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch Biochem Biophys* 1983;221(1):281-90.

733. Ingold K, Webb A, Witter D*, et al.* Vitamin E remains the major lipid soluble chain breaking anti-oxidant in human plasma even in individuals suffering severe vitamin E deficiency. *Arch Biochem Biophys* 1987;259:224-225.

734. Brigelius-Flohe R. Vitamin E: the shrew waiting to be tamed. *Free Radic Biol Med* 2009;46(5):543-54.

735. Devaraj S, Li D, Jialal I. The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. *J Clin Invest* 1996;98(3):756-63.

736. Devaraj S, Jialal I. Low-density lipoprotein postsecretory modification, monocyte function, and circulating adhesion molecules in type 2 diabetic patients with and without macrovascular complications: the effect of alpha-tocopherol supplementation. *Circulation* 2000;102(2):191-6.

737. Wu D, Koga T, Martin KR*, et al.* Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. *Atherosclerosis* 1999;147(2):297-307.

738. Noguchi N, Hanyu R, Nonaka A*, et al.* Inhibition of THP-1 cell adhesion to endothelial cells by alpha-tocopherol and alpha-tocotrienol is dependent on intracellular concentration of the antioxidants. *Free Radic Biol Med* 2003;34(12):1614-20.

739. Yoshida N, Yoshikawa T, Manabe H*, et al.* Vitamin E protects against polymorphonuclear leukocyte-dependent adhesion to endothelial cells. *J Leukoc Biol* 1999;65(6):757-63.

740. Faruqi R, de la Motte C, DiCorleto PE. Alpha-tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells. *J Clin Invest* 1994;94(2):592-600.

741. van Dam B, van Hinsbergh VW, Stehouwer CD*, et al.* Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects. *Cardiovasc Res* 2003;57(2):563-71.

742. Desideri G, Croce G, Marinucci MC*, et al.* Prolonged, low dose alpha-tocopherol therapy counteracts intercellular cell adhesion molecule-1 activation. *Clin Chim Acta* 2002;320(1-2):5-9.

743. Desideri G, Marinucci MC, Tomassoni G*, et al.* Vitamin E supplementation reduces plasma vascular cell adhesion molecule-1 and von Willebrand factor levels and increases nitric oxide concentrations in hypercholesterolemic patients. *J Clin Endocrinol Metab* 2002;87(6):2940-5.

744. Upritchard JE, Sutherland WH, Mann JI. Effect of supplementation with tomato juice, vitamin E, and vitamin C on LDL oxidation and products of inflammatory activity in type 2 diabetes. *Diabetes Care* 2000;23(6):733-8.

745. Devaraj S, Jialal I. Alpha tocopherol supplementation decreases serum C-reactive protein and monocyte interleukin-6 levels in normal volunteers and type 2 diabetic patients. *Free Radic Biol Med* 2000;29(8):790-2.

746. Suzuki YJ, Packer L. Inhibition of NF-kappa B DNA binding activity by alpha-tocopheryl succinate. *Biochem Mol Biol Int* 1993;31(4):693-700.

747. Ricciarelli R, Tasinato A, Clement S*, et al.* alpha-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. *Biochem J* 1998;334 (Pt 1):243-9.

748. Boscoboinik D, Szewczyk A, Hensey C*, et al.* Inhibition of cell proliferation by alpha-tocopherol. Role of protein kinase C. *J Biol Chem* 1991;266(10):6188-94.

749. Azzi A, Boscoboinik D, Clement S*, et al.* Alpha-tocopherol as a modulator of smooth muscle cell proliferation. *Prostaglandins Leukot Essent Fatty Acids* 1997;57(4-5):507-14.

750. O'Leary KA, de Pascual-Tereasa S, Needs PW*, et al.* Effect of flavonoids and vitamin E on cyclooxygenase-2 (COX-2) transcription. *Mutat Res* 2004;551(1-2):245-54.

751. Grammas P, Hamdheydari L, Benaksas EJ*, et al.* Anti-inflammatory effects of tocopherol metabolites. *Biochem Biophys Res Commun* 2004;319(3):1047-52.

752. Jiang Q, Elson-Schwab I, Courtemanche C*, et al.* Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc Natl Acad Sci U S A* 2000;97(21):11494-9.

753. Antoniades C, Tousoulis D, Tentolouris C*, et al.* Effects of antioxidant vitamins C and E on endothelial function and thrombosis/fibrinolysis system in smokers. *Thromb Haemost* 2003;89(6):990-5.

754. Devaraj S, Chan AV, Jr., Jialal I. alpha-Tocopherol supplementation decreases plasminogen activator inhibitor-1 and P-selectin levels in type 2 diabetic patients. *Diabetes Care* 2002;25(3):524-9.

755. Bonfigli AR, Pieri C, Manfrini S*, et al.* Vitamin E intake reduces plasminogen activator inhibitor type 1 in T2DM patients. *Diabetes Nutr Metab* 2001;14(2):71-7.

756. Miyamoto S, Kawano H, Takazoe K*, et al.* Vitamin E improves fibrinolytic activity in patients with coronary spastic angina. *Thromb Res* 2004;113(6):345-51.

757. Aznar J, Estelles A, Tormo G*, et al.* Plasminogen activator inhibitor activity and other fibrinolytic variables in patients with coronary artery disease. *Br Heart J* 1988;59(5):535-41.

758. Folsom AR, Aleksic N, Park E*, et al.* Prospective study of fibrinolytic factors and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) Study. *Arterioscler Thromb Vasc Biol* 2001;21(4):611-7.

759. Sobel BE, Woodcock-Mitchell J, Schneider DJ*, et al.* Increased plasminogen activator inhibitor type 1 in coronary artery atherectomy specimens from type 2 diabetic compared with nondiabetic patients: a potential factor predisposing to thrombosis and its persistence. *Circulation* 1998;97(22):2213-21.

760. Schneiderman J, Sawdey MS, Keeton MR*, et al.* Increased type 1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proc Natl Acad Sci U S A* 1992;89(15):6998-7002.

761. Lupu F, Bergonzelli GE, Heim DA*, et al.* Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. *Arterioscler Thromb* 1993;13(7):1090-100.

762. Sawa H, Sobel BE, Fujii S. Potentiation by hypercholesterolemia of the induction of aortic intramural synthesis of plasminogen activator inhibitor type 1 by endothelial injury. *Circ Res* 1993;73(4):671-80.

763. Lusis AJ. Atherosclerosis. *Nature* 2000;407(6801):233-41.

764. Davi G, Romano M, Mezzetti A*, et al.* Increased levels of soluble P-selectin in hypercholesterolemic patients. *Circulation* 1998;97(10):953-7.

765. Blann AD, Lip GY, Beevers DG*, et al.* Soluble P-selectin in atherosclerosis: a comparison with endothelial cell and platelet markers. *Thromb Haemost* 1997;77(6):1077-80.

766. Blann AD, Lip GY. Hypothesis: is soluble P-selectin a new marker of platelet activation? *Atherosclerosis* 1997;128(2):135-8.

767. Dowd P, Zheng ZB. On the mechanism of the anticlotting action of vitamin E quinone. *Proc Natl Acad Sci U S A* 1995;92(18):8171-5.

768. Booth SL, Golly I, Sacheck JM*, et al.* Effect of vitamin E supplementation on vitamin K status in adults with normal coagulation status. *Am J Clin Nutr* 2004;80(1):143-8.

769. Ageno W, Gallus AS, Wittkowsky A*, et al.* Oral anticoagulant therapy: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest* 2012;141(2 Suppl):e44S-88S.

770. Meydani SN, Meydani M, Blumberg JB*, et al.* Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. *Am J Clin Nutr* 1998;68(2):311-8.

771. Corrigan JJ, Jr. Coagulation problems relating to vitamin E. *Am J Pediatr Hematol Oncol* 1979;1(2):169-73.

772. Corrigan JJ, Jr., Ulfers LL. Effect of vitamin E on prothrombin levels in warfarin-induced vitamin K deficiency. *Am J Clin Nutr* 1981;34(9):1701-5.

773. Cranenburg EC, Schurgers LJ, Uiterwijk HH*, et al.* Vitamin K intake and status are low in hemodialysis patients. *Kidney Int* 2012;82(5):605-10.

774. Farbstein D, Kozak-Blickstein A, Levy AP. Antioxidant vitamins and their use in preventing cardiovascular disease. *Molecules* 2010;15(11):8098-110.

775. Wu D, Liu L, Meydani M*, et al.* Effect of vitamin E on prostacyclin (PGI2) and prostaglandin (PG) E2 production by human aorta endothelial cells: mechanism of action. *Ann N Y Acad Sci* 2004;1031:425-7.

776. Suttorp N, Toepfer W, Roka L. Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. *Am J Physiol* 1986;251(5 Pt 1):C671-80.

777. Keaney JF, Jr., Guo Y, Cunningham D*, et al.* Vascular incorporation of alpha-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. *J Clin Invest* 1996;98(2):386-94.

778. Losonczy KG, Harris TB, Havlik RJ. Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. *Am J Clin Nutr* 1996;64(2):190-6.

779. Meyer F, Bairati I, Dagenais GR. Lower ischemic heart disease incidence and mortality among vitamin supplement users. *Can J Cardiol* 1996;12(10):930-4.

780. Knekt P, Reunanen A, Jarvinen R*, et al.* Antioxidant vitamin intake and coronary mortality in a longitudinal population study. *Am J Epidemiol* 1994;139(12):1180-9.

781. Stampfer MJ, Hennekens CH, Manson JE*, et al.* Vitamin E consumption and the risk of coronary disease in women. *N Engl J Med* 1993;328(20):1444-9.

782. Rimm EB, Stampfer MJ, Ascherio A*, et al.* Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med* 1993;328(20):1450-6.

783. Sesso HD, Buring JE, Christen WG*, et al.* Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA* 2008;300(18):2123-33.

784. de Gaetano G. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. *Lancet* 2001;357(9250):89-95.

785. Virtamo J, Rapola JM, Ripatti S*, et al.* Effect of vitamin E and beta carotene on the incidence of primary nonfatal myocardial infarction and fatal coronary heart disease. *Arch Intern Med* 1998;158(6):668-75.

786. Lee IM, Cook NR, Gaziano JM*, et al.* Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. *JAMA* 2005;294(1):56-65.

787. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354(9177):447-55.

788. Waters DD, Alderman EL, Hsia J*, et al.* Effects of hormone replacement therapy and antioxidant vitamin supplements on coronary atherosclerosis in postmenopausal women: a randomized controlled trial. *JAMA* 2002;288(19):2432-40.

789. Cook NR, Albert CM, Gaziano JM*, et al.* A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study. *Arch Intern Med* 2007;167(15):1610-8.

790. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 2002;360(9326):23-33.

791. Rapola JM, Virtamo J, Ripatti S*, et al.* Effects of alpha tocopherol and beta carotene supplements on symptoms, progression, and prognosis of angina pectoris. *Heart* 1998;79(5):454-8.

792. Lonn E, Bosch J, Yusuf S*, et al.* Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA* 2005;293(11):1338-47.

793. Stephens NG, Parsons A, Schofield PM*, et al.* Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 1996;347(9004):781-6.

794. Yusuf S, Dagenais G, Pogue J*, et al.* Vitamin E supplementation and cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 2000;342(3):154-60.

795. Bjelakovic G, Nikolova D, Gluud LL*, et al.* Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA* 2007;297(8):842-57.

796. Vivekananthan DP, Penn MS, Sapp SK*, et al.* Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials. *Lancet* 2003;361(9374):2017-23.

797. Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. *FASEB J* 1999;13(10):1145-55.

798. Hoppe PP, Krennrich G. Bioavailability and potency of natural-source and all-racemic alpha-tocopherol in the human: a dispute. *Eur J Nutr* 2000;39(5):183-93.

799. Galli F, Buoncristiani U, Conte C*, et al.* Vitamin E in uremia and dialysis patients. *Ann N Y Acad Sci* 2004;1031:348-51.

800. Galli F, Floridi AG, Floridi A*, et al.* Accumulation of vitamin E metabolites in the blood of renal failure patients. *Clin Nutr* 2004;23(2):205-12.

801. Traber MG, Kayden HJ. Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins. *Am J Clin Nutr* 1989;49(3):517-26.

802. Bjorneboe A, Bjorneboe GE, Drevon CA. Serum half-life, distribution, hepatic uptake and biliary excretion of alpha-tocopherol in rats. *Biochim Biophys Acta* 1987;921(2):175-81.

803. Baldi S, Innocenti M, Frascerra S*, et al.* Effects of hemodialysis and vitamin E supplementation on low-density lipoprotein oxidizability in end-stage renal failure. *J Nephrol* 2012:0.

804. Sato M, Matsumoto Y, Morita H*, et al.* Effects of vitamin supplementation on microcirculatory disturbance in hemodialysis patients without peripheral arterial disease. *Clin Nephrol* 2003;60(1):28-34.

805. Yalcin AS, Yurtkuran M, Dilek K*, et al.* The effect of vitamin E therapy on plasma and erythrocyte lipid peroxidation in chronic hemodialysis patients. *Clin Chim Acta* 1989;185(1):109-12.

806. Taccone-Gallucci M, Giardini O, Ausiello C*, et al.* Vitamin E supplementation in hemodialysis patients: effects on peripheral blood mononuclear cells lipid peroxidation and immune response. *Clin Nephrol* 1986;25(2):81-6.

807. Giray B, Kan E, Bali M*, et al.* The effect of vitamin E supplementation on antioxidant enzyme activities and lipid peroxidation levels in hemodialysis patients. *Clin Chim Acta* 2003;338(1-2):91-8.

808. Antoniadi G, Eleftheriadis T, Liakopoulos V*, et al.* Prolonged alfa-tocopherol administration increases oxidative stress markers and anticardiolipin antibody levels in hemodialysis (HD) patients [Abstract]. *Blood Purif* 2004;22(4):398.

809. Lubrano R, Taccone-Gallucci M, Piazza A*, et al.* Vitamin E supplementation and oxidative status of peripheral blood mononuclear cells and lymphocyte subsets in hemodialysis patients. *Nutrition* 1992;8(2):94-7.

810. Antoniadi G, Eleftheriadis T, Liakopoulos V*, et al.* Effect of one-year oral alpha-tocopherol administration on the antioxidant defense system in hemodialysis patients. *Ther Apher Dial* 2008;12(3):237-42.

811. Lubrano R, Taccone-Gallucci M, Mazzarella V*, et al.* Relationship between red blood cell lipid peroxidation, plasma hemoglobin, and red blood cell osmotic resistance before and after vitamin E supplementation in hemodialysis patients. *Artif Organs* 1986;10(3):245-8.

812. Giardini O, Taccone-Gallucci M, Lubrano R*, et al.* Effects of alpha-tocopherol administration on red blood cell membrane lipid peroxidation in hemodialysis patients. *Clin Nephrol* 1984;21(3):174-7.

813. Nemeth I, Turi S, Haszon I*, et al.* Vitamin E alleviates the oxidative stress of erythropoietin in uremic children on hemodialysis. *Pediatr Nephrol* 2000;14(1):13-7.

814. Islam KN, O'Byrne D, Devaraj S*, et al.* Alpha-tocopherol supplementation decreases the oxidative susceptibility of LDL in renal failure patients on dialysis therapy. *Atherosclerosis* 2000;150(1):217-24.

815. Badiou S, Cristol J-P, Morena M*, et al.* Vitamin E supplementation increases LDL resistance to ex vivo oxidation in hemodialysis patients. *Int J Vitam Nutr Res* 2003;73(4):290-6.

816. Uzum A, Toprak O, Gumustas MK*, et al.* Effect of vitamin E therapy on oxidative stress and erythrocyte osmotic fragility in patients on peritoneal dialysis and hemodialysis. *J Nephrol* 2006;19(6):739-45.

817. Roozbeh J, Shahriyari B, Akmali M*, et al.* Comparative effects of silymarin and vitamin E supplementation on oxidative stress markers, and hemoglobin levels among patients on hemodialysis. *Ren Fail* 2011;33(2):118-23.

818. Turi S, Nemeth I, Dobos E*, et al.* The effect of vitamin E on uraemic erythrocytes in the early period of erythropoietin treatment [Abstract]. *Pediatr Nephrol* 1992;6:C168.

819. Yeksan M, Polat M, Turk S*, et al.* Effect of vitamin E on erythropoietin levels in uraemic haemodialysis patients [Abstract]. *Nephrol Dial Transplant* 1991;6:830.

820. Ono K. Reduction of osmotic haemolysis and anaemia by high dose vitamin E supplementation in regular haemodialysis patients. *Proc Eur Dial Transplant Assoc Eur Ren Assoc* 1985;21:296-9.

821. Cristol JP, Bosc JY, Badiou S*, et al.* Erythropoietin and oxidative stress in haemodialysis: beneficial effects of vitamin E supplementation. *Nephrol Dial Transplant* 1997;12(11):2312-7.

822. Kamgar M, Zaldivar F, Vaziri ND*, et al.* Antioxidant therapy does not ameliorate oxidative stress and inflammation in patients with end-stage renal disease. *J Natl Med Assoc* 2009;101(4):336-44.

823. Smith KS, Lee C-L, Ridlington JW*, et al.* Vitamin E supplementation increases circulating vitamin E metabolites tenfold in end-stage renal disease patients. *Lipids* 2003;38(8):813-9.

824. Hodkova M, Dusilova-Sulkova S, Kalousova M*, et al.* Influence of oral vitamin E therapy on micro-inflammation and cardiovascular disease markers in chronic hemodialysis patients. *Ren Fail* 2006;28(5):395-9.

825. Boaz M, Smetana S, Weinstein T*, et al.* Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease (SPACE): randomised placebo-controlled trial. *Lancet* 2000;356(9237):1213-8.

826. Tepel M, van der Giet M, Statz M*, et al.* The antioxidant acetylcysteine reduces cardiovascular events in patients with end-stage renal failure: a randomized, controlled trial. *Circulation* 2003;107(7):992-5.

827. Mydlik M, Derzsiova K, Racz O*, et al.* A modified dialyzer with vitamin E and antioxidant defense parameters. *Kidney Int Suppl* 2001;78:S144-7.

828. Hakim RM. Clinical implications of hemodialysis membrane biocompatibility. *Kidney Int* 1993;44(3):484-94.

829. Varela MP, Kimmel PL, Phillips TM*, et al.* Biocompatibility of hemodialysis membranes: interrelations between plasma complement and cytokine levels. *Blood Purif* 2001;19(4):370-9.

830. Boure T, Vanholder R. Which dialyser membrane to choose? *Nephrol Dial Transplant* 2004;19(2):293-6.

831. Hakim RM, Held PJ, Stannard DC*, et al.* Effect of the dialysis membrane on mortality of chronic hemodialysis patients. *Kidney Int* 1996;50(2):566-70.

832. Bloembergen WE, Hakim RM, Stannard DC*, et al.* Relationship of dialysis membrane and cause-specific mortality. *Am J Kidney Dis* 1999;33(1):1-10.

833. Yokoyama H, Kawaguchi T, Wada T*, et al.* Biocompatibility and permeability of dialyzer membranes do not affect anemia, erythropoietin dosage or mortality in japanese patients on chronic non-reuse hemodialysis: a prospective cohort study from the J-DOPPS II study. *Nephron Clin Pract* 2008;109(2):c100-8.

834. Krane V, Krieter DH, Olschewski M*, et al.* Dialyzer membrane characteristics and outcome of patients with type 2 diabetes on maintenance hemodialysis. *Am J Kidney Dis* 2007;49(2):267-75.

835. Grooteman MP, Nube MJ. Impact of the type of dialyser on the clinical outcome in chronic haemodialysis patients: does it really matter? *Nephrol Dial Transplant* 2004;19(12):2965-70.

836. MacLeod AM, Campbell MK, Cody JD*, et al.* Cellulose, modified cellulose and synthetic membranes in the haemodialysis of patients with end-stage renal disease. *Cochrane Database Syst Rev* 2005;3:CD003234.

837. Grooteman MP, Nube MJ, van Limbeek J*, et al.* Biocompatibility and performance of a modified cellulosic and a synthetic high flux dialyzer. A randomized crossover comparison between cellulose triacetate and polysulphon. *ASAIO J* 1995;41(2):215-20.

838. Panichi V, Rizza GM, Taccola D*, et al.* C-reactive protein in patients on chronic hemodialysis with different techniques and different membranes. *Biomed Pharmacother* 2006;60(1):14-7.

839. Port FK, Wolfe RA, Hulbert-Shearon TE*, et al.* Mortality risk by hemodialyzer reuse practice and dialyzer membrane characteristics: results from the usrds dialysis morbidity and mortality study. *Am J Kidney Dis* 2001;37(2):276-86.

840. Finelli L, Miller JT, Tokars JI*, et al.* National surveillance of dialysis-associated diseases in the United States, 2002. *Semin Dial* 2005;18(1):52-61.

841. Zhang DL, Liu J, Cui WY*, et al.* Differences in Bio-incompatibility among Four Biocompatible Dialyzer Membranes Using in Maintenance Hemodialysis Patients. *Ren Fail* 2011;33(7):682-91.

842. Biasioli S, Schiavon R, Petrosino L*, et al.* Role of cellulosic and noncellulosic membranes in hyperhomocysteinemia and oxidative stress. *ASAIO J* 2000;46(5):625-34.

843. Slowik-Zylka D, Safranow K, Dziedziejko V*, et al.* The effect of dialysis membrane type on plasma concentrations of pentosidine, a marker of carbonyl stress, in hemodialyzed patients. *Pol Arch Med Wewn* 2009;119(10):614-20.

844. Muller C, Eisenbrand G, Gradinger M*, et al.* Effects of hemodialysis, dialyser type and iron infusion on oxidative stress in uremic patients. *Free Radic Res* 2004;38(10):1093-100.

845. Lucchi L, Bergamini S, Botti B*, et al.* Influence of different hemodialysis membranes on red blood cell susceptibility to oxidative stress. *Artif Organs* 2000;24(1):1-6.

846. Yavuz O, Bicik Z, Cinar Y*, et al.* The effect of different dialysis membranes on oxidative stress and selenium status. *Clin Chim Acta* 2004;346(2):153-60.

847. Salamunic I, Juretic D, Ljutic D. Effect of different dialysis membranes on erythrocyte antioxidant enzyme levels and scavenger systems related to free hemoglobin in serum of hemodialysis patients. *Clin Chem Lab Med* 2003;41(7):904-7.

848. Schonermarck U, Dengler C, Ebeling F*, et al.* Comparative evaluation of oxidative and antioxidative capacity during high-flux hemodialysis using two different membranes. *Clin Nephrol* 2006;66(5):357-63.

849. Sasaki M, Hosoya N, Saruhashi M. Vitamin E modified cellulose membrane. *Artif Organs* 2000;24(10):779-89.

850. Schiecke G, Gwinner W, Radermacher J*, et al.* Long-term effects of vitamin E-bonded dialysis membrane on mononuclear cell activation, malondialdehyde generation and endothelial function in ESRD patients. *Contrib Nephrol* 1999;127:243-50.

851. Sanaka T, Omata M, Nishimura H*, et al.* Suppressive effects of vitamin E-coated dialysis membrane on hemodialysis-induced neutrophil activation. *Contrib Nephrol* 1999;127:261-8.

852. Shimazu T, Kondo S, Toyama K*, et al.* Effect of vitamin E-modified regenerative cellulose membrane on neutrophil superoxide anion radical production and lipid peroxidation. *Contrib Nephrol* 1999;127:251-60.

853. Hara T. Reduction of oxidized low-density lipoprotein by the long-term use of vitamin E-coated dialyzers in hemodialysis patients. *Dialysis Transplant* 2004;33:197-207.

854. Takouli L, Hadjiyannakos D, Metaxaki P*, et al.* Vitamin E-coated cellulose acetate dialysis membrane: long-term effect on inflammation and oxidative stress. *Ren Fail* 2010;32(3):287-93.

855. Odetti P, Robaudo C, Valentini S*, et al.* Effect of a new vitamin E-coated membrane on glycoxidation during hemodialysis. *Contrib Nephrol* 1999;127:192-9.

856. Taccone-Gallucci M, Meloni C, Lubrano R*, et al.* Chronic haemolysis and erythrocyte survival in haemodialysis patients treated with vitamin E-modified dialysis filters. *Contrib Nephrol* 1999;127:44-8.

857. Omata M, Higuchi C, Demura R*, et al.* Reduction of neutrophil activation by vitamin E modified dialyzer membranes. *Nephron* 2000;85(3):221-31.

858. Kirmizis D, Papagianni A, Belechri AM*, et al.* Effects of vitamin E-coated membrane dialyser on markers of oxidative stress and inflammation in patients on chronic haemodialysis. *Nephrol Dial Transplant* 2011;26(7):2296-301.

859. Buoncristiani U, Galli F, Rovidati S*, et al.* Oxidative damage during hemodialysis using a vitamin-E-modified dialysis membrane: a preliminary characterization. *Nephron* 1997;77(1):57-61.

860. Sommerburg, Sostmann K, Grune T*, et al.* Oxidative stress in hemodialysis patients treated with a dialysis membrane which has alpha-tocopherol bonded to its surface. *Biofactors* 1999;10(2-3):121-4.

861. Usberti M, Bufano G, Lima G*, et al.* Increased red blood cell survival reduces the need of erythropoietin in hemodialyzed patients treated with exogenous glutathione and vitamin E-modified membrane. *Contrib Nephrol* 1999;127:208-14.

862. Mune M, Yukawa S, Kishino M*, et al.* Effect of vitamin E on lipid metabolism and atherosclerosis in ESRD patients. *Kidney Int Suppl* 1999;71:S126-9.

863. Tarng DC, Huang TP, Liu TY*, et al.* Effect of vitamin E-bonded membrane on the 8-hydroxy 2'-deoxyguanosine level in leukocyte DNA of hemodialysis patients. *Kidney Int* 2000;58(2):790-9.

864. Galli F, Varga Z, Balla J*, et al.* Vitamin E, lipid profile, and peroxidation in hemodialysis patients. *Kidney Int Suppl* 2001;78:S148-54.

865. Satoh M, Yamasaki Y, Nagake Y*, et al.* Oxidative stress is reduced by the long-term use of vitamin E-coated dialysis filters. *Kidney Int* 2001;59(5):1943-50.

866. Eiselt J, Racek J, Trefil L*, et al.* Effects of a vitamin E-modified dialysis membrane and vitamin C infusion on oxidative stress in hemodialysis patients. *Artif Organs* 2001;25(6):430-6.

867. Tsuruoka S, Kawaguchi A, Nishiki K*, et al.* Vitamin E-bonded hemodialyzer improves neutrophil function and oxidative stress in patients with end-stage renal failure. *Am J Kidney Dis* 2002;39(1):127-33.

868. Usberti M, Gerardi G, Bufano G*, et al.* Effects of erythropoietin and vitamin E-modified membrane on plasma oxidative stress markers and anemia of hemodialyzed patients. *Am J Kidney Dis* 2002;40(3):590-9.

869. Usberti M, Gerardi G, Micheli A*, et al.* Effects of a vitamin E-bonded membrane and of glutathione on anemia and erythropoietin requirements in hemodialysis patients. *J Nephrol* 2002;15(5):558-64.

870. Triolo L, Malaguti M, Ansali F*, et al.* Vitamin E-bonded cellulose membrane, lipoperoxidation, and anemia in hemodialysis patients. *Artif Cells Blood Substit Immobil Biotechnol* 2003;31(2):185-91.

871. Bufano G, Usberti M, Mandolfo S*, et al.* Von Willebrand factor and autoantibodies against oxidized LDL in hemodialysis patients treated with vitamin E-modified dialyzers. *Int J Artif Organs* 2004;27(3):214-21.

872. Mydlik M, Derzsiova K, Racz O*, et al.* Vitamin E-coated dialyzer and antioxidant defense parameters: three-month study. *Semin Nephrol* 2004;24(5):525-31.

873. Galli F, Rovidati S, Chiarantini L*, et al.* Bioreactivity and biocompatibility of a vitamin E-modified multi-layer hemodialysis filter. *Kidney Int* 1998;54(2):580-9.

874. Odetti P, Traverso N, Monacelli F*, et al.* Vitamin E-coated filter decreases levels of free 4-hydroxyl-2-nonenal during haemodialysis sessions. *Free Radic Res* 2006;40(2):207-12.

875. Calzavara P, De Angeli S, Gatto C*, et al.* Morphologic evaluation of red blood cells using vitamin E-modified dialysis filters. *Contrib Nephrol* 1999;127:172-6.

876. Cruz DN, De Cal M, Garzotto F*, et al.* Effect of vitamin E-coated dialysis membranes on anemia in patients with chronic kidney disease: an Italian multicenter study. *Int J Artif Organs* 2008;31(6):545-52.

877. Huraib S, Tanimu D, Shaheen F*, et al.* Effect of vitamin-E-modified dialysers on dialyser clotting, erythropoietin and heparin dosage: a comparative crossover study. *Am J Nephrol* 2000;20(5):364-8.

878. Nakatan T, Takemoto Y, Tsuchida AK. The effect of vitamin E-bonded dialyzer membrane on red blood cell survival in hemodialyzed patients. *Artif Organs* 2003;27(3):214-7.

879. Kobayashi S, Moriya H, Aso K*, et al.* Vitamin E-bonded hemodialyzer improves atherosclerosis associated with a rheological improvement of circulating red blood cells. *Kidney Int* 2003;63(5):1881-7.

880. Girndt M, Lengler S, Kaul H*, et al.* Prospective crossover trial of the influence of vitamin E-coated dialyzer membranes on T-cell activation and cytokine induction. *Am J Kidney Dis* 2000;35(1):95-104.

881. Pertosa G, Grandaliano G, Valente M*, et al.* In vivo evaluation of biocompatibility of a new dialyzer employing the vitamin E-modified cellulose membrane 'Excebrane E': study of mechanisms involved in mononuclear cell activation. *Contrib Nephrol* 1999;127:200-7.

882. Senatore M, Nicoletti A, Rizzuto G. Is the bioreactivity of vitamin-E-modified dialyzer an expression of increased plasmatic vitamin E concentration? *Nephron* 2002;92(2):487-9.

883. Pertosa G, Grandaliano G, Soccio M*, et al.* Vitamin E-modified filters modulate Jun N-terminal kinase activation in peripheral blood mononuclear cells. *Kidney Int* 2002;62(2):602-10.

884. Sosa MA, Balk EM, Lau J*, et al.* A systematic review of the effect of the Excebrane dialyser on biomarkers of lipid peroxidation. *Nephrol Dial Transplant* 2006;21(10):2825-33.

885. Sasaki M. Development of vitamin E-modified polysulfone membrane dialyzers. *J Artif Organs* 2006;9(1):50-60.

886. Dahe GJ, Teotia RS, Kadam SS*, et al.* The biocompatibility and separation performance of antioxidative polysulfone/vitamin E TPGS composite hollow fiber membranes. *Biomaterials* 2011;32(2):352-65.

887. Yamamoto K, Matsuda M, Okuoka M*, et al.* Antioxidation property of vitamin E-coated polyulfone dialysis membrane and recovery of oxidized vitamin E by vitamin C treatment. *J Memb Sci* 2007;302:115-8.

888. Floridi A, Piroddi M, Pilolli F*, et al.* Analysis method and characterization of the antioxidant capacity of vitamin E-interactive polysulfone hemodialyzers. *Acta Biomater* 2009;5(8):2974-82.

889. Mandolfo S, Corradi B, Bucci R*, et al.* Evaluation of the impact of a new synthetic vitamin E-bonded membrane on anemia and rHuEPO requirement in ESRD patients with central venous catheters: a pilot study. *Int Urol Nephrol* 2012;44(5):1493-500.

890. Aoun B, Janssen-Lozinska Y, Ulinski T. Effect of vitamin E coated dialyzers on anticoagulation requirement in hemodialyzed children. *Saudi J Kidney Dis Transpl* 2010;21(3):466-70.

891. Calo LA, Naso A, D'Angelo A*, et al.* Molecular biology-based assessment of vitamin E-coated dialyzer effects on oxidative stress, inflammation, and vascular remodeling. *Artif Organs* 2011;35(2):E33-9.

892. Matsumura M, Sasaki H, Sekizuka K*, et al.* Improved management of intradialytic hypotension (IDH) using vitamin E-bonded polysulfone membrane dialyzer. *Int J Artif Organs* 2010;33(3):147-53.

893. Panichi V, Rosati A, Paoletti S*, et al.* A Vitamin E-Coated Polysulfone Membrane Reduces Serum Levels of Inflammatory Markers and Resistance to Erythropoietin-Stimulating Agents in Hemodialysis Patients: Results of a Randomized Cross-Over Multicenter Trial. *Blood Purif* 2011;32(1):7-14.

894. Andrulli S, Di Filippo S, Manzoni C*, et al.* Effect of synthetic vitamin E-bonded membrane on responsiveness to erythropoiesis-stimulating agents in hemodialysis patients: a pilot study. *Nephron Clin Pract* 2010;115(1):c82-9.

895. Morimoto H, Nakao K, Fukuoka K*, et al.* Long-term use of vitamin E-coated polysulfone membrane reduces oxidative stress markers in haemodialysis patients. *Nephrol Dial Transplant* 2005;20(12):2775-82.

896. Sanaka T, Mochizuki T, Kinugasa E*, et al.* Randomized Controlled Open-Label Trial of Vitamin E-Bonded Polysulfone Dialyzer and Erythropoiesis-Stimulating Agent Response. *Clin J Am Soc Nephrol* 2013;8(6):969-78.

897. The Renal Association. UK Renal Registry: The Twelfth Annual Report. 2009.

898. Davies SJ, Phillips L, Naish PF*, et al.* Quantifying comorbidity in peritoneal dialysis patients and its relationship to other predictors of survival. *Nephrol Dial Transplant* 2002;17(6):1085-92.

899. Davies SJ, Bryan J, Phillips L*, et al.* The predictive value of KT/V and peritoneal solute transport in CAPD patients is dependent on the type of comorbidity present. *Perit Dial Int* 1996;16 Suppl 1:S158-62.

900. Davies SJ, Russell L, Bryan J*, et al.* Comorbidity, urea kinetics, and appetite in continuous ambulatory peritoneal dialysis patients: their interrelationship and prediction of survival. *Am J Kidney Dis* 1995;26(2):353-61.

901. Lines SW, Lindley EJ, Tattersall JE*, et al.* A predictive algorithm for the management of anaemia in haemodialysis patients based on ESA pharmacodynamics: better results for less work. *Nephrol Dial Transplant* 2012;27(6):2425-9.

902. Will EJ, Richardson D, Tolman C*, et al.* Development and exploitation of a clinical decision support system for the management of renal anaemia. *Nephrol Dial Transplant* 2007;22 Suppl 4:iv31-iv36.

903. Tolman C, Richardson D, Bartlett C*, et al.* Structured conversion from thrice weekly to weekly erythropoietic regimens using a computerized decision-support system: a randomized clinical study. *J Am Soc Nephrol* 2005;16(5):1463-70.

904. National Institute for Health and Clinical Excellence, UK. Anaemia management in chronic kidney disease (Clinical Guideline 39). *London: Naitional Institute for Health and Clinical Excellence* 2006.

905. Koubaa N, Nakbi A, Smaoui M*, et al.* Hyperhomocysteinemia and elevated ox-LDL in Tunisian type 2 diabetic patients: role of genetic and dietary factors. *Clin Biochem* 2007;40(13-14):1007-14.

906. Jentzsch AM, Bachmann H, Furst P*, et al.* Improved analysis of malondialdehyde in human body fluids. *Free Radic Biol Med* 1996;20(2):251-6.

907. Lepage G, Munoz G, Champagne J*, et al.* Preparative steps necessary for the accurate measurement of malondialdehyde by high-performance liquid chromatography. *Anal Biochem* 1991;197(2):277-83.

908. Wasowicz W, Neve J, Peretz A. Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum: importance of extraction pH and influence of sample preservation and storage. *Clin Chem* 1993;39(12):2522-6.

909. Grotto D, Santa Maria LD, Boeira S*, et al.* Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. *J Pharm Biomed Anal* 2007;43(2):619-24.

910. Hendriks T, Assmann RF. On the fluorometric assay of circulating lipoperoxides. *Clin Chim Acta* 1988;174(3):263-9.

911. Somani R, Richardson VR, Standeven KF*, et al.* Elevated properdin and enhanced complement activation in first-degree relatives of South Asian subjects with type 2 diabetes. *Diabetes Care* 2012;35(4):894-9.

912. Mollnes TE, Lea T, Harboe M*, et al.* Monoclonal antibodies recognizing a neoantigen of poly(C9) detect the human terminal complement complex in tissue and plasma. *Scand J Immunol* 1985;22(2):183-95.

913. Mollnes TE, Lea T, Froland SS*, et al.* Quantification of the terminal complement complex in human plasma by an enzyme-linked immunosorbent assay based on monoclonal antibodies against a neoantigen of the complex. *Scand J Immunol* 1985;22(2):197-202.

914. Pfeifer PH, Kawahara MS, Hugli TE. Possible mechanism for in vitro complement activation in blood and plasma samples: futhan/EDTA controls in vitro complement activation. *Clin Chem* 1999;45(8 Pt 1):1190-9.

915. Altman DG. *Practical Statistics for Medical Research*. London: Chapman & Hall; 1991.

916. Mickey RM, Greenland S. The impact of confounder selection criteria on effect estimation. *Am J Epidemiol* 1989;129(1):125-37.

917. Bland JM, Altman DG. Transformations, means, and confidence intervals. *BMJ* 1996;312(7038):1079.

918. de Oliveira RM, de los Santos CA, Antonello I*, et al.* Warning: an anemia outbreak due to chloramine exposure in a clean hemodialysis unit--an issue to be revisited. *Ren Fail* 2009;31(1):81-3.

919. Schneider A, Drechsler C, Krane V*, et al.* The effect of high-flux hemodialysis on hemoglobin concentrations in patients with CKD: results of the MINOXIS study. *Clin J Am Soc Nephrol* 2012;7(1):52-9.

920. Ford BA, Coyne DW, Eby CS*, et al.* Variability of ferritin measurements in chronic kidney disease; implications for iron management. *Kidney Int* 2009;75(1):104-10.

921. The Renal Association. UK Renal Registry: The Thirteenth Annual Report. 2010.

922. Barany P, Divino Filho JC, Bergstrom J. High C-reactive protein is a strong predictor of resistance to erythropoietin in hemodialysis patients. *Am J Kidney Dis* 1997;29(4):565-8.

923. Gunnell J, Yeun JY, Depner TA*, et al.* Acute-phase response predicts erythropoietin resistance in hemodialysis and peritoneal dialysis patients. *Am J Kidney Dis* 1999;33(1):63-72.

924. Kalantar-Zadeh K, McAllister CJ, Lehn RS*, et al.* Effect of malnutrition-inflammation complex syndrome on EPO hyporesponsiveness in maintenance hemodialysis patients. *Am J Kidney Dis* 2003;42(4):761-73.

925. Panichi V, Rosati A, Bigazzi R*, et al.* Anaemia and resistance to erythropoiesis-stimulating agents as prognostic factors in haemodialysis patients: results from the RISCAVID study. *Nephrol Dial Transplant* 2011;26(8):2641-8.

926. Locatelli F, Altieri P, Andrulli S*, et al.* Predictors of haemoglobin levels and resistance to erythropoiesis-stimulating agents in patients treated with low-flux haemodialysis, haemofiltration and haemodiafiltration: results of a multicentre randomized and controlled trial. *Nephrol Dial Transplant* 2012.

927. Bratosin D, Mazurier J, Tissier JP*, et al.* Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. *Biochimie* 1998;80(2):173-95.

928. Nemeth E, Rivera S, Gabayan V*, et al.* IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004;113(9):1271-6.

929. Movilli E, Brunori G, Camerini C*, et al.* The kind of vascular access influences the baseline inflammatory status and epoetin response in chronic hemodialysis patients. *Blood Purif* 2006;24(4):387-93.

930. Chand DH, Teo BW, Fatica RA*, et al.* Influence of vascular access type on outcome measures in patients on maintenance hemodialysis. *Nephron Clin Pract* 2008;108(2):c91-8.

931. Charlson ME, Pompei P, Ales KL*, et al.* A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987;40(5):373-83.

932. Ifudu O, Uribarri J, Rajwani I*, et al.* Gender modulates responsiveness to recombinant erythropoietin. *Am J Kidney Dis* 2001;38(3):518-22.

933. Pan WH, Habicht JP. The non-iron-deficiency-related difference in hemoglobin concentration distribution between blacks and whites and between men and women. *Am J Epidemiol* 1991;134(12):1410-6.

934. Reeves WB, Fairman RM, Haurani FI. Influence of hormones on the release of iron by macrophages. *J Reticuloendothel Soc* 1981;29(3):173-9.

935. Fried W, Jonasson O, Lang G*, et al.* The hematologic effect of androgen in uremic patients. Study of packed cell volume and erythropoietin responses. *Ann Intern Med* 1973;79(6):823-7.

936. Holley JL, Schmidt RJ, Bender FH*, et al.* Gynecologic and reproductive issues in women on dialysis. *Am J Kidney Dis* 1997;29(5):685-90.

937. Servilla KS, Singh AK, Hunt WC*, et al.* Anemia management and association of race with mortality and hospitalization in a large not-for-profit dialysis organization. *Am J Kidney Dis* 2009;54(3):498-510.

938. Lacson E, Jr., Rogus J, Teng M*, et al.* The association of race with erythropoietin dose in patients on long-term hemodialysis. *Am J Kidney Dis* 2008;52(6):1104-14.

939. Petronis KR, Carroll CE, Held PJ*, et al.* Effect of race on access to recombinant human erythropoietin in long-term hemodialysis patients. *JAMA* 1994;271(22):1760-3.

940. Kurian AK, Cardarelli KM. Racial and ethnic differences in cardiovascular disease risk factors: a systematic review. *Ethn Dis* 2007;17(1):143-52.

941. Zakai NA, McClure LA, Prineas R*, et al.* Correlates of anemia in American blacks and whites: the REGARDS Renal Ancillary Study. *Am J Epidemiol* 2009;169(3):355-64.

942. Al-Jondeby MS, Cabaguing IT, Pajarillo AA*, et al.* Comparative crossover controlled study using polysulphone and vitamin E coated dialyzers. *Saudi Med J* 2003;24(3):265-8.

943. Westhuyzen J, Saltissi D, Stanbury V. Oxidative stress and erythrocyte integrity in end-stage renal failure patients hemodialysed using a vitamin E-modified membrane. *Ann Clin Lab Sci* 2003;33(1):3-10.

944. Opatrny K, Jr., Reischig T, Vienken J*, et al.* Does treatment modality have an impact on anemia in patients with chronic renal failure? Effect of low- and high-flux biocompatible dialysis. *Artif Organs* 2002;26(2):181-8.

945. Locatelli F, Andrulli S, Pecchini F*, et al.* Effect of high-flux dialysis on the anaemia of haemodialysis patients. *Nephrol Dial Transplant* 2000;15(9):1399-409.

946. Richardson D, Lindley EJ, Bartlett C*, et al.* A randomized, controlled study of the consequences of hemodialysis membrane composition on erythropoietic response. *Am J Kidney Dis* 2003;42(3):551-60.

947. Scott SD. Dose conversion from recombinant human erythropoietin to darbepoetin alfa: recommendations from clinical studies. *Pharmacotherapy* 2002;22(9 Pt 2):160S-165S.

948. Bonafont X, Bock A, Carter D*, et al.* A meta-analysis of the relative doses of erythropoiesis-stimulating agents in patients undergoing dialysis. *NDT Plus* 2009;2(5):347-353.

949. Itabe H. Oxidized low-density lipoprotein as a biomarker of in vivo oxidative stress: from atherosclerosis to periodontitis. *J Clin Biochem Nutr* 2012;51(1):1-8.

950. An WS, Kim SE, Kim KH*, et al.* Associations between oxidized LDL to LDL ratio, HDL and vascular calcification in the feet of hemodialysis patients. *J Korean Med Sci* 2009;24 Suppl:S115-20.

951. Honda H, Ueda M, Kojima S*, et al.* Oxidized high-density lipoprotein as a risk factor for cardiovascular events in prevalent hemodialysis patients. *Atherosclerosis* 2012;220(2):493-501.

952. Honda H, Ueda M, Kojima S*, et al.* Oxidized high-density lipoprotein is associated with protein-energy wasting in maintenance hemodialysis patients. *Clin J Am Soc Nephrol* 2010;5(6):1021-8.

953. Kirmizis D, Papagianni A, Dogrammatzi F*, et al.* Effects of simvastatin on markers of inflammation, oxidative stress and endothelial cell apoptosis in patients on chronic hemodialysis. *J Atheroscler Thromb* 2010;17(12):1256-65.

954. Osorio A, Ortega E, de Haro T*, et al.* Lipid profiles and oxidative stress parameters in male and female hemodialysis patients. *Mol Cell Biochem* 2011;353(1-2):59-63.

955. Ribeiro S, Faria MD, Silva G*, et al.* Oxidized low-density lipoprotein and lipoprotein(a) levels in chronic kidney disease patients under hemodialysis: Influence of adiponectin and of a polymorphism in the apolipoprotein(a) gene. *Hemodial Int* 2012;16(4):481-90.

956. Sevinc Ok E, Kircelli F, Asci G*, et al.* Neither oxidized nor anti-oxidized low-density lipoprotein level is associated with atherosclerosis or mortality in hemodialysis patients. *Hemodial Int* 2012;16(3):334-341.

957. Pfutzner A, Efstrathios K, Lobig M*, et al.* Differences in the results and interpretation of oxidized LDL cholesterol by two ELISA assays--an evaluation with samples from the PIOstat study. *Clin Lab* 2009;55(7-8):275-81.

958. Yagi K. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* 1976;15(2):212-6.

959. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic Biol Med* 1990;9(6):515-40.

960. Armstrong D, Browne R. The analysis of free radicals, lipid peroxides, antioxidant enzymes and compounds related to oxidative stress as applied to the clinical chemistry laboratory. *Adv Exp Med Biol* 1994;366:43-58.

961. Knight JA, Pieper RK, McClellan L. Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation. *Clin Chem* 1988;34(12):2433-8.

962. Bird RP, Draper HH. Comparative studies on different methods of malonaldehyde determination. *Methods Enzymol* 1984;105:299-305.

963. Hamberg M, Samuelsson B. On the mechanism of the biosynthesis of prostaglandins E-1 and F-1-alpha. *J Biol Chem* 1967;242(22):5336-43.

964. Diczfalusy U, Falardeau P, Hammarstrom S. Conversion of prostaglandin endoperoxides to C17-hydroxy acids catalyzed by human platelet thromboxane synthase. *FEBS Lett* 1977;84(2):271-4.

965. Bonnefont-Rousselot D, Lehmann E, Jaudon MC*, et al.* Blood oxidative stress and lipoprotein oxidizability in haemodialysis patients: effect of the use of a vitamin E-coated dialysis membrane. *Nephrol Dial Transplant* 2000;15(12):2020-8.

966. Wang YC, Lee WC, Liao SC*, et al.* Mitochondrial DNA copy number correlates with oxidative stress and predicts mortality in nondiabetic hemodialysis patients. *J Nephrol* 2011;24(3):351-8.

967. Khaira A, Mahajan S, Kumar A*, et al.* Endothelial function and oxidative stress in chronic kidney disease of varying severity and the effect of acute hemodialysis. *Ren Fail* 2011;33(4):411-7.

968. Gocmen AY, Sahin E, Kocak H*, et al.* Levels of asymmetric dimethylarginine, nitric oxide and lipid peroxidation markers in patients with end-stage renal disease having peritoneal dialysis treatment. *Clin Biochem* 2008;41(10-11):836-40.

969. Boero L, Cuniberti L, Magnani N*, et al.* Increased oxidized low density lipoprotein associated with high ceruloplasmin activity in patients with active acromegaly. *Clin Endocrinol* 2010;72(5):654-60.

970. Parthasarathy S, Raghavamenon A, Garelnabi MO*, et al.* Oxidized low-density lipoprotein. *Methods Mol Biol* 2010;610:403-17.

971. Seres DS, Strain GW, Hashim SA*, et al.* Improvement of plasma lipoprotein profiles during high-flux dialysis. *J Am Soc Nephrol* 1993;3(7):1409-15.

972. Goldberg IJ, Kaufman AM, Lavarias VA*, et al.* High flux dialysis membranes improve plasma lipoprotein profiles in patients with end-stage renal disease. *Nephrol Dial Transplant* 1996;11 Suppl 2:104-7.

973. Blankestijn PJ, Vos PF, Rabelink TJ*, et al.* High-flux dialysis membranes improve lipid profile in chronic hemodialysis patients. *J Am Soc Nephrol* 1995;5(9):1703-8.

974. Josephson MA, Fellner SK, Dasgupta A. Improved lipid profiles in patients undergoing high-flux hemodialysis. *Am J Kidney Dis* 1992;20(4):361-6.

975. House AA, Wells GA, Donnelly JG*, et al.* Randomized trial of high-flux vs low-flux haemodialysis: effects on homocysteine and lipids. *Nephrol Dial Transplant* 2000;15(7):1029-34.

976. Fisher WR, Hammond MG, Warmke GL. Measurements of the molecular weight variability of plasma low density lipoproteins among normals and subjects with hyper- -lipoproteinemia. Demonstration of macromolecular heterogeneity. *Biochemistry* 1972;11(4):519-25.

977. Fisher WR, Hammond MG, Mengel MC*, et al.* A genetic determinant of the phenotypic variance of the molecular weight of low density lipoprotein. *Proc Natl Acad Sci U S A* 1975;72(6):2347-51.

978. Huang XJ, Guduru D, Xu ZK*, et al.* Immobilization of heparin on polysulfone surface for selective adsorption of low-density lipoprotein (LDL). *Acta Biomater* 2010;6(3):1099-106.

979. Toshima S, Hasegawa A, Kurabayashi M*, et al.* Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. *Arterioscler Thromb Vasc Biol* 2000;20(10):2243-7.

980. Holvoet P, Vanhaecke J, Janssens S*, et al.* Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998;98(15):1487-94.

981. Zhang YC, Wei JJ, Wang F*, et al.* Elevated levels of oxidized low-density lipoprotein correlate positively with C-reactive protein in patients with acute coronary syndrome. *Cell Biochem Biophys* 2012;62(2):365-72.

982. Koenig W, Karakas M, Zierer A*, et al.* Oxidized LDL and the risk of coronary heart disease: results from the MONICA/KORA Augsburg Study. *Clin Chem* 2011;57(8):1196-200.

983. Meisinger C, Baumert J, Khuseyinova N*, et al.* Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation* 2005;112(5):651-7.

984. Li D, Yang B, Mehta JL. Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas. *Am J Physiol* 1998;275(2 Pt 2):H568-76.

985. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol* 2000;20(4):1116-22.

986. Morel DW, Hessler JR, Chisolm GM. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J Lipid Res* 1983;24(8):1070-6.

987. Khoo JC, Miller E, Pio F*, et al.* Monoclonal antibodies against LDL further enhance macrophage uptake of LDL aggregates. *Arterioscler Thromb* 1992;12(11):1258-66.

988. Han J, Hajjar DP, Febbraio M*, et al.* Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *J Biol Chem* 1997;272(34):21654-9.

989. Li D, Mehta JL. Antisense to LOX-1 inhibits oxidized LDL-mediated upregulation of monocyte chemoattractant protein-1 and monocyte adhesion to human coronary artery endothelial cells. *Circulation* 2000;101(25):2889-95.

990. Cominacini L, Pasini AF, Garbin U*, et al.* Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB

through an increased production of intracellular reactive oxygen species. *J Biol Chem* 2000;275(17):12633-8.

991. Drueke T, Witko-Sarsat V, Massy Z*, et al.* Iron therapy, advanced oxidation protein products, and carotid artery intima-media thickness in end-stage renal disease. *Circulation* 2002;106(17):2212-7.

992. Cesari M, Kritchevsky SB, Nicklas BJ*, et al.* Lipoprotein peroxidation and mobility limitation: results from the Health, Aging, and Body Composition Study. *Arch Intern Med* 2005;165(18):2148-54.

993. Tsuzura S, Ikeda Y, Suehiro T*, et al.* Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes. *Metabolism* 2004;53(3):297-302.

994. van Tits LJ, van Himbergen TM, Lemmers HL*, et al.* Proportion of oxidized LDL relative to plasma apolipoprotein B does not change during statin therapy in patients with heterozygous familial hypercholesterolemia. *Atherosclerosis* 2006;185(2):307-12.

995. Shoji T, Nishizawa Y, Fukumoto M*, et al.* Inverse relationship between circulating oxidized low density lipoprotein (oxLDL) and anti-oxLDL antibody levels in healthy subjects. *Atherosclerosis* 2000;148(1):171-7.

996. van der Zwan LP, Teerlink T, Dekker JM*, et al.* Circulating oxidized LDL: determinants and association with brachial flow-mediated dilation. *J Lipid Res* 2009;50(2):342-9.

997. Futatsuyama M, Oiwa T, Komatsu Y. Correlation between oxidized low-density lipoprotein and other factors in patients on peritoneal dialysis. *Adv Perit Dial* 2002;18:192-4.

998. Kimak E, Halabis M, Baranowicz-Gaszczyk I*, et al.* Association between moderately oxidized low-density lipoprotein and high-density lipoprotein particle subclass distribution in hemodialyzed and post-renal transplant patients. *J Zhejiang Univ Sci B* 2011;12(5):365-71.

999. Brouwers A, Langlois M, Delanghe J*, et al.* Oxidized low-density lipoprotein, iron stores, and haptoglobin polymorphism. *Atherosclerosis* 2004;176(1):189-95.

1000. Ikeda Y, Suehiro T, Yamanaka S*, et al.* Association between serum ferritin and circulating oxidized low-density lipoprotein levels in patients with type 2 diabetes. *Endocr J* 2006;53(5):665-70.

1001. Halliwell B. The role of oxygen radicals in human disease, with particular reference to the vascular system. *Haemostasis* 1993;23 Suppl 1:118-26.

1002. Puntarulo S. Iron, oxidative stress and human health. *Mol Aspects Med* 2005;26(4-5):299-312.

1003. Jomova K, Valko M. Importance of iron chelation in free radical-induced oxidative stress and human disease. *Curr Pharm Des* 2011;17(31):3460-73.

1004. Juckett MB, Balla J, Balla G*, et al.* Ferritin protects endothelial cells from oxidized low density lipoprotein in vitro. *Am J Pathol* 1995;147(3):782-9.

1005. Agarwal R, Vasavada N, Sachs NG*, et al.* Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. *Kidney Int* 2004;65(6):2279-89.

1006. Leehey DJ, Palubiak DJ, Chebrolu S*, et al.* Sodium ferric gluconate causes oxidative stress but not acute renal injury in patients with chronic kidney disease: a pilot study. *Nephrol Dial Transplant* 2005;20(1):135-40.

1007. Trevisan M, Browne R, Ram M*, et al.* Correlates of markers of oxidative status in the general population. *Am J Epidemiol* 2001;154(4):348-56.

1008. Garner B, Waldeck AR, Witting PK*, et al.* Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. *J Biol Chem* 1998;273(11):6088-95.

1009. Tabet F, Rye KA. High-density lipoproteins, inflammation and oxidative stress. *Clin Sci* 2009;116(2):87-98.

1010. Vaziri ND, Navab M, Fogelman AM. HDL metabolism and activity in chronic kidney disease. *Nat Rev Nephrol* 2010;6(5):287-96.

1011. Navab M, Hama SY, Hough GP*, et al.* High density associated enzymes: their role in vascular biology. *Curr Opin Lipidol* 1998;9(5):449-56.

1012. Laplaud PM, Dantoine T, Chapman MJ. Paraoxonase as a risk marker for cardiovascular disease: facts and hypotheses. *Clin Chem Lab Med* 1998;36(7):431-41.

1013. McMahon M, Grossman J, FitzGerald J*, et al.* Proinflammatory high-density lipoprotein as a biomarker for atherosclerosis in patients with systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Rheum* 2006;54(8):2541-9.

1014. Vekic J, Kotur-Stevuljevic J, Jelic-Ivanovic Z*, et al.* Association of oxidative stress and PON1 with LDL and HDL particle size in middle-aged subjects. *Eur J Clin Invest* 2007;37(9):715-23.

1015. Morena M, Cristol JP, Dantoine T*, et al.* Protective effects of high-density lipoprotein against oxidative stress are impaired in haemodialysis patients. *Nephrol Dial Transplant* 2000;15(3):389-95.

1016. Johansen JS, Harris AK, Rychly DJ*, et al.* Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovasc Diabetol* 2005;4(1):5.

1017. Coombes JS, Fassett RG. Antioxidant therapy in hemodialysis patients: a systematic review. *Kidney Int* 2012;81(3):233-46.

1018. Sharma A, Kharb S, Chugh SN*, et al.* Evaluation of oxidative stress before and after control of glycemia and after vitamin E supplementation in diabetic patients. *Metabolism* 2000;49(2):160-2.

1019. Van Dam PS, Van Asbeck BS, Erkelens DW*, et al.* The role of oxidative stress in neuropathy and other diabetic complications. *Diabetes Metab Rev* 1995;11(3):181-92.

1020. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 1999;48(1):1-9.

1021. Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. *Diabetes Care* 1996;19(3):257-67.

1022. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991;40(4):405-12.

1023. Chugh SN, Kakkar R, Kalra S*, et al.* An evaluation of oxidative stress in diabetes mellitus during uncontrolled and controlled state and after vitamin E supplementation. *J Assoc Physicians India* 1999;47(4):380-3.

1024. Dursun E, Dursun B, Suleymanlar G*, et al.* Effect of haemodialysis on the oxidative stress and antioxidants in diabetes mellitus. *Acta Diabetologica* 2005;42(3):123-8.

1025. Koken T, Serteser M, Kahraman A*, et al.* Changes in serum markers of oxidative stress with varying periods of haemodialysis. *Nephrology* 2004;9(2):77-82.

1026. Kalantar-Zadeh K, Kopple JD, Block G*, et al.* Association among SF36 quality of life measures and nutrition, hospitalization, and mortality in hemodialysis. *J Am Soc Nephrol* 2001;12(12):2797-806.

1027. Kushner I. The phenomenon of the acute phase response. *Ann N Y Acad Sci* 1982;389:39-48.

1028. Borawski J, Naumnik B, Mysliwiec M. Serum alpha1-antitrypsin but not complement C3 and C4 predicts chronic inflammation in hemodialysis patients. *Ren Fail* 2003;25(4):589-93.

1029. Goldstein SL, Ikizler TA, Zappitelli M*, et al.* Non-infected hemodialysis catheters are associated with increased inflammation compared to arteriovenous fistulas. *Kidney Int* 2009;76(10):1063-9.

1030. Shoji T, Emoto M, Shinohara K*, et al.* Diabetes mellitus, aortic stiffness, and cardiovascular mortality in end-stage renal disease. *J Am Soc Nephrol* 2001;12(10):2117-24.

1031. Miller M, Zhan M, Havas S. High attributable risk of elevated C-reactive protein level to conventional coronary heart disease risk factors: the Third National Health and Nutrition Examination Survey. *Arch Intern Med* 2005;165(18):2063-8.

1032. Muscari A, Massarelli G, Bastagli L*, et al.* Relationship between serum C3 levels and traditional risk factors for myocardial infarction. *Acta Cardiol* 1998;53(6):345-54.

1033. Morimoto Y, Taniguchi H, Yamashiro Y*, et al.* Complements in diabetes mellitus: activation of complement system evidenced by C3d elevation in IDDM. *Diabetes Res Clin Pract* 1988;5(4):309-12.

1034. McMurray DN, Beskitt PA, Newmark SR. Immunologic status in severe obesity. *Int J Obes* 1982;6(1):61-8.

1035. Ajjan R, Carter AM, Somani R*, et al.* Ethnic differences in cardiovascular risk factors in healthy Caucasian and South Asian individuals with the metabolic syndrome. *J Thromb Haemost* 2007;5(4):754-60.

1036. Siezenga MA, Chandie Shaw PK, van der Geest RN*, et al.* Enhanced complement activation is part of the unfavourable cardiovascular risk profile in South Asians. *Clin Exp Immunol* 2009;157(1):98-103.

1037. Hernandez-Mijares A, Jarabo-Bueno MM, Lopez-Ruiz A*, et al.* Levels of C3 in patients with severe, morbid and extreme obesity: its relationship to insulin resistance and different cardiovascular risk factors. *Int J Obes* 2007;31(6):927-32.

1038. Warnberg J, Nova E, Moreno LA*, et al.* Inflammatory proteins are related to total and abdominal adiposity in a healthy adolescent population: the AVENA Study. *Am J Clin Nutr* 2006;84(3):505-12.

1039. Onat A, Can G, Rezvani R*, et al.* Complement C3 and cleavage products in cardiometabolic risk. *Clin Chim Acta* 2011;412(13-14):1171-9.

1040. Gabrielsson BG, Johansson JM, Lonn M*, et al.* High expression of complement components in omental adipose tissue in obese men. *Obes Res* 2003;11(6):699-708.

1041. Brasier AR, Recinos A, 3rd, Eledrisi MS. Vascular inflammation and the renin-angiotensin system. *Arterioscler Thromb Vasc Biol* 2002;22(8):1257-66.

1042. Prasad K. C-reactive protein (CRP)-lowering agents. *Cardiovasc Drug Rev* 2006;24(1):33-50.

1043. Eiserich JP, Baldus S, Brennan ML*, et al.* Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 2002;296(5577):2391-4.

1044. Janssen-Heininger YM, Poynter ME, Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med* 2000;28(9):1317-27.

1045. Mares J, Richtrova P, Hricinova A*, et al.* Proteomic profiling of blood-dialyzer interactome reveals involvement of lectin complement pathway in hemodialysis-induced inflammatory response. *Proteomics Clin Appl* 2010;4(10-11):829-38.

1046. Mares J, Thongboonkerd V, Tuma Z*, et al.* Specific adsorption of some complement activation proteins to polysulfone dialysis membranes during hemodialysis. *Kidney Int* 2009;76(4):404-13.

1047. Derzsy Z, Prohaszka Z, Rigo J, Jr.*, et al.* Activation of the complement system in normal pregnancy and preeclampsia. *Mol Immunol* 2010;47(7-8):1500-6.

1048. Wamba PC, Mi J, Zhao XY*, et al.* Acylation stimulating protein but not complement C3 associates with metabolic syndrome components in Chinese children and adolescents. *Eur J Endocrinol* 2008;159(6):781-90.

1049. Cianflone K, Xia Z, Chen LY. Critical review of acylation-stimulating protein physiology in humans and rodents. *Biochim Biophys Acta* 2003;1609(2):127-43.

1050. Greenstein JD, Peake PW, Charlesworth JA. The kinetics and distribution of C9 and SC5b-9 in vivo: effects of complement activation. *Clin Exp Immunol* 1995;100(1):40-6.

1051. Baumgartner-Parzer SM, Seyfert UT, Mannhalter C. Possible clinical effects of the interaction of hemodialysis membranes with adhesion proteins. *Kidney Int* 1995;47(4):1115-20.

1052. Ohi H, Sakamoto K, Hatano M. S protein (vitronectin) in hemodialysis patients. *Nephron* 1990;54(2):190-1.

1053. Aparicio M, Cano N, Chauveau P*, et al.* Nutritional status of haemodialysis patients: a French national cooperative study. French Study Group for Nutrition in Dialysis. *Nephrol Dial Transplant* 1999;14(7):1679-86.

1054. Forte WC, Forte AC, Leao RC. Complement system in malnutrition. *Allergol Immunopathol* 1992;20(4):157-60.

1055. Haller L, Zubler RH, Lambert PH. Plasma levels of complement components and complement haemolytic activity in protein-energy malnutrition. *Clin Exp Immunol* 1978;34(2):248-52.

1056. Petersen BH, Watson RD, Holmes DH. Protein Malnutrition and complement activity in guinea pigs, germ-free and conventional rats. *J Nutr* 1980;110(11):2159-65.

1057. Lonnemann G, Novick D, Rubinstein M*, et al.* A switch to high-flux helixone membranes reverses suppressed interferon-gamma production in patients on low-flux dialysis. *Blood Purif* 2003;21(3):225-31.

1058. Amann K, Breitbach M, Ritz E*, et al.* Myocyte/capillary mismatch in the heart of uremic patients. *J Am Soc Nephrol* 1998;9(6):1018-22.

1059. Parfrey PS. Cardiac disease in dialysis patients: diagnosis, burden of disease, prognosis, risk factors and management. *Nephrol Dial Transplant* 2000;15 Suppl 5:58-68.

1060. Harnett JD, Foley RN, Kent GM*, et al.* Congestive heart failure in dialysis patients: prevalence, incidence, prognosis and risk factors. *Kidney Int* 1995;47(3):884-90.

1061. Silberberg JS, Barre PE, Prichard SS*, et al.* Impact of left ventricular hypertrophy on survival in end-stage renal disease. *Kidney Int* 1989;36(2):286-90.

1062. Drueke TB, Massy ZA. Atherosclerosis in CKD: differences from the general population. *Nat Rev Nephrol* 2010;6(12):723-35.

1063. Figueroa JE, Densen P. Infectious diseases associated with complement deficiencies. *Clin Microbiol Rev* 1991;4(3):359-95.

1064. Gajos G, Zalewski J, Rostoff P*, et al.* Reduced Thrombin Formation and Altered Fibrin Clot Properties Induced by Polyunsaturated Omega-3 Fatty Acids on Top of Dual Antiplatelet Therapy in Patients Undergoing Percutaneous Coronary Intervention (OMEGA-PCI Clot). *Arterioscler Thromb Vasc Biol* 2011;31(7):1696-702.

1065. Hirsh J, Anand SS, Halperin JL*, et al.* Guide to anticoagulant therapy: Heparin : a statement for healthcare professionals from the American Heart Association. *Circulation* 2001;103(24):2994-3018.

1066. Undas A, Wiek I, Stepien E*, et al.* Hyperglycemia is associated with enhanced thrombin formation, platelet activation, and fibrin clot resistance to lysis in patients with acute coronary syndrome. *Diabetes Care* 2008;31(8):1590-5.

1067. Kolev K, Tenekedjiev K, Komorowicz E*, et al.* Functional evaluation of the structural features of proteases and their substrate in fibrin surface degradation. *J Biol Chem* 1997;272(21):13666-75.

1068. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. *J Biol Chem* 1992;267(34):24259-63.

1069. Collet JP, Soria J, Mirshahi M*, et al.* Dusart syndrome: a new concept of the relationship between fibrin clot architecture and fibrin clot degradability: hypofibrinolysis related to an abnormal clot structure. *Blood* 1993;82(8):2462-9.

1070. Shah GA, Nair CH, Dhall DP. Comparison of fibrin networks in plasma and fibrinogen solution. *Thromb Res* 1987;45(3):257-64.

1071. Nair CH, Azhar A, Dhall DP. Studies on fibrin network structure in human plasma. Part One: Methods for clinical application. *Thromb Res* 1991;64(4):455-76.

1072. Chernysh IN, Weisel JW. Dynamic imaging of fibrin network formation correlated with other measures of polymerization. *Blood* 2008;111(10):4854-61.

1073. Nair CH, Dhall DP. Studies on fibrin network structure: the effect of some plasma proteins. *Thromb Res* 1991;61(3):315-25.

1074. Shah GA, Ferguson IA, Dhall TZ*, et al.* Polydispersion in the diameter of fibers in fibrin networks: consequences on the measurement of mass-length ratio by permeability and turbidity. *Biopolymers* 1982;21(6):1037-47.

1075. van Gelder JM, Nair CH, Dhall DP. The permeability of fibrin network developed in human plasma. *Blood Coagul Fibrinolysis* 1995;6(4):293-301.

1076. Shah GA, Nair CH, Dhall DP. Physiological studies on fibrin network structure. *Thromb Res* 1985;40(2):181-8.

1077. Lowe GD, Rumley A, Mackie IJ. Plasma fibrinogen. *Ann Clin Biochem* 2004;41(Pt 6):430-40.

1078. Stec JJ, Silbershatz H, Tofler GH*, et al.* Association of fibrinogen with cardiovascular risk factors and cardiovascular disease in the Framingham Offspring Population. *Circulation* 2000;102(14):1634-8.

1079. Zoccali C, Benedetto FA, Mallamaci F*, et al.* Fibrinogen, inflammation and concentric left ventricular hypertrophy in chronic renal failure. *Eur J Clin Invest* 2003;33(7):561-6.

1080. Hooper JM, Stuijver DJ, Orme SM*, et al.* Thyroid dysfunction and fibrin network structure: a mechanism for increased thrombotic risk in hyperthyroid individuals. *J Clin Endocrinol Metab* 2012;97(5):1463-73.

1081. Sundsmo JS, Fair DS. Relationships among the complement, kinin, coagulation, and fibrinolytic systems. *Springer Semin Immunopathol* 1983;6(2-3):231-58.

1082. Nydegger UE, Suter S. Pathophysiology of the interaction between complement and non-complement proteases. *Adv Exp Med Biol* 1984;167:227-34.

1083. Kazatchkine MD, Jouvin MH. Interactions between the alternative complement pathway and proteases of the coagulation system. *Adv Exp Med Biol* 1984;167:235-9.

1084. Roger M, Hogasen K, Holme PA*, et al.* The Fluid-phase SC5b-9 Terminal Complement Complex Binds to the GPIIb/IIIa Complex of Thrombin-stimulated Human Blood Platelets Inhibiting Platelet Aggregation. *Platelets* 1995;6(3):160-8.

1085. Lutjens A, te Velde AA, vd Veen EA*, et al.* Glycosylation of human fibrinogen in vivo. *Diabetologia* 1985;28(2):87-9.

1086. Pieters M, van Zyl DG, Rheeder P*, et al.* Glycation of fibrinogen in uncontrolled diabetic patients and the effects of glycaemic control on fibrinogen glycation. *Thromb Res* 2007;120(3):439-46.

1087. Hammer MR, John PN, Flynn MD*, et al.* Glycated fibrinogen: a new index of short-term diabetic control. *Ann Clin Biochem* 1989;26 (Pt 1):58-62.

1088. Henschen-Edman AH. Fibrinogen non-inherited heterogeneity and its relationship to function in health and disease. *Ann N Y Acad Sci* 2001;936:580-93.

1089. Coffey CS, Asselbergs FW, Hebert PR*, et al.* The Association of the Metabolic Syndrome with PAI-1 and t-PA Levels. *Cardiol Res Pract* 2011;2011:541467.

1090. McGill JB, Schneider DJ, Arfken CL*, et al.* Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. *Diabetes* 1994;43(1):104-9.

1091. Juhan-Vague I, Roul C, Alessi MC*, et al.* Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients--relationship with plasma insulin. *Thromb Haemost* 1989;61(3):370-3.

1092. Gray RP, Yudkin JS, Patterson DL. Plasminogen activator inhibitor: a risk factor for myocardial infarction in diabetic patients. *Br Heart J* 1993;69(3):228-32.

1093. Auwerx J, Bouillon R, Collen D*, et al.* Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. *Arteriosclerosis* 1988;8(1):68-72.

1094. Dunn EJ, Philippou H, Ariens RA*, et al.* Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia* 2006;49(5):1071-80.

1095. Mansfield MW, Stickland MH, Grant PJ. Environmental and genetic factors in relation to elevated circulating levels of plasminogen activator inhibitor-1 in Caucasian patients with non-insulin-dependent diabetes mellitus. *Thromb Haemost* 1995;74(3):842-7.

1096. Raiko JR, Oikonen M, Wendelin-Saarenhovi M*, et al.* Plasminogen activator inhitor-1 associates with cardiovascular risk factors in healthy young adults in the Cardiovascular Risk in Young Finns Study. *Atherosclerosis* 2012.

1097. Lindahl B, Asplund K, Eliasson M*, et al.* Insulin resistance syndrome and fibrinolytic activity: the northern Sweden MONICA study. *Int J Epidemiol* 1996;25(2):291-9.

1098. Akanji AO, Abdullah A, Tahzeeb S. Lipoprotein(a), tissue plasminogen activator and plasminogen activator inhibitor 1 levels in hyperlipidaemic patients in Kuwait. *Eur J Clin Invest* 1997;27(5):380-6.

1099. Talens S, Leebeek FW, Demmers JA*, et al.* Identification of fibrin clot-bound plasma proteins. *PLoS One* 2012;7(8):e41966.

1100. Akanji AO, Al-Isa AN, Thalib L. Determinants of blood levels of some thrombogenic biomarkers in healthy Arab adolescent subjects. *Clin Chem Lab Med* 2011;49(10):1681-90.

1101. Margaglione M, Cappucci G, d'Addedda M*, et al.* PAI-1 plasma levels in a general population without clinical evidence of atherosclerosis: relation to environmental and genetic determinants. *Arterioscler Thromb Vasc Biol* 1998;18(4):562-7.

1102. Eliasson M, Evrin PE, Lundblad D*, et al.* Influence of gender, age and sampling time on plasma fibrinolytic variables and fibrinogen: A population study. *Fibrinolysis* 1993;7(5):316-323.

1103. Libetta C, Zucchi M, Gori E*, et al.* Prevalence of hyperhomocysteinemia in male hemodialysis patients. *Kidney Int* 2003;64(4):1531.

1104. Nygard O, Vollset SE, Refsum H*, et al.* Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. *JAMA* 1995;274(19):1526-33.

1105. Undas A, Brozek J, Jankowski M*, et al.* Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. *Arterioscler Thromb Vasc Biol* 2006;26(6):1397-404.

1106. Shacter E, Williams JA, Lim M*, et al.* Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. *Free Radic Biol Med* 1994;17(5):429-37.

1107. Mori TA, Beilin LJ. Omega-3 fatty acids and inflammation. *Curr Atheroscler Rep* 2004;6(6):461-7.

1108. Vanschoonbeek K, Wouters K, van der Meijden PE*, et al.* Anticoagulant effect of dietary fish oil in hyperlipidemia: a study of hepatic gene expression in APOE2 knock-in mice. *Arterioscler Thromb Vasc Biol* 2008;28(11):2023-9.

1109. Nordoy A, Bonaa KH, Sandset PM*, et al.* Effect of omega-3 fatty acids and simvastatin on hemostatic risk factors and postprandial hyperlipemia in patients with combined hyperlipemia. *Arterioscler Thromb Vasc Biol* 2000;20(1):259-65.

1110. Barcelli U, Glas-Greenwalt P, Pollak VE. Enhancing effect of dietary supplementation with omega-3 fatty acids on plasma fibrinolysis in normal subjects. *Thromb Res* 1985;39(3):307-12.

1111. Kardys I, Uitterlinden AG, Hofman A*, et al.* Fibrinogen gene haplotypes in relation to risk of coronary events and coronary and extracoronary atherosclerosis: the Rotterdam Study. *Thromb Haemost* 2007;97(2):288-95.

1112. Keavney B, Danesh J, Parish S*, et al.* Fibrinogen and coronary heart disease: test of causality by 'Mendelian randomization'. *Int J Epidemiol* 2006;35(4):935-43.

1113. Carty CL, Cushman M, Jones D*, et al.* Associations between common fibrinogen gene polymorphisms and cardiovascular disease in older adults. The Cardiovascular Health Study. *Thromb Haemost* 2008;99(2):388-95.

1114. Smith GD, Harbord R, Milton J*, et al.* Does elevated plasma fibrinogen increase the risk of coronary heart disease? Evidence from a meta-analysis of genetic association studies. *Arterioscler Thromb Vasc Biol* 2005;25(10):2228-33.

1115. Valenzuela R, Shainoff JR, DiBello PM*, et al.* Immunoelectrophoretic and immunohistochemical characterizations of fibrinogen derivatives in atherosclerotic aortic intimas and vascular prosthesis pseudo-intimas. *Am J Pathol* 1992;141(4):861-80.

1116. Smith EB, Keen GA, Grant A*, et al.* Fate of fibrinogen in human arterial intima. *Arteriosclerosis* 1990;10(2):263-75.

1117. Tavora F, Cresswell N, Li L*, et al.* Immunolocalisation of fibrin in coronary atherosclerosis: implications for necrotic core development. *Pathology* 2010;42(1):15-22.

1118. Hicks RC, Golledge J, Mir-Hasseine R*, et al.* Vasoactive effects of fibrinogen on saphenous vein. *Nature* 1996;379(6568):818-20.

1119. Patibandla PK, Tyagi N, Dean WL*, et al.* Fibrinogen induces alterations of endothelial cell tight junction proteins. *J Cell Physiol* 2009;221(1):195-203.

1120. Tyagi N, Roberts AM, Dean WL*, et al.* Fibrinogen induces endothelial cell permeability. *Mol Cell Biochem* 2008;307(1-2):13-22.

1121. Naito M, Hayashi T, Kuzuya M*, et al.* Effects of fibrinogen and fibrin on the migration of vascular smooth muscle cells in vitro. *Atherosclerosis* 1990;83(1):9-14.

1122. Thompson WD, McGuigan CJ, Snyder C*, et al.* Mitogenic activity in human atherosclerotic lesions. *Atherosclerosis* 1987;66(1-2):85-93.

1123. Stirk CM, Kochhar A, Smith EB*, et al.* Presence of growth-stimulating fibrin degradation products containing fragment E in human atherosclerotic plaques. *Atherosclerosis* 1993;103(2):159-69.

1124. Naito M, Stirk CM, Smith EB*, et al.* Smooth muscle cell outgrowth stimulated by fibrin degradation products. The potential role of fibrin fragment E in restenosis and atherogenesis. *Thromb Res* 2000;98(2):165-74.

1125. Forsyth CB, Solovjov DA, Ugarova TP*, et al.* Integrin alpha(M)beta(2)-mediated cell migration to fibrinogen and its recognition peptides. *J Exp Med* 2001;193(10):1123-33.

1126. Retzinger GS, DeAnglis AP, Patuto SJ. Adsorption of fibrinogen to droplets of liquid hydrophobic phases. Functionality of the bound protein and biological implications. *Arterioscler Thromb Vasc Biol* 1998;18(12):1948-57.

1127. Rabbani LE, Loscalzo J. Recent observations on the role of hemostatic determinants in the development of the atherothrombotic plaque. *Atherosclerosis* 1994;105(1):1-7.

1128. Bennett JS. Platelet-fibrinogen interactions. *Ann N Y Acad Sci* 2001;936:340-54.

1129. Saha SA, Kizhakepunnur LG, Bahekar A*, et al.* The role of fibrates in the prevention of cardiovascular disease--a pooled meta-analysis of long-term randomized placebo-controlled clinical trials. *Am Heart J* 2007;154(5):943-53.

1130. Jun M, Foote C, Lv J*, et al.* Effects of fibrates on cardiovascular outcomes: a systematic review and meta-analysis. *Lancet* 2010;375(9729):1875-84.

1131. Staels B, Dallongeville J, Auwerx J*, et al.* Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* 1998;98(19):2088-93.

1132. Sugo T, Nakamikawa C, Takebe M*, et al.* Factor XIIIa cross-linking of the Marburg fibrin: formation of alpham.gamman-heteromultimers and the alpha-chain-linked albumin. gamma complex, and disturbed protofibril assembly resulting in acquisition of plasmin resistance relevant to thrombophila. *Blood* 1998;91(9):3282-8.

1133. Marchi R, Mirshahi SS, Soria C*, et al.* Thrombotic dysfibrinogenemia. Fibrinogen "Caracas V" relation between very tight fibrin network and defective clot degradability. *Thromb Res* 2000;99(2):187-93.

1134. Marchi R, Lundberg U, Grimbergen J*, et al.* Fibrinogen Caracas V, an abnormal fibrinogen with an Aalpha 532 Ser-->Cys substitution associated with thrombosis. *Thromb Haemost* 2000;84(2):263-70.

1135. Lefebvre P, Velasco PT, Dear A*, et al.* Severe hypodysfibrinogenemia in compound heterozygotes of the fibrinogen AalphaIVS4 + 1G>T mutation and an AalphaGln328 truncation (fibrinogen Keokuk). *Blood* 2004;103(7):2571-6.

1136. Collet JP, Woodhead JL, Soria J*, et al.* Fibrinogen Dusart: electron microscopy of molecules, fibers and clots, and viscoelastic properties of clots. *Biophys J* 1996;70(1):500-10.

1137. Carter AM, Catto AJ, Kohler HP*, et al.* alpha-fibrinogen Thr312Ala polymorphism and venous thromboembolism. *Blood* 2000;96(3):1177-9.

1138. Carter AM, Catto AJ, Grant PJ. Association of the alpha-fibrinogen Thr312Ala polymorphism with poststroke mortality in subjects with atrial fibrillation. *Circulation* 1999;99(18):2423-6.

1139. Standeven KF, Grant PJ, Carter AM*, et al.* Functional analysis of the fibrinogen Aalpha Thr312Ala polymorphism: effects on fibrin structure and function. *Circulation* 2003;107(18):2326-30.

1140. Ariens RA, Philippou H, Nagaswami C*, et al.* The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. *Blood* 2000;96(3):988-95.

1141. Elbaz A, Poirier O, Canaple S*, et al.* The association between the Val34Leu polymorphism in the factor XIII gene and brain infarction. *Blood* 2000;95(2):586-91.

1142. Franco RF, Pazin-Filho A, Tavella MH*, et al.* Factor XIII val34leu and the risk of myocardial infarction. *Haematologica* 2000;85(1):67-71.

1143. Wartiovaara U, Perola M, Mikkola H*, et al.* Association of FXIII Val34Leu with decreased risk of myocardial infarction in Finnish males. *Atherosclerosis* 1999;142(2):295-300.

1144. Kohler HP, Stickland MH, Ossei-Gerning N*, et al.* Association of a common polymorphism in the factor XIII gene with myocardial infarction. *Thromb Haemost* 1998;79(1):8-13.

1145. Lim BC, Ariens RA, Carter AM*, et al.* Genetic regulation of fibrin structure and function: complex gene-environment interactions may modulate vascular risk. *Lancet* 2003;361(9367):1424-31.

1146. Gersh KC, Nagaswami C, Weisel JW. Fibrin network structure and clot mechanical properties are altered by incorporation of erythrocytes. *Thromb Haemost* 2009;102(6):1169-75.

1147. Collet JP, Moen JL, Veklich YI*, et al.* The alphaC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis. *Blood* 2005;106(12):3824-30.

1148. Huijbregts HJ, Bots ML, Wittens CH*, et al.* Hemodialysis arteriovenous fistula patency revisited: results of a prospective, multicenter initiative. *Clin J Am Soc Nephrol.* 2008;3(3):714-9.

1149. Paraskevas KI, Mikhailidis DP, Roussas N*, et al.* Effect of Antiplatelet Agents, Statins, and Other Drugs on Vascular Access Patency Rates. *Angiology* 2012;63(1):5-8.

1150. Panichi V, Rizza GM, Paoletti S*, et al.* Chronic inflammation and mortality in haemodialysis: effect of different renal replacement therapies. Results from the RISCAVID study. *Nephrol Dial Transplant* 2008;23(7):2337-43.

1151. Abdalla MY, Hassan IM, Mustafa NH*, et al.* Total body glutathione depletion induces oxidative stress and disrupts the immune function in mice. *Toxicol Environ Chem* 2010;93(1):157-170.

1152. Lopez O, Bonnefont-Rousselot D, Mollereau M*, et al.* Increased plasma thiobarbituric acid-reactive substances (TBARS) before opportunistic infection symptoms in HIV infected individuals. *Clin Chim Acta* 1996;247(1-2):181-7.

1153. Mailloux LU, Bellucci AG, Napolitano B*, et al.* Survival estimates for 683 patients starting dialysis from 1970 through 1989: identification of risk factors for survival. *Clin Nephrol* 1994;42(2):127-35.

1154. Nairz M, Sonnweber T, Schroll A*, et al.* The pleiotropic effects of erythropoietin in infection and inflammation. *Microbes Infect* 2012;14(3):238-46.

1155. Nairz M, Schroll A, Moschen AR*, et al.* Erythropoietin contrastingly affects bacterial infection and experimental colitis by inhibiting nuclear factor-kappaB-inducible immune pathways. *Immunity* 2011;34(1):61-74.

1156. Sarnak MJ, Jaber BL. Mortality caused by sepsis in patients with end-stage renal disease compared with the general population. *Kidney Int* 2000;58(4):1758-64.

1157. Hauser AB, Stinghen AE, Kato S*, et al.* Characteristics and causes of immune dysfunction related to uremia and dialysis. *Perit Dial Int* 2008;28 Suppl 3:S183-7.

1158. Cohen G, Haag-Weber M, Horl WH. Immune dysfunction in uremia. *Kidney Int Suppl* 1997;62:S79-82.

1159. Kaplowitz LG, Comstock JA, Landwehr DM*, et al.* A prospective study of infections in hemodialysis patients: patient hygiene and other risk factors for infection. *Infect Control Hosp Epidemiol* 1988;9(12):534-41.

1160. Powe NR, Jaar B, Furth SL*, et al.* Septicemia in dialysis patients: incidence, risk factors, and prognosis. *Kidney Int* 1999;55(3):1081-90.

1161. Slinin Y, Foley RN, Collins AJ. Clinical epidemiology of pneumonia in hemodialysis patients: the USRDS waves 1, 3, and 4 study. *Kidney Int* 2006;70(6):1135-41.

1162. Sarnak MJ, Jaber BL. Pulmonary infectious mortality among patients with end-stage renal disease. *Chest* 2001;120(6):1883-7.

1163. Ng LJ, Chen F, Pisoni RL*, et al.* Hospitalization risks related to vascular access type among incident US hemodialysis patients. *Nephrol Dial Transplant* 2011;26(11):3659-66.

1164. Lee H, Manns B, Taub K*, et al.* Cost analysis of ongoing care of patients with end-stage renal disease: the impact of dialysis modality and dialysis access. *Am J Kidney Dis* 2002;40(3):611-22.

1165. Okumiya T, Jiao Y, Saibara T*, et al.* Sensitive enzymatic assay for erythrocyte creatine with production of methylene blue. *Clin Chem* 1998;44(7):1489-96.

1166. Torato T, Doi K, Negishi K*, et al.* Efficacy of vitamin E-bonded polysulfone dialyzer and polysulfone dialyzer on a series of non-anticoagulant hemodialysis. *ASAIO J* 2013;59(3):284-5.

1167. Vilar E, Wellsted D, Chandna SM*, et al.* Residual renal function improves outcome in incremental haemodialysis despite reduced dialysis dose. *Nephrol Dial Transplant* 2009;24(8):2502-10.

1168. Aguilera A, Teruel JL, Villatruela JJ*, et al.* Effect of vitamin E administration on erythropoietin values and anaemia in hemodialysis patients. *Nephrol Dial Transplant* 1993;8(4):379.

1169. Ardalan MR, Tubbs RS, Shoja MM. Vitamin E and selenium co-supplementation attenuates oxidative stress in haemodialysis patients receiving intra-dialysis iron infusion. *Nephrol Dial Transplant* 2007;22(3):973-5.

1170. Diepeveen SHA, Verhoeven GWHE, Van Der Palen J*, et al.* Effects of atorvastatin and vitamin E on lipoproteins and oxidative stress in dialysis patients: a randomised-controlled trial. *J Intern Med* 2005;257(5):438-45.

1171. Himmelfarb J, Phinney S, Ikizler TA*, et al.* Gamma-tocopherol and docosahexaenoic acid decrease inflammation in dialysis patients. *J Ren Nutr* 2007;17(5):296-304.

1172. Hodkova M, Dusilova-Sulkova S, Skalicka A*, et al.* Influence of parenteral iron therapy and oral vitamin E supplementation on neutrophil respiratory burst in chronic hemodialysis patients. *Ren Fail* 2005;27(2):135-41.

1173. Inal M, Kanbak G, Sen S*, et al.* Antioxidant status and lipid peroxidation in hemodialysis patients undergoing erythropoietin and erythropoietin-vitamin E combined therapy. *Free Radic Res* 1999;31(3):211-6.

1174. Lillo-Ferez M, Allain B, Dupommereulle C*, et al.* Inefficacy of vitamin E supplementation on anemia in hemodialysis patients. *Nephron* 1987;45(1):79-80.

1175. Lu L, Erhard P, Salomon RG*, et al.* Serum vitamin E and oxidative protein modification in hemodialysis: a randomized clinical trial. *Am J Kidney Dis* 2007;50(2):305-13.

1176. Mafra D, Santos FR, Lobo JC*, et al.* Alpha-tocopherol supplementation decreases electronegative low-density lipoprotein concentration [LDL(-)] in haemodialysis patients. *Nephrol Dial Transplant* 2009;24(5):1587-92.

1177. Sanaka T, Takahashi C, Sanaka M*, et al.* Accumulation of phosphatydilcholine-hydroperoxide in dialysis patients with diabetic nephropathy. *Clin Nephrol* 1995;44 Suppl 1:S33-7.

1178. Sinsakul V, Drake JR, Leavitt JN, Jr.*, et al.* Lack of effect of vitamin E therapy on the anemia of patients receiving hemodialysis. *Am J Clin Nutr* 1984;39(2):223-6.

1179. Yukawa S, Hibino A, Maeda T*, et al.* Effect of alpha-tocopherol on in vitro and in vivo metabolism of low-density lipoproteins in haemodialysis patients. *Nephrol Dial Transplant* 1995;10 Suppl 3:1-3.

1180. Baragetti I, Furiani S, Vettoretti S*, et al.* Role of vitamin E-coated membrane in reducing advanced glycation end products in hemodialysis patients: a pilot study. *Blood Purif* 2006;24(4):369-76.

1181. Clermont G, Lecour S, Cabanne JF*, et al.* Vitamin E-coated dialyzer reduces oxidative stress in hemodialysis patients. *Free Radic Biol Med* 2001;31(2):233-41.

1182. Dhondt A, Vanholder R, Glorieux G*, et al.* Vitamin E-bonded cellulose membrane and hemodialysis bioincompatibility: absence of an acute benefit on expression of leukocyte surface molecules. *Am J Kidney Dis* 2000;36(6):1140-6.

1183. Kojima K, Oda K, Homma H*, et al.* Effect of vitamin E-bonded dialyzer on eosinophilia in haemodialysis patients. *Nephrol Dial Transplant* 2005;20(9):1932-5.

1184. Miyazaki H, Matsuoka H, Itabe H*, et al.* Hemodialysis impairs endothelial function via oxidative stress: effects of vitamin E-coated dialyzer. *Circulation* 2000;101(9):1002-6.

1185. Morena M, Gausson V, Mothu N*, et al.* Regional variations of low-density lipoprotein oxidizability in hemodialysis patients may explain discrepancies in interventional therapy on oxidative profile. *Blood Purif* 2008;26(3):300-10.

1186. Sato M, Morita H, Ema H*, et al.* Effect of different dialyzer membranes on cutaneous microcirculation during hemodialysis. *Clin Nephrol* 2006;66(6):426-32.

1187. Shimazu T, Ominato M, Toyama K*, et al.* Effects of a vitamin E-modified dialysis membrane on neutrophil superoxide anion radical production. *Kidney Int Suppl* 2001;78:S137-43.

1188. Yang CC, Hsu SP, Wu MS*, et al.* Effects of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress. *Kidney Int* 2006;69(4):706-14.

1189. Zaluska WT, Ksiazek A, Roliski J. Effect of vitamin E modified cellulose membrane on human lymphocyte, monocyte, and granulocyte CD11b/CD18 adhesion molecule expression during hemodialysis. *ASAIO J* 2001;47(6):619-22.

1190. National Institute for Health and Clinical Excellence, UK. Anaemia management in people with chronic kidney disease (Clinical Guideline 114). *London: Naitional Institute for Health and Clinical Excellence* 2011.

1191. Kalicki RM, Uehlinger DE. Red cell survival in relation to changes in the hematocrit: more important than you think. *Blood Purif* 2008;26(4):355-60.

1192. Uehlinger DE, Gotch FA, Sheiner LB. A pharmacodynamic model of erythropoietin therapy for uremic anemia. *Clin Pharmacol Ther* 1992;51(1):76-89.

Appendices

Appendix A Supplementary data tables

Table 52 - Studies of vitamin E supplementation in dialysis patients - effects on oxidative stress, inflammation, anaemia, lipids and cardiovascular outcomes.

Study	*Duration (days)*	*Dose*	*No. HD patients receiving VE*	*Reported effects of Vitamin E*
Aguilera *et al*, 1993 [1168]	90	400 mg	24	Hb→, ESA →, Hct →
Antoniadi *et al*, 2004 [808]	365	500 mg αT	27	↓TAC, ↓SOD
Antoniadi *et al*, 2008 [810]	365	500 mg αT	27	↓TAC, ↓SOD
Ardalan *et al*, 2007[1] [1169]	1	400 IU	19	→MDA
Badiou *et al*, 2003 [815]	180	500 mg	14	↓TBARS and ↓LDL susceptibility to oxidation
Baldi *et al*., 2012 [803]	84	800 IU	10	↓LDL susceptibility to oxidation
Boaz *et al*, 2000 [825]	519	800 IU	97	↓composite CV events endpoint, →mortality
Cristol *et al*, 1997 [821]	180	500 mg	7	↓ESA requirements
Diepeveen *et al*, 2005[2] [1170]	84	800 IU	10	↑ox-LDL
Galli *et al*, 2001[3] [864]	21	800 mg	7	→TBARS, ↑polyunsaturated:saturated fatty acids ratio
Gallucci *et al*, 1986 [806]	15	300 mg IM	10	↓MDA
Giardini *et al*, 1984 [812]	15	300 mg IM	19	↓MDA, ↑Hct
Giray *et al*, 2003 [807]	98	600 mg	36	↓TBARS, ↑SOD, ↑GPx
Himmelfarb *et al*, 2003 [389]	14	300 mg	9 αT / 5 γT enriched	γT: ↓CRP, →IL-6 αT: →CRP, ↑IL-6
Himmelfarb *et al*, 2007[4] [1171]	56	308 mg γT	31	→PGF2α, →carbonyl levels, ↓IL-6, →CRP
Hodkova *et al*, 2005[5] [1172]	7	200 mg	7	No effect on neutrophil respiratory burst activity
Hodkova *et al*, 2006 [824]	35	400 mg αT	15	→CRP, →ICAM-1, →E-selectin
Inal *et al*, 1999 [1173]	120	300 mg	36	↑SOD, ↓ESA
Islam *et al*, 2000 [814]	84	800 IU	33	↓LDL susceptibility to oxidation
Kamgar *et al*, 2009[6] [822]	56	800 IU	20	→PGF2α, →CRP, →IL-6
Lilli-Ferez *et al*, 1987 [1174]	30	600 mg	10	→Hb, →Hct, →Retics
Lu *et al*, 2007 [1175]	180	800 IU	13	No change in oxidative protein damage
Lubrano *et al*, 1986 [811]	15	300 mg IM	9	↓MDA, ↓EOF

Lubrano *et al*, 1992 [809]	15	300 mg IM	10	↓PBMC oxidation
Mafra *et al*, 2009 [1176]	120	400 IU	19	↓Total cholesterol, ↓LDL-cholesterol, ↓LDL(-)
Mydlik *et al*, 2001 [827]	21	400 mg	8	→MDA, →TAC
Nemeth *et al*, 2000[7] [813]	14	15 mg/kg	10	↓oxidised glutathione
Ono *et al*, 1985 [820]	30	600mg	15	↑Hct, ↓EOF
Roob *et al*, 2000[5] [368]	1	800 IU	22	↓Iron-induced lipid peroxidation
Roozbeh *et al*, 2011 [817]	21	400 IU	20	↑GPx, →MDA, ↑Hb
Sanaka *et al*, 1995[8] [1177]	365	500 mg	11	No effect on lipid peroxidation
Sato *et al*, 2003[9] [804]	180	600 mg	8	↓TBARS
Sinsakul *et al*, 1984 [1178]	140	800 IU	16	→Hct, →Blood transfusion requirements
Smith *et al*, 2003 [823]	60	400 IU	11	→ PGF2α, ↑Hct, →IL-6, →CRP, →TNF-α
Turi *et al*, 1992[10] [818]	14	15mg/kg	10	↑Hb, ↑Hct
Uzum *et al*, 2006 [816]	140	300 mg	19	↓MDA, ↓EOF
Yalcin *et al*, 1989 [805]	30	300 mg	20	↓MDA
Yeksan *et al*, 1991 [819]	56	300 mg	12	↑Hb, ↑serum EPO
Yukawa *et al*, 1995 [1179]	14	600 mg	5	Improved MDA-LDL metabolism

Notes:
1. Given 6 hours post intravenous iron in conjunction with selenium 600 µg
2. Cohort included patients on haemodialysis and peritoneal dialysis
3. Patients with low levels of vitamin E at baseline
4. Vitamin E supplement principally contained γ-tocopherol and patients also received docosahexaenoic acid
5. In conjunction with intravenous iron
6. Supplements also contained Vitamins C, B & folic acid
7. Paediatric study. Supplemented from 2 weeks after commencing ESA
8. Diabetics
9. Patients with evidence of microcirculatory disturbance. Supplement also contained vitamin C
10. Paediatric study. Supplementation followed 4 week washout from ESA therapy

αT/γT: Alpha-/Gamma-tocopherol; CRP: C-reactive protein; CV: Cardiovascular; EOF: Erythrocyte osmotic fragility; EPO: Erythropoietin; ESA: Erythropoietin stimulating agent (dose); GPx: glutathione peroxidase; Hb: Haemoglobin; Hct: Haematocrit; ICAM: Inter-cellular adhesion molecule 1; IL: Interleukin; IU: International Units; iv Fe: intravenous iron; LDL: Low-density lipoprotein; MDA: Malondialdehyde; PBMC: Peripheral blood mononuclear cell; PGF2α: Prostaglandin $F_{2\alpha}$; SOD: Superoxide dismutase activity; TAC: Total anti-oxidant capacity; TBARS: Thiobarbituric acid reactive species; TNF-α: Tumour necrosis factor-α; VE: Vitamin E.

Table 53 - Studies examining the effects of vitamin E bonded modified cellulose dialysis membranes on oxidative stress, inflammation, anaemia, leukocyte function and complement activation.

Study	*Duration*	*No. pts on VEM*	*Reported effects of VE membrane*
Al-Jondeby *et al*, 2003 [942]	4 weeks	75	→Hb, ↑dialyser clotting
Baragetti *et al*, 2006 [1180]	42 weeks	8	↓Free- and protein bound pentosidine, ↓AGEs, →endothelial function, →ox-LDL, →Homocysteine
Bonnefont-Rousselot *et al*, 2000 [965]	3 months	12	→TBARS, →TAS, →SOD, →GPx
Bufano *et al*, 2004 [871]	6 months	16	↓ox-LDL
Buoncristiani *et al*, 1997 [859]	30 days	10	↓MDA, ↑GSH
Calzavara *et al*, 1999 [875]	1 month	5	↓dysmorphic RBCs
Clermont *et al*, 2000 [1181]	1 month	16	↓ascorbyl free radical / vitamin C ratio
Cruz *et al*, 2008 [876]	1 year	172	↑Hb, ↓ESA dose
Dhondt *et al*, 2000 [1182]	4 weeks	10	↑leukopenia, ↑CD11b, CD11c & CD45 expression, ↓leukocyte responsiveness
Eiselt *et al*, 2001 [866]	4 weeks	20	↓TBARS
Galli *et al*, 1998 [873]	3 months	15	↑GSH, ↑leukocyte responsiveness
Galli *et al*, 2001 [864]	3 months	15	↓TBARS
Girndt *et al*, 2000 [880]	4 weeks	21	↓IL-6 production
Hara *et al*, 2004 [853]	1 year	13	↓ox-LDL
Huraib *et al*, 2000[1] [877]	2 months	20	↓Dialyser clotting, ↓ESA, ↑Hb (Only with low-flux membranes. No significant effects for patients on high-flux membranes)
Kirmizis *et al*, 2011 [858]	6 months	35	↓CRP, ↓IL-6, ↓sICAM, ↓ox-LDL, ↓TBARS, ↓TAS
Kobayashi *et al*, 2003[2] [879]	1 year	34	↓Carotid IMT, ↓RBC viscosity, ↓ESA, ↓dysmorphic RBCs
Kojima *et al*, 2005 [1183]	4 weeks	7	↓Eosinphilia, ↓IL-5, →IgE, ↓CD4 +ve lymphocytes
Miyazaki *et al*, 2000 [1184]	single session	12	→ox-LDL, → endothelial dysfunction[3]
Morena *et al*, 2008 [1185]	62 patients: 33 for 3 months / 29 for 6 months		→ox-LDL, →total glutathione, →AOPP
Muller *et al*, 2004 [844]	4 weeks	9	↓oxidative DNA damage, ↑MDA (compared to polysulfone, data not provided in paper)
Mune *et al*, 1999 [862]	2 years	25	↓ox-LDL, ↓MDA, ↓increase in aortic caclification

Mydlik *et al*, 2004 [872]	3 months	14	↓MDA, ↑TAC, ↑GSH
Mydlik *et al*, 2001 [827]	3 weeks	8	↓MDA, ↑TAC
Nakatan *et al*, 2003 [878]	12 months	18	↑Hb, ↑HCt, ↑RBC count, ↑RBC lifespan
Odetti *et al*, 2006 [874]	single session	8	↓free 4-hydroxyl-2-nonenal
Odetti *et al*, 1999 [855]	single session	8	↓TBARS, →Protein carbonyl groups, ↓protein glycoxidation
Omata *et al*, 2000 [857]	10 weeks	7	↓Neutropenia, ↓CD11b expression, ↓MPO, ↓C3a
Pertosa *et al*, 1999 [881]	3 months	6	↓Pre- & post-HD sC5b-9 & complement Bb fragment, ↓intradialytic PBMC activation
Pertosa *et al*, 2002 [883]	3 months	8	↓Jun N-terminal kinase activation, ↓C5b-9 generation
Sanaka *et al*, 1999 [851]	5 weeks	7	↓leukopenia, ↓MPO, ↓C3a
Sato *et al*, 2006 [1186]	single session	11	→TBARS[4]
Satoh *et al*, 2001 [865]	6 months	18	↓post-dialysis MDA, AGE, 8-OhdG; ↓basal AGE and 8-OhdG
Schieke *et al*, 1999 [850]	18 weeks	12	↓MDA, →endothelial function, →spontaneous & ↑inducible PBMC activity
Senatore *et al*, 2002 [882]	6 months	30	↓β_2-microglobulin, ↓ferritin, ↓IgG, Normalisation of C3
Shimazu *et al*, 2001 [852, 1187]	9 months	6	↓MDA, ↓ox-LDL, ↑superoxide anion radical producing ability, ↑plasma hydroxyl radical producing ability, ↓superoxide anion scavenging activity
Sommerburg *et al*, 1999 [860]	6 weeks	10	↓MDA
Taccone-Gallucci *et al*, 1999 [856]	12 months	10	→Hb, →Hct, →RBC count, ↑Reticulocytes, ↓RBC MDA
Takouli *et al*, 2010 [854]	3 months	9	↓dROMS, ↑TAC, ↓SOD, ↓CRP, ↓IL-6
Tarng *et al*, 2000 [863]	8 weeks	36	↓8-OhdG (only compared to cellulose membrane)
Triolo *et al*, 2003 [870]	12 months	10	↓MDA, →Hb, RBC count, Hct, ESA
Tsuruoka *et al*, 2002 [867]	12 weeks	10	↓ox-LDL, ↓LDL, ↓MDA, ↓predialysis PMN superoxide production, ↓leukopenia
Usberti *et al*, 1999[5] [861]	10 months	11	↑RBC lifespan, ↓dROMS, ↓MDA, ↓ESA
Usberti *et al*, 2002 [868]	7-9 months	9	↓MDA, ↑RBC survival (for pts on low dose ESA), ↓Thiol levels, ↑"anaemia correction"

Usberti *et al*, 2002[5] [869]	7 months	38	↓ox-LDL, ↓MDA, →TAS, →Thiols, ↑RBC survival, ↓ESA
Westhuyzen *et al*, 2003 [943]	13 weeks	12	→Hb, →ESA, →SOD, →GSH, ↑GPx, ↓erythrocyte susceptibility to haemolysis[6], ↓Saturated & ↑polyunsaturated lipid composition of erythrocyte membrane
Yang *et al*, 2006 [1188]	2 months	40[7]	→Plasma H_2O_2 activity, →TAS, ↓plasma & erythrocyte PCOOH, →CRP
Zaluska *et al*, 2001 [1189]	20 HD sessions	10	↓post-dialysis monocyte and granulocyte CD11b/CD18 expression

Notes:
1. Patients with frequent dialyser clotting. 10 pts on low-flux and 10 pts on high-flux membranes
2. Patients with sustained eosinophilia at baseline selected (>700 /µL)
3. Dialysis with non vitamin E coated membrane led to ↑ox-LDL and ↑endothelial dysfunction. Endothelial dysfunction measured by flow-mediated vasodilation of brachial artery.
4. Patients on Vitamin E membrane for 2 weeks but only TBARS levels pre- and post- single dialysis session at end of 2 week period reported.
5. Patients were also given reduced glutathione 1200 mg intravenously after every dialysis session.
6. Reduced erythrocyte susceptibility to haemolysis observed at 6 weeks compared to baseline but 13 week samples returned to baseline values
7. 20/40 patients also received 1g vitamin C infusion on dialysis

8-OhdG: 8-hydroxy-2'-deoxyguanosine; AGEs: Advanced glycation end products; AOPP: Advanced oxidation protein products; dROMS: Reactive oxygen metabolites and derivatives; GPx: Glutathione peroxidise activity; GSH: Reduced glutathione; IgG/IgE: Immunoglobulin G/E; H_2O_2: Hydrogen peroxide; IMT: Intima media thickness; MDA: Malondialdehyde; MPO: myeloperoxidase; PBMC: Peripheral blood mononuclear cell; PCOOH: Phosphatidylcholine hydroperoxide; RBC: Red blood cell; SOD: Superoxide dismutase activity; TAC: Total antioxidant capacity; TAS: Total antioxidant status; VEM: Vitamin E membrane

Appendix B Responsibilities of core research team members

Team member (*Job role*)	**Responsibilities**
Dr Simon Lines (*Principal Investigator*)	Principal Investigator for study Study design and study protocol Obtaining ethical approval and LTHT Research and Development sponsorship Securing study funding Adoption of study on to the National Institute for Health Research (NIHR) portfolio Patient screening for study eligibility Consenting patients Study visits including blood sampling and data collection Collection of hospital admission and mortality data Sample processing Laboratory assays All data analysis and interpretation
Dr Mark Wright (*Research Supervisor*)	Securing study funding Input into study design – clinical aspects
Dr Angela Carter (*Research Supervisor*)	Input into study design – laboratory aspects Laboratory supervision
Dr Emma Dunn (*Research Supervisor*)	Input into study design – clinical and laboratory aspects
Ms Victoria Richardson (*PhD student*)	Technician assistance: C3 ELISA and TBARS assay
Ms Jia-Ying Lee (*Visiting PhD student*)	Technician assistance: C3 ELISA, initial properdin ELISA optimisation assay
Rosalyn Wheatley (Lead *Research Nurse*)	Obtaining informed patient consent Co-ordinated study visits Undertook study visits and blood sampling Co-ordinated and undertook dialyser adherence monitoring
Shyama Rughooputh (*Research Nurse*)	Undertook study visits and blood sampling
Stuart Turner (*Research Nurse*)	Undertook study visits and blood sampling Monitoring of dialyser adherence
Emma Giddings (*Research Nurse*)	Obtaining informed patient consent Undertook study visits and blood sampling
Frank Lee (*Clerical support*)	Entered recruitment data on to NIHR database Monitoring of dialyser adherence Administrative support

Appendix C Leeds teaching hospitals laboratory assays

Siemens ADVIA 1800 and 2400 Biochemistry analysers

- Urea and electrolytes
- Lipids (Cholesterol, LDL, HDL, Triglycerides)
- C-reactive protein
- Calcium
- Phosphate
- Bicarbonate

Siemens ADVIA Centaur Immunoassay analyser

- Parathyroid hormone (2-site sandwich immunoassay)

Bayer 2120 Analyser

- Full blood count

Instrumentation Laboratory Automated Coagulation Laboratory (ACL TOP)

- Activated partial thromboplastin time, prothrombin time
- Fibrinogen (Clauss method)

Appendix D Anaemia management algorithm

D.1 ESA dosing

The ESA dosing for all study patients was carried out by means of a predictive algorithm. The details of the algorithm and the LTHT experience of using it have been published in 2012 [901]. The ESA used for all study patients was darbepoetin alfa (Aranesp®, Amgen, UK) and the target haemoglobin concentration was 10.5-12.5 g/dL in keeping with the contemporaneous National Institute for Health and Clinical Excellence (NICE) recommendations [904]. (Since this study commenced, NICE have issued new guidance recommending a target haemoglobin range of 10-12 g/dL [1190]). The main tenet of the algorithm is that it takes approximately three months for the haemoglobin level to reach steady state following the initiation of an ESA or a change in the dose. The steady state haemoglobin concentration is predicted using linear projection of two haemoglobin levels measured at one and two months following a change in ESA dose. This three months window is based on the pharmacodynamics of ESAs and the altered red blood cell lifespan in haemodialysis patients [1191, 1192]. No changes to the ESA dose are recommended if insufficient time has elapsed to predict the steady-state haemoglobin level, which in practice prevents dose changes at intervals of less than 2 months. This helps to ensure that the effects of an ESA dose change are fully evaluated before another change is made. The magnitude of the dose change is proportional to the difference between the predicted steady state haemoglobin level and the population mean target of 11.5 g/dL, as detailed in Table 54 and Table 55. All doses are rounded up or down to the nearest pre-filled syringe sizes: 10 µg, 15 µg, 20 µg, 30 µg, 40 µg, 50 µg, 60 µg, 80 µg, 100 µg, 130 µg, 150 µg, 300 µg and 500 µg.

Table 54 - Definitions used in ESA dosing algorithm.

Hb_{target}
- Mid point of target range, i.e. 11.5 g/dL

$Hb_{steady\ state}$
- Measured Hb >90 days after last ESA dose change

OR
- Predicted Hb at 90 days after last ESA dose change by linear extrapolation of Hb levels measured >=14 days and >= 42 days following a change in dose

ΔHb
- Hb_{target} - $Hb_{steady\ state}$

Hb: Haemoglobin concentration

Table 55 - Darbepoetin alfa algorithmic dose adjustments.

Current dose	*New dose*
Starting dose	
0 mcg/wk	10 x ΔHb [Maximum starting dose 30 mcg/wk]
Dose increases	
2.5 mcg/wk	5 mcg/wk
5 mcg/wk	7.5 mcg/wk if ΔHb <3 g/dl, otherwise 10 mcg/wk
10 mcg/wk	15 mcg/wk if ΔHb <3 g/dl, otherwise 20 mcg/wk
15 mcg/wk	20 mcg/wk if ΔHb <3 g/dl, otherwise 30 mcg/wk
20 mcg/wk	30 mcg/wk
>20 mcg/wk	((ΔHb x 0.17) + 1) x Current dose [Max increase 50%]
Dose reductions	
2.5 mcg/wk*	Stop ESA
5 mcg/wk	2.5 mcg/wk
> 5 mcg/wk	((ΔHb x 0.17) + 1) x Current dose [Max decrease 50%]

*The lowest dose is 10 mcg given every 4 weeks i.e. 2.5 mcg/wk

D.2 Iron dosing

Iron deficiency results in anaemia which is poorly responsive to correction by ESAs but identifying iron deficiency in HD patients is not straightforward as they have functional iron deficiency (see 1.3.2.1) and frequently employed indices of iron stores, such as ferritin, are subject to variation from influences such as inflammation. Administration of intravenous iron to HD patients has been shown to reduce ESA requirements [268], even in patients with high ferritin levels [264, 269], and published guidelines recommend the regular administration of parenteral iron to chronic haemodialysis patients. For over a decade, iron dosing for LTHT haemodialysis patients has been determined by a computer aided decision support system based on the monthly measurement of ferritin and haemoglobin levels, the percentage of hypochromic red blood cells and the mean cell volume (MCV). The iron preparation used was iron sucrose (Venofer®, Syner-Med Pharmaceuticals, UK). The default position was for all patients to receive a maintenance dose of 100 mg of iron sucrose fortnightly on dialysis. This dose was augmented or withheld if patients were deemed iron deficient or at risk of iron overload as set out below.

For the purposes of the decision support system, patients were classified as iron deficient according to the criteria set out in Table 56. One point was scored for each criterion

satisfied and if a patients scored ≥3 points and had a haemoglobin level <12.5 g/dL they were classed as iron deficient. Scoring was based on measurements made within the last 91 days and if more than two measurements had been made, separated by at least 21 days, an average value was calculated and processed by the algorithm. If patients were classed as iron deficient, an ESA was not commenced or the dose increased until they had received intravenous iron treatment for at least a 42 day period (i.e. minimum of 1 g intravenous iron).

Table 56 - Identification of iron deficiency by iron dosing decision support system.

Haemoglobin < 12.5 g/dl	***Mandatory***
Ferritin < 200 μg/l	1 point
Ferritin < 100 μg/l	1 point
Mean red cell volume <85 fl	1 point
Mean red cell volume <75 fl	1 point
Hypochromic red cells >6%	1 point
Hypochromic red cells >13%	1 point
Haemoglobin <10.5 g/dl	1 point
≥3 points = iron deficiency	

As already discussed, in the presence of inflammation ferritin levels are an unreliable indicator of iron status. Patients were considered by the decision support system to have active inflammation if they had two consecutive CRP measurements, separated by at least 21 days, of greater than 27 mg/L (upper quartile of the dialysis population) in the last 91 days. In these patients, the ferritin criteria in Table 56 were disregarded and the patients classed as iron deficient if they scored ≥2 points from the remaining criteria. Patients were deemed to be at risk of iron overload if their ferrtin was >650 μg/L and would only be prescribed intravenous iron at the discretion of the supervising physician. A summary of the decision support system iron dosing algorithm is set out in Table 57.

Table 57 - Summary of intravenous iron dosing algorithm.

Maintenance dose
℞ 100 mg/fortnight
Iron deficiency + Hb<12.5 g/dl + patient receiving ESA + ferritin ≤ 650 µg/L
℞ 100 mg/twice weekly
Iron deficiency + Hb<12.5 g/dl + patient receiving ESA + ferritin > 650 µg/L
℞ 100 mg/fortnight
No iron deficiency + ferritin 500-650 µg/L
℞ 100 mg/month
Continue until:
Ferritin <350 µg/L
℞ 100 mg/fortnight
Ferritin >650 µg/L
℞ Withhold iron
Hb≥12.5 g/dL + No ESA for ≥91 days
℞ Withhold iron
Hb >14 g/dL
℞ Withhold iron
Ferritin >650 µg/L + No iron deficiency
℞ Withhold iron

Hb: Haemoglobin concentration, ℞: Prescribe

Appendix E Buffers

In this appendix, the quantities of salts in the buffers refers to their composition at the time of preparation prior to pH adjustment.

E.1 Turbidimetric fibrin clot structure and function assays

Tris-buffer
50 mM Tris-HCl, 100 mM NaCl, pH 7.4

E.2 Thiobarbituric acid reactive species assays

Thiobarbituric acid (TBA)
70 mM thiobarbituric acid dissolved in 0.1 M NaOH

Butylated hydroxyl toluene (BHT)
70 mM butylated hydroxytoluene in ethanol

1,1,3,3,-tetramethoxypropane (TMP)
5 mM in 0.1 M HCl

E.3 C3 ELISA

Phosphate buffered saline (PBS)
2.5 mM $NaH_2PO_4.2H_2O$, 7.5 mM $Na_2HPO_4.2H_2O$, 140 mM NaCl, pH 7.2

PBS-Tween (PBS-T)
2.5 mM $NaH_2PO_4.2H_2O$, 7.5 mM $Na_2HPO_4.2H_2O$, 0.5 M NaCl, 0.2% Tween-20, pH 7.2

E.4 Properdin, factor D and sC5b9 ELISAs

Coating buffer
50 mM Na_2CO_3, pH 9.6

Washing buffer
50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5

Sample diluting buffer
50 mM Tris-HCl, 150 mM NaCl, 10mM EDTA*, 0.05% Tween-20, pH 7.5

Blocking buffer
50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, 1% BSA, pH 7.5

Diluting buffer
50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5

Diethanolamine
1 M Diethanolamine, 0.5 mM $MgCl_2$, pH 9.8

*The initial optimisation experiments were carried out using sample dilution buffer which did not contain EDTA as detailed in the methods section. The sample dilution buffer used in the finalised ELISA protocols did contain EDTA.

Appendix F ICH-GCP certificate

The Faculty of Pharmaceutical Medicine has accredited this course for 9 Continuing Professional Development credits.

GCP web-based training course designed and developed by

INFONETICA

Ashford and St. Peter's Hospitals NHS
NHS Trust

Certificate Of Achievement

This is to certify that
Simon Lines
of Leeds Teaching Hospitals NHS Trust

has successfully passed a web-based examination covering all aspects of the
International Conference on Harmonisation – Good Clinical Practice Guideline Course

29 June 2009
(Recommended renewal date: 29 June 2011)

Certificate No: 3877-1-5297

Course Director
Dr Isaac John, Hon Lecturer
Royal Holloway, University of London
Assistant Director Research & Development
Ashford & St Peter's Hospitals NHS Trust

Royal Holloway
University of London

Endorsed by
Professor George Dickson
Head, School of Biological Sciences
Royal Holloway, University of London