THROMBOTIC RISK ASSESSMENT IN END STAGE RENAL DISEASE PATIENTS ON RENAL REPLACEMENT THERAPY

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I do not have any conflicts of interest to declare. Professor Gorog is related through family to a company director in Montrose Diagnostics Ltd, but has no financial involvement or equity interest in, and has received no financial assistance, support, or grant from the aforementioned company.

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ABSTRACT

Aims:

End stage renal disease (ESRD) patients have an excess cardiovascular risk, above that predicted by traditional risk factor models. Despite the advances in both Cardiovascular disease (CVD) management and renal replacement therapy (RRT), there still is a major burden of cardiovascular mortality and morbidity in the chronic kidney disease (CKD) population. Declining renal function itself represents a continuum of cardiovascular risk and in those individuals who survive to reach ESRD, the risk of suffering a cardiac event is uncomfortably and unacceptably high. Pro-thrombotic status may contribute to this increased risk. Global thrombotic status assessment, including measurement of occlusion time (OT) the time taken to form an occlusive platelet rich thrombus and thrombolytic status (time taken to lyse such thrombus) as assessed by measuring Lysis Time (LT), may identify vulnerable patients. The aim of this study was to assess overall thrombotic status in ESRD and relate this to cardiovascular and peripheral thrombotic risk. Small sub studies were also planned to establish the effect of RRT modality on the thrombotic status.

Methods and results

Thrombotic and thrombolytic status of ESRD patients (n = 216) on haemodialysis and 100 healthy volunteers was assessed using the Global Thrombosis Test (GTT). This novel, near-patient test measures the time required (OT) to form and time required to lyse (lysis time, LT) an occlusive platelet thrombus. Patients were followed-up for a minimum 1 year for major adverse cardiovascular events (MACE; composite of cardiovascular death, non-fatal MI, or stroke). Peripheral arterial or arterio-venous fistula thrombosis was a secondary endpoint. The 100 healthy volunteers did not have any renal impairment, and were not taking any medications. There were 55 males and 45 females, and mean age was 38±11 years (range 22-76, IQR 11). OT was normally distributed with mean OT 378 sec (200-550 sec). LT demonstrated a skewed distribution ranging from 457 to 2934 sec. A median of 1053 sec (600-2000s) was established for LT. OT was prolonged (491±177 vs. 378±96 s, P<0.001) and endogenous thrombolysis was impaired (LT median 1820 vs.1053 s, P< 0.001) in ESRD compared with normal subjects. LT≥3000 s occurred in 42% of ESRD patients, and none of the controls. Impaired endogenous thrombolysis (LT≥3000 s) was strongly associated MACE (HR-4.25, 95% CI 1.58– 11.46, p=0.004), non-fatal MI and stroke (HR-14.28, 95% CI 1.86–109.90, p=0.01), and peripheral thrombosis (HR -9.08,

95% CI 2.08–39.75, p=0.003). No association was found between OT and MACE.

Small sub studies were performed on patients receiving peritoneal dialysis (PD) and also post renal transplant recipients to compare the thrombotic status assessed by GTT in patients with different modalities of RRT. These patients did not have any clinical follow-up. The global thrombotic status of renal transplant recipients or PD patients was not different to that of the HD patients suggesting that the modality of RRT does not alter the thrombotic status in ESRD.

Conclusion:

Impaired endogenous thrombolysis, as measured by using the near patient, bed-side novel test, is a novel risk factor in ESRD. It is strongly associated with cardiovascular events. Further studies are required to confirm the role of impaired endogenous thrombolysis as an independent risk factor for CVD in CKD patients.

Thesis Outline

This thesis has been divided into 8 chapters. In the first chapter I have discussed the burden of CKD and the magnitude of CVD in ESRD patients and reviewed the possible risk factors implicated in this. I have then discussed the mechanism of thrombotic events in general and the possible explanation of the differences found in ESRD compared to patients with normal renal function. I have then reviewed the available platelet function tests, drawbacks in currently available tests and thus the need for a new test.

In chapter 2 and 3 I have outlined the hypothesis, aims and methodology used to perform this study. In this chapter I have also detailed the main test used to perform the study –GTT. It also lists the reasons for choosing peripheral venous sample as compared to fistula sample in HD patients for GTT analysis which is then further discussed in detail as a sub-study in chapter 4

Chapter 5 outlines the demographics of the main study group (HD patients) and the OT and LT values observed in the HD group. This showed significantly prolonged LT in HD population compared to the healthy volunteers. The effects of demographic profile, medical co-morbidities and medications on the OT and LT of HD patients are then studied in this chapter. Following this the relationship between OT and

LT and the study endpoints is established. This showed that significantly prolonged LT (>3000s) was an independent risk factors in this study group for MACE and secondary end-points.

Chapter 6 and 7 are establishes the OT and LT in small groups of patients receiving PD or post renal transplant and then a comparison is made with the main study population.

Chapter 8 is a general discussion about the methodology, rationale of the study, possible explanations of the results and limitations of the study. It also briefly highlights possible future work.

In the end are the references and the appendices which include the ethical approval letters, data collection sheets, patient and GP information sheets.

Publications and Presentations generated from this work

Publications:

- **Impaired thrombolysis: a novel cardiovascular risk factor in end-stage renal disease.**
  Sumeet Sharma, Ken Farrington, Robert Kozarski, Christos Christopoulos, Maria NeisPialoswka-steuden, Daniel Moffat, Diana A Gorog, European Heart Journal; 2013 Feb;34(5):354-63.

- **Impaired endogenous thrombolysis predicts fistula thrombosis in end stage renal disease**. Sumeet Sharma, Ken Farrington, Christos Christopoulos, Robert Kozarski, Diana Gorog. J Am Coll Cardiol; 50(13s1):E2097.doi:10.1016/s0735-1097 (12) 62098-1 (Published Abstract).

Posters:

- **Impaired endogenous thrombolysis predicts fistula thrombosis in end stage renal disease** Sumeet Sharma**,** Ken Farrington, Christos Christopoulos, Robert Kozarski and Diana Gorog- Presented at American college of Cardiology **(ACC.12)**, Chicago 2012

- **Impaired Thrombolysis: a novel predictor of cardiovascular and peripheral thrombotic events in ESRD** Sumeet Sharma**,** Ken Farrington, Christos Christopoulos, Robert Kozarski and Diana Gorog **–** presented at British Renal Society **(BRS)**, Birmingham 2011

- **Global thrombotic status predicts cardiovascular events in end-stage renal failure**
  S. Sharma, D. Moffat, C. Christopoulous, M. Klonizakis, D. Wellstead, K. Farrington, DA. Gorog- Presented at European Society of Cardiology **(ESC)**, Stockholm 2010

- **Deficiency in primary haemostasis, but no pro-thrombotic tendency in ESRF patients, compared to normal volunteers-**Presented at British Renal society **(BRS)**, Birmingham, July 2009**.** Sumeet Sharma**,** Smriti Saraf, Ken Farrington, Diana A Gorog

- **Clopidogrel Resistance as Assessed by P2Y (12) Receptor Inhibition Is Not Reflective of Global Thrombotic Status –** presented at American college of Cardiology **(ACC)**, Orlando March 2009. Smriti Saraf, Sumeet Sharma, Imen Ben Salha, Diana A. Gorog

Oral Presentations:

- **Markedly impaired endogenous thrombolytic status in ESRF may explain the increased thrombotic events in this group**- Sumeet Sharma, British Renal society **(BRS)**, Birmingham 2009

ABBREVIATIONS:

AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
ARB	Angiotensin receptor blockers
ATP	Adenosine tri phosphate
AV	Arterio-venous
BP	Blood pressure
BT	Bleeding Time
CAD	Coronary artery disease
CAPD	Continuous Ambulatory PD
CCPD	Continuous cycling PD
CI	Confidence intervals
CKD	Chronic kidney disease
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
eGFR	Estimated Glomerular Filtration Rate
ESRD	End stage renal disease
GP	Glycoprotein
GTT	Global Thrombosis Test

HD	Haemodialysis
HR	Hazard ratio
LDL	Low-density lipoprotein
LT	Lysis Time
LTA	Light transmission aggregometry
LV	Left ventricle
MACE	Major adverse cardiovascular events
MACCE	Major adverse cardiovascular and cerebrovascular events
MI	Myocardial Infarction
MIA	Malnutrition, inflammation and atherosclerosis
MICS	Malnutrition-inflammation complex syndrome
MPV	Mean platelet volume
NHANES	National Health and Nutrition Examination Survey
NKFDOQI	National Kidney Foundation Kidney Disease Outcomes Quality Initiative
NO	Nitric oxide
NRI	Net reclassification improvement
OT	Occlusion time
PAI-I	Plasminogen activator inhibitor-I
PD	Peritoneal dialysis
PEM	Protein-energy malnutrition
PFA	Platelet function analyser

PH	Proportional hazard
PMP	per million population
PPI	Proton pump inhibitor
PRP	Platelet-rich plasma
PVD	Peripheral vascular disease
RAS	Renin angiotensin system
ROC	Receiver operating characteristic
ROTEM	Rotation Thromboelastometry
RRT	Renal replacement therapy
Sec	Seconds
TXA2	Thromboxane A2
TRAP	Thrombin receptor activating peptide
TEG	Thrombelastography
TF	Tissue factor
TMA	Thrombotic microangiopathy
t-PA	Tissue type plasminogen activator
USRDS	United States renal data system
VASP	Vasodilator stimulated phosphoprotein
VHA	Viscoelastic point-of-care haemostatic assays
vWd	von Willebrand disease
vWf	von Willebrand factor

Chapter 1: Background

1.1 Introduction

This chapter has two parts. In the first part of this chapter I will discuss the burden of chronic kidney disease (CKD) and the magnitude of (CVD) in end stage renal disease (ESRD) patients and review the possible risk factors implicated in this. I will then go on to explain the mechanism of thrombotic events in general and the possible explanation of the differences found in ESRD compared to patients with normal renal function. In the second part of this chapter I will review the available literature on the platelet function testing, drawbacks in currently available tests and thus the need for a new test.

1.1.1 CKD prevalence

The prevalence of CKD is high and rising, affecting 13% of the population in the United States in 1999–2004 as compared with 11% in 1988–1994, according to data from the National Health and Nutrition Examination Survey (1). More or less similar prevalence estimates have been reported in Australia (2), Norway (3) and the United Kingdom (4). In a UK-based cross-sectional study, the overall prevalence of CKD estimated Glomerular Filtration Rate (eGFR) <60 ml/min/1.73 m^2) in a population of older people aged 75 years or more was 56.1% (95% CI: 55.3–57.0). The prevalence of eGFR less than 45 and 30 ml/min/1.73 m^2 was 17.7% (95% CI: 17.1–18.4) and

2.7% (95% CI: 2.4–2.9), respectively (5). The prevalence may be even higher among institutionalized older people, with 82% of a residential home population in the UK having a GFR equivalent to stage 3 CKD or worse, and 40% with stage 3B CKD or worse (6).

CKD typically evolves over many years, with a long latent period when the disease is clinically silent and therefore diagnosis, evaluation and treatment is based mainly on biomarkers that assess kidney function. In 2002, the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI) instituted new guidelines that established a novel CKD staging paradigm. In this model, CKD was categorized into five stages based on eGFR range: I, II III IV and V (7) eGFR more than 90 mL/min per 1·73 m^2 (stage 1), 60–89 mL/min per 1·73 m^2 (stage 2), 30–59 mL/min per 1·73 m^2 (stage 3), 15–29 mL/min per 1·73 m^2 (stage 4), and less than 15 mL/min per 1·73 m^2 (stage 5)

Some CKD patients are able to maintain stable, albeit diminished glomerular filtration rates over several years, the so-called "non-progressors" or "asymptomatic" CKD patients, whereas, other CKD patients, for often unclear reasons, have an apparent enhanced propensity to progressively lose GFR over time, the so-called "progressors" or "symptomatic" CKD (8). The natural history of progression in certain conditions such as polycystic kidney disease and

diabetic nephropathy is well documented with these diseases, often following a predictable linear decline. However, much less is known about the rate and nature of progression in general population-based cohorts of CKD patients, particularly in the elderly. Studies have consistently shown that in patients with CKD, death is far more common than progression of kidney disease (9).

Keith et al. (10) identified 27,998 patients who had eGFR <90 ml/min/1.73 m^2 measured on at least 2 occasions separated by 90 days. Subjects were followed-up for 66 months or until death or initiation of renal replacement therapy (RRT). Death was found to be significantly more common at each stage of CKD compared with the frequency of RRT: 19.5% in stage 2, 24.3% in stage 3 and 45.7% in stage 4 died compared with rates of RRT of 1.1, 1.3 and 19.9% in each stage, respectively. Two other population-based studies have also demonstrated that the risk of death far exceed the risk of progression to RRT. Drey et al. (11) found that only 4% of those with creatinine >150 µmol/l progressed to RRT compared with 69% who died during the 5.5-year follow-up. Foley et al. (12) examined 5% of the Medicare population and found that the rate of death per 100 patient-years were significantly higher than the risk of progression to RRT in CKD patients with and without diabetes. The risk of mortality, principally from CVD,

in older people with CKD outweighs the risk of progression to ESRD (13).

Patients with ESRD consume a disproportionate amount of health care resources. However, despite the magnitude of the resources committed to the treatment of ESRD and the substantial improvements in the quality of dialysis therapy, these patients continue to experience significant mortality and morbidity, and a reduced quality of life.

The modalities of RRT available for the treatment of (ESRD) include renal transplantation, haemodialysis (HD), and peritoneal dialysis (PD). HD is subdivided into centre HD, the most commonly used modality, and home HD. PD comprises both continuous ambulatory PD (CAPD), and continuous cycling PD (CCPD), in addition to a small subgroup of other forms of PD. Renal transplantation may be from a living donor (either a blood relative or other unrelated donor) or a cadaveric donor. During the course of their treatment for ESRD, patients may switch a number of times between different modalities of renal replacement therapy. For example, a given patient might move from CAPD to transplantation and, after transplant failure, to haemodialysis and perhaps to a second transplant.

According to the United States renal data system 2014 annual report (14) the use of PD and pre-emptive kidney transplant were relatively

more common in younger patients. The use of home dialysis in the USA has increased significantly since 2002 and was 35% higher in 2012 (c.f 2002). The majority of patients receiving home dialysis (95%) were receiving PD.

The numbers of incident case using various modalities of RRT in US in the year 2012 based on age, race, ethnicity, sex and aetiology of ESRD is shown in the table 1.1 adapted from the USRDS annual report.

Table 1.1

Table 1.1 : Number and percentage of incident cases of HD, PD and transplantation (Tx) by age, sex, race, ethnicity, and primary ESRD diagnosis, in the U.S. population, 2012

	HD		PD		Tx	
	N	%	N	%	N	%
Age						
0-19	506	0.5	349	3.8	245	8.7
20-44	10,375	10.5	1,650	18.0	661	23.6
45-64	38,268	38.7	3,952	43.1	1,355	48.3
65-74	24,528	24.8	1,900	20.7	485	17.3
75+	25,277	25.5	1,324	14.4	57	2.0
Sex						
Male	56,847	57.4	5,197	56.6	1,612	57.5

Female	42,107	42.6	3,978	43.4	1,191	42.5
Race						
White	65,430	66.1	6,415	69.9	2,288	81.6
Black/ African Am	28,659	29.0	2,137	23.3	292	10.4
Native American	1,139	1.2	87	0.9	29	1.0
Asian	3,726	3.8	536	5.8	194	6.9
Ethnicity						
Hispanic	13,702	13.8	1,251	13.6	420	15.0
Non-Hispanic	85,252	86.2	7,924	86.4	2,383	85.0
Primary cause of ESRD						
Diabetes	43,922	44.4	3,783	41.2	441	15.7
Hyperten-sion	29,111	29.4	2,373	25.9	257	9.2
Glomerulo-nephritis	6,889	7.0	1,387	15.1	553	19.7
Cystic kidney	1,551	1.6	476	5.2	429	15.3
Other urologic	410	0.4	61	0.7	54	1.9
Other Cause	10,762	10.9	682	7.4	501	17.9
Unknown/ missing	6,309	6.4	413	4.5	568	20.3
All	98,954	100.0	9,175	100.0	2,803	100.0

ADAPTED FROM USRDDS DATABASE (www.usrds.org/2014)

Data Source: Special analyses, USRDS ESRD Database. Abbreviation: African Am, African American; ESRD, end-stage renal disease.

In comparison, the UK renal registry 17th annual report stated that there were 56,940 adult patients receiving RRT in the UK on 31st December 2013, an absolute increase of 4.0% from 2012. The actual number of patients increased 1.2% for HD, 7.1% for those with a functioning transplant but decreased 3.3% for PD. The UK adult prevalence of RRT was 888 per million population (pmp) compared to 523 pmp. in the year 2000. The number of patients receiving home HD increased by 3% from 1,080 patients in 2012 to 1,113 patients in 2013. The median age of these patients was 58.4 years (HD 66.9 years, PD 63.7 years, transplant 52.8 years). In 2000 the median age was 55 years (HD 63 years, PD 58 years, transplant 48 years). The percentage of RRT patients aged greater than 70 years old increased from 19.2% in 2000 to 25% in 2013. For all ages, the prevalence rate in men exceeded that in women, peaking in age group 75–79 years at 3,010 p.m.p. in men and for women at 1,560 p.m.p.

Unlike the US, the most common identifiable renal diagnosis was glomerulonephritis (19.0%), followed by uncertain aetiology (16.0%) and diabetes (15.9%). Transplantation continued to be the most common treatment modality (52%), HD was used in 41.6% and PD in 6.4% of RRT patients. Prevalence rates in patients aged > 85 years

continued to increase between 2012 and 2013 (983 age related pmp to 1,020 age related pmp).

In 2013, 21.1% of the prevalent UK RRT populations (with ethnicity assigned) were from ethnic minorities compared to 14.9% in 2007.

The UK numbers are much smaller than those from the US but are comparable to northern European countries according to the UKRDS report.

1.1.2 Cardiovascular risk in CKD

CKD affects around 10–13% of the general population, with only a small proportion of these patients exhibiting ESRD, either on dialysis or awaiting renal transplantation (1). It is well documented that CKD patients have an extremely high risk of developing CVD compared with the general population, so much so that in the early stages of CKD patients are more likely to develop CVD than they are to progress to ESRD.

A number of studies in the past have demonstrated that as eGFR falls, the incidence of CVD increases, independent of associated risk factors (15) Go et al. investigated the association between GFR, CVD and death in 1,120,295 adults. On multivariate analysis, the adjusted hazard ratio for CVD was 1.4 with an estimated GFR of 45–59 ml/min/1.73 m^2 (95% CI, 1.1–1.2) compared with 3.4 (95% CI, 3.1–

3.8) with an estimated GFR of < 15 ml/min/1.73 m^2. Similar findings have been reproduced in other large studies and few of these are summarized in the table 1.2.

Table1.2*: Studies investigating the association between eGFR and CVD

- Table modified and adapted from (16).

Study	Outcome	Number of participants	Comparison	Result (CI; p-value)
Go et al.	Any cardiovascular events	1,120,290	eGFR 45–59 vs. > 60 ml/min/1.73 m^2 eGFR 30–44 vs. > 60 ml/min/1.73 m^2 eGFR 15–29 vs. > 60 ml/min/1.73 m^2 eGFR < 15 vs. > 60 ml/min/1.73 m^2	HR 1.4 (CI 1.4–1.5) HR 2.0 (CI 1.9–2.1) HR 2.8 (CI 2.6–2.9) HR 3.4 (CI 3.1–3.8)
Muntner et al. *	Coronary heart disease	14,856	eGFR 15–59 vs. > 90 ml/min/1.73 m^2	RR 4.27 (CI 2.09–8.71 p < 0.001)
Manjunath et al. **	Atherosclerotic cardiovascular disease	15,350	eGFR 15–59 vs. 90–150 ml/min/1.73 m^2	HR 1.38 (CI 1.02–1.87 p = 0.038)
Manjunath et al. **	Atherosclerotic cardiovascular disease	15,350	Every 10 unit reduction in eGFR	HR 1.05 (CI 1.02–1.09 p = 0.006)
Manjunath et al. **	Cardiovascular disease	5135	eGFR 15–59 vs. 90–150 ml/min/1.73 m^2	HR 1.31 (CI 1.06–1.62 p = 0.013)
Manjunath et al. **	Cardiovascular disease	5135	Every 10 unit reduction in eGFR	HR 1.05 (CI 1.02–1.09 p = 0.005)

CHD, coronary heart disease; eGFR, estimated glomerular filtration rate; NIDDM, none insulin dependent diabetes mellitus.

* (17)

** (18)

As early as 1970s it was recognized that there is high burden of cardiovascular disease in HD patients (19) Presence of angiographically significant coronary artery disease ranges from 25% in young non-diabetic HD patients to 85% in older, diabetics with ESRD (20) When compared to patients without CKD who undergo evaluation for coronary artery disease (CAD), those with ESRD have substantially more numerous and severe coronary artery lesions, as well as more severe left ventricular dysfunction (21,22)

The 5-year survival of men > 64 year old starting dialysis is worse than that of men with colon cancer and prostate cancer (*). The 5-year survival of women >64 year old starting dialysis is worse than that of women with breast cancer and colon cancer (*). (* = U.S. Renal Data System: USRDS 1998 Annual Data Report, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, April 1998).

CVD accounts for 40%-50% of deaths in dialysis populations (8). Overall, the risk of cardiac mortality is 10 to 20-fold greater in dialysis patients than in age and sex-matched controls without CKD.

Despite this, dialysis patients often undergo fewer investigations and invasive procedures for CVD, and are prescribed fewer medications for these conditions compared with age-matched non-ESRD patients. These discrepancies can be explained by the paucity of large trials for

evidence-based treatment strategies in this population, but there is concern that this attitude may be impacting on the medical care of these patients.

Recently, Roberts et al used registry data to compare age- and era-specific CVD mortality rates in Australian dialysis patients with rates in the Australian general population. The encouraging finding of this study is that CVD mortality rates have decreased, in this population, over time in both dialysis patients and the general population. The concerning finding was that the relative risk of CVD mortality in dialysis patients compared with the general population increased. In addition, statistical analyses confirmed an interaction between era and dialysis status with CVD mortality, indicating significantly higher relative risk for dialysis patients in more recent eras (23). On the basis of these observations the authors concluded that despite decreasing cardiovascular mortality rates in some dialysis patients, the excess cardiovascular risk compared with the general population is increasing (24).

The interpretation of this comparison may or may not be extrapolated to other populations and certainly has limitations. However it does highlight that despite the advances in both CVD management and RRT, there still is a major burden of cardiovascular mortality and morbidity

in the CKD population. Declining renal function itself represents a continuum of cardiovascular risk and in those individuals who survive to reach ESRD, the risk of suffering a cardiac event is uncomfortably and unacceptably high. It also raises the question of why the rates of CVD in dialysis patients are not decreasing at the same rate as in the general population.

1.1.3 Risk factors of CVD in CKD

Although atherosclerosis on its own is predominantly a clinically silent process, its consequences and presentations can be catastrophic, and in the vast majority of patients may even be the first presentation of the disease process. Clinical presentations of atherosclerosis include ischemic heart disease, namely, angina, myocardial infarction, and sudden cardiac death, which is common in CKD, and cerebrovascular disease, peripheral vascular disease, or heart failure. The cardiovascular burden in dialysis patients now is recognized to include not only accelerated atherosclerosis, but also arteriosclerosis with stiff and noncompliant arteries, as well as a high prevalence of cardiomyopathy. Thus in practice, it is seen that, although the risk of myocardial infarction is increased in patients on dialysis, other cardiovascular events, such as sudden cardiac death and heart failure are also common in this cohort (25).

1.1.4 Traditional Risk Factors

Most of the traditional CVD risk factors (as defined in the Framingham population), such as older age, diabetes mellitus, systolic hypertension and high low-density lipoprotein (LDL) cholesterol, are highly prevalent in CKD. However, the relationship between these risk factors and cardiovascular events in people with kidney disease often differs from that in the general population and does not explain the increased risk. It is also true that intervening on these traditional risk factors, such as lipid lowering with statin therapy have not shown any major benefit of cardiovascular event reduction in this population.

Traditional risk factors for CVD and mortality in the general population such as body mass, serum cholesterol, and blood pressure are also found to relate to outcome in patients receiving maintenance dialysis, but often in an opposite direction. Obesity, hypercholesterolemia, and hypertension appear to have protective features that may be associated with a greater survival among dialysis patients. These findings are in contrast to the well-known association between *over*-nutrition and poor outcome in the general population. The association between *under*-nutrition and adverse cardiovascular outcome in dialysis patients, which appears to contrast to that seen in non-ESRD individuals, has been referred to as "reverse epidemiology." The

etiology of this inverse association is not clear. Several possible causes are hypothesized including survival bias (only a small number of patients with CKD survive long enough to reach ESRD, and the presence of the "malnutrition-inflammation complex syndrome" (MICS) in dialysis patients (26). Both protein-energy malnutrition (PEM) and inflammation or a combination of these two, are much more common in dialysis patients than in the general population. The degree to which PEM in dialysis patients is caused by inflammation is not clear. Some studies suggest that PEM and inflammation each independently contribute to hypoalbuminemia and subsequently increase morbidity and mortality. Since both PEM and inflammation are strongly associated with each other and can change many nutritional measures in the same direction, and because the relative contributions of measures of these two conditions to each other and to outcomes in dialysis patients are not yet well defined, MICS has been suggested to denote the important contribution of both of these conditions to ESRD outcome (27).

The possible causes of MICS include comorbid illnesses, oxidative and carbonyl stress, nutrient loss through dialysis, anorexia and a low nutrient intake, uremic toxins, a decreased clearance of the inflammatory cytokines, a volume overload, and dialysis-related

factors. Many elements of MICS, such as a low weight-for-height, hypocholesterolaemia, or hypocreatininaemia, are the known risk factors of a poor outcome in dialysis patients (28). Because MICS leads to a low body mass index, hypocholesterolaemia, hypocreatininaemia and hypohomocysteinaemia, a "reverse epidemiology" of the cardiovascular risks has been seen in the dialysis patients.

The AURORA trial, was a large international, multicenter, randomized, double-blind, prospective trial involving 2776 patients, 50 to 80 years of age, who had been undergoing maintenance HD for at least 3 months. Patients were randomly assigned to receive rosuvastatin, 10 mg daily, or placebo. The primary end point was the time to a major cardiovascular event, defined as a nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes (29). In this trial, the statin therapy failed to achieve any benefit on the primary outcome or its subsets individually, despite achieving a mean 43% reduction in the LDL cholesterol level at 3 months.

Similar findings were seen in the 4D study, where 1225 patients with type 2 diabetes undergoing maintenance HD received either atorvastatin at a dose of 20 mg or placebo. Although the median LDL cholesterol level was reduced by 42% with atorvastatin, there was no

significant reduction in the composite primary cardiovascular end point (30). The lack of benefit of statin therapy in the 4D and AURORA studies suggests that CVD risk in patients undergoing HD differs from that in other patients.

The much larger, more recent SHARP study, which included nearly 10,000 patients with CKD (of whom 3,023 were on maintenance dialysis therapy), reported that allocation to simvastatin plus ezetimibe achieved a 17% reduction in major atherosclerotic events compared to placebo. However, the majority of patients in SHARP were not on dialysis and analysis of the subgroup of patients on HD did not show a benefit of lipid-lowering therapy (31). Thus for prevention of CVD, there is strong clinical evidence supporting the benefit of using statins in the early stages of CKD, but there is no clear advantage to their use in dialysis patients, a particularly high risk population.

Similarly, blood pressure lowering in CKD patients has not shown any major impact on CVD in these patients. The "U shaped" relationship between hypertension and mortality is more difficult to explain, not least because of the difficulty in defining hypertension in this population as there is tremendous variation in BP throughout the dialysis cycle making it difficult to establish which BP is most important. Also the high prevalence of cardiac dysfunction may account for the

increased mortality at low-normal levels of BPThere have been a number studies which have indicated that, similar to the risk seen in the general population, high systolic or diastolic blood pressure (BP) is associated with an increased risk of death in dialysis patients (32). A number of large epidemiological studies have paradoxically indicated inverse (33) or U-shaped (34) associations between BP and mortality in dialysis patients. It has been argued that discrepancies in these studies including the so-called "reverse epidemiology" of BP (or hypertension paradox) are related to differences in various clinical characteristics including comorbid conditions of the studied patient populations (35).

Agents acting via the renin angiotensin system (RAS), including angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), are often recommended as first-line treatment on the basis of RCTs demonstrating a reduction in renal events (for e.g. kidney failure or doubling serum creatinine) in patients who have proteinuria, either with (36, 37) or without diabetes (Angiotensin-Converting Enzyme Inhibition in Progressive Renal Insufficiency (AIPRD) study (38), Ramipril Efficacy in Nephropathy study(REIN); (39, 40), or advanced CKD(SCr >3 mg/dl) (41).

Most of the blood pressure lowering trials are driven by renal outcomes and the evidence for cardiovascular outcomes is based mainly on sub

group analysis of large trials studying ACE–inhibitors, such as the ADVANCE (42), PROGRESS (43), HOPE (44), EUROPA (45) studies and PEACE STUDY (46).

A post hoc analysis of the ALLHAT study demonstrated that lisinopril was not superior to chlorthalidone or amlodipine in preventing coronary heart disease, stroke, or combined CVD in patients with estimated GFR<60 ml/min/1.73 m^2 and chlorthalidone was superior to both for preventing heart failure, independent of renal function (47).
Thus far, despite the increased risk of death and cardiovascular events in individuals undergoing dialysis there is no consensus about the treatments proven to reduce cardiovascular outcomes, as one would expect, by modifying the so-called traditional risk factors.

1.1.5 Novel or non-traditional risk factors

Due to the discrepancy in the outcomes and burden of traditional risk factors in end stage renal disease, the search for novel or non-traditional risk factors that may be involved in the pathogenesis of CVD in ESRD has been an area of intense ongoing research. Interventions such as dialysis prescription modification (48), homocysteine lowering, (49) mineral metabolism modification (50, 51, 52) and haemoglobin normalization (53) have been assessed in

randomized trials and systematic reviews, but there is no clear evidence to show that any of these approaches except possibly more frequent dialysis (54) reduces the risk of death or major cardiovascular events.

1.1.6 Atherosclerosis in ESRD

The metabolic environment that results from renal dysfunction appears to accelerate the atherosclerotic process by decades in patients with ESRD. Atherosclerotic disease may not follow identical pathways in people with ESRD compared with those with normal renal function, and indeed may be accelerated (55). It has been shown in studies with CT calcium scoring that coronary artery calcification occurs more frequently in young adults with end-stage renal disease than in either normal subjects of the same age and sex or older adults with normal renal function (56, 57).

The accelerated atherosclerotic process of ESRD may involve several inter-related processes, such as oxidative stress, endothelial dysfunction and vascular calcification, in a milieu of constant low-grade inflammation (58). Indeed, recent evidence suggests that the uremic milieu may affect both the quality and quantity of the atherosclerotic plaques. Schwarz et al (59) have shown that coronary plaques in uremic patients are characterized by increased media thickness,

infiltration and activation of macrophages and marked calcification. Importantly, the striking difference compared with non-renal controls did not concern the size but rather the composition of the plaque. Thus, it could be postulated that heavily calcified and inflamed plaques contribute to the excessive cardiovascular risk in ESRD patients.

The inflammation in ESRD is multifactorial and, while it may reflect underlying CVD, an acute-phase reaction may also be a direct cause of vascular injury via several pathogenic mechanisms. Available data suggest that pro-inflammatory cytokines play a central role in the genesis of both malnutrition and CVD in ESRD (60). Thus, it could be speculated that suppression of the vicious cycle of malnutrition, inflammation and atherosclerosis (MIA syndrome) would improve survival in dialysis patients.

1.1.7 Platelets in athero-thrombosis

Platelets are cells which circulate in blood. They are of pivotal importance in blood clot formation, affecting thrombosis and haemostasis. By rapidly altering the activation and expression of surface receptors, platelets are able to quickly undergo structural and phenotypic changes in response to stimulation, such as collagen exposure from beneath injured vascular endothelium. The main trigger for the formation of a haemostatic thrombus is the loss of the

endothelial cell barrier between extracellular matrix components and flowing blood. This exposes platelets to the thrombogenic components of the sub-endothelium. Among the main components exposed are proteoglycans, collagen type IV, entactin, laminin, fibulin and von Willebrand factor (vWF) (61).

In response to stimulation platelets become adhesive, aggregate to form a thrombus, and release a variety of mediators affecting coagulation, inflammation, and chemotaxis at the site of injury. Platelet adhesion under conditions of high shear stress, as occurs in stenotic atherosclerotic arteries, is central to the development of arterial thrombosis; therefore, platelet activation and adhesion must be prevented in order to maintain blood fluidity and to prevent thrombotic or haemorrhagic complications. The main inducers of platelet activation are collagen, vWF, alpha-thrombin, adenosine diphosphate (ADP) and thromboxane A2. ADP is essential to the platelet response because after its secretion from the platelet dense granules where it is stored, it amplifies the responses induced by other agonists.

Formation of the platelet plug in response to vascular injury can be thought of as occurring in three stages: initiation, extension, and perpetuation (Fig1.1) (62).

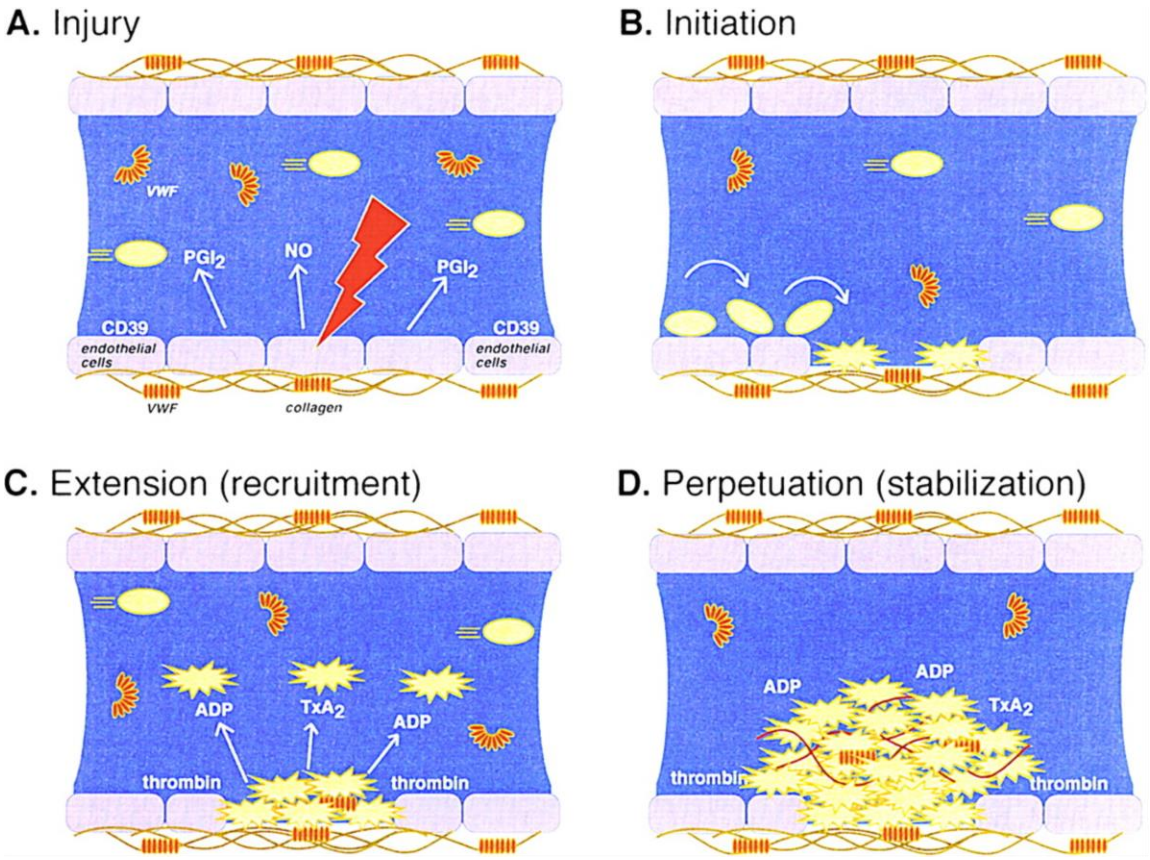


Figure 1.1 Stages in platelet plug formation. A: Prior to vascular injury, platelet activation is prevented by inhibitory factors that include PGI_2 and NO released from endothelial cells, the presence of CD39 on the surface of endothelial cells, and the inability of normal plasma VWF to bind spontaneously to the platelet surface. B: The development of the platelet plug can be initiated by the exposure of collagen and VWF in the vessel wall, and by the local generation of thrombin, a process that occurs more rapidly on the surface of activated platelets. C: Rolling platelets adhere and spread on the collagen matrix, forming a monolayer of activated platelets that can act as a surface for subsequent recruitment of platelets by thrombin, ADP, and TxA_2. D: During the perpetuation stage, close contact between platelets promote the growth and stabilization of the hemostatic plug, in part through contact-dependent signaling mechanisms.

Initiation begins with the tethering, rolling, and arrest of moving platelets on collagen and their subsequent activation to form a platelet monolayer. Large VWF multimers are essential to this process, particularly under high shear conditions in the arterial circulation, but thrombin can also help to initiate platelet activation. Extension refers to the recruitment of additional platelets through the local accumulation of thrombin, ADP and TxA_2. Perpetuation refers to the events that stabilize the platelet plug, some of which involves molecules on the platelet surface that are capable of generating intracellular signals only after platelets have come into sustained contact with each other. The net result is the formation of a fibrin-anchored platelet plug, a structure in which platelet/platelet interactions are supported by the binding of fibrinogen and fibrin to the integrin αIIbβ3 (also known as glycoprotein [GP] IIb-IIIa) and by VWF bound to GP Ib and $\alpha_{IIb}\beta_3$ (Fig1.2).

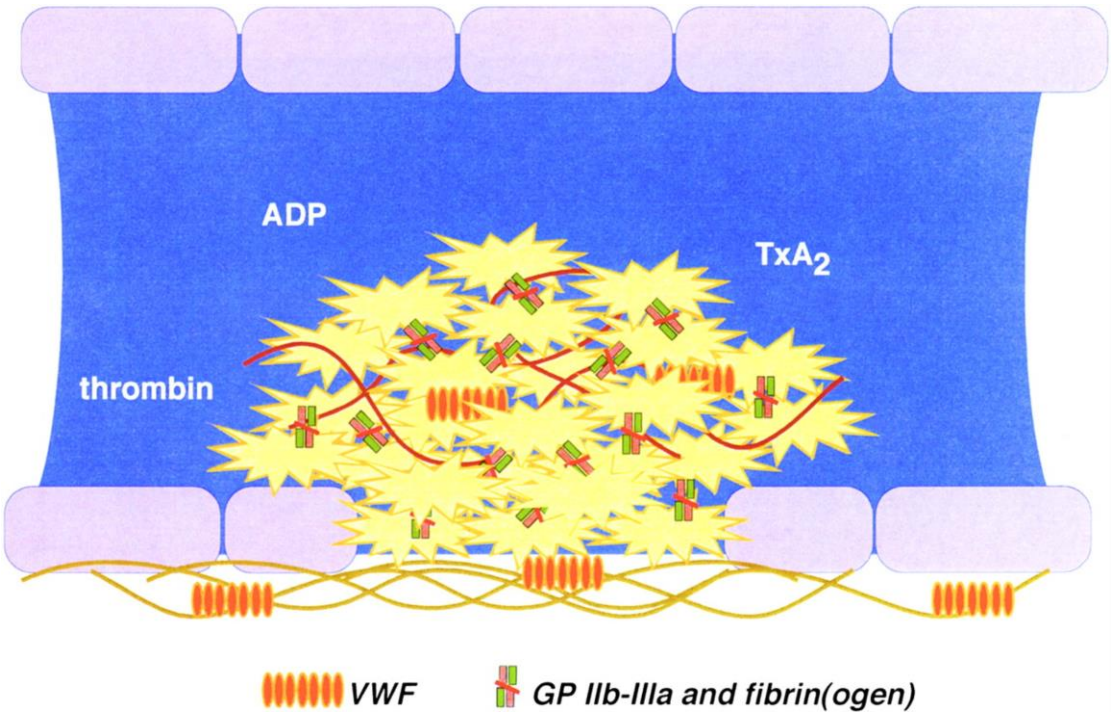


Figure 1.2

Anatomy of a platelet plug: an enlarged view of the assembled platelet plug, highlighting platelet/platelet interactions mediated by the binding of fibrinogen, fibrin, and VWF to activated GP IIb-IIIa ($\alpha_{IIb}\beta_3$) and the binding of VWF to GP Ibα.

The contribution of platelets to haemostasis is different in arteries and veins. Blood circulates at much higher velocity at the center of a vessel compared to near the wall, and this creates are shearing effect between adjacent layers of fluid that is greatest at the wall. In the venous system, low-flow rates and stasis permit the accumulation of activated coagulation factors and the local generation of thrombin, with a less prominent contribution from platelets. Venous thrombi

contain platelets, but the dominant cellular component consists of trapped erythrocytes. In the arterial circulation, higher flow rates limit fibrin formation by washing out soluble clotting factors. Platelets, which contribute most to thrombosis at higher shear rates, help to form a physical barrier against further blood loss and, at the same time, provide a surface on which thrombin is generated and fibrin can accumulate.

Shear stress is particularly increased over atherosclerotic lesions because significant lumen reduction by obstructive plaques causes a local increase in blood flow velocity. Animal studies have shown that platelets are not only involved in occlusive thrombus formation leading to clinical conditions like myocardial infarction or stroke but may also play a prominent role in the promotion of atherosclerotic lesions (63).

1.2. Platelet Function testing

With platelets being key players in athero-thrombotic events, platelet inhibition has been of immense therapeutic interest. The ideal antiplatelet drug would be able to overcome the deleterious effects of platelets in athero-thrombosis without affecting haemostatic functionality. Despite multiple antiplatelet drugs being available for therapeutic use, the clinical response to these drugs is variable and thus there is a demand for tests to assess platelet function. Platelet

function can be assessed in a wide range of clinical scenarios, for e.g. in patients with bleeding disorders, in patients with previous thrombo-embolic events to assess the response to anti-platelet medication, in transfusion recipients, in the preoperative setting to assess bleeding risk. Cardiologists, particularly, are interested in the prediction of thrombotic events, especially in patients undergoing percutaneous coronary intervention and using the most appropriate antiplatelet drug to minimize future coronary thrombotic events, such as acute stent thrombosis. On the other hand, surgeons are mostly interested in knowing about the bleeding tendency, post-operative bleeding risk and similar haemostatic issue. Multiple tests are available but identification of a single test to answer a question related to platelet function and clinical outcome has still not been possible.

The first stage of assessing platelet function involves measuring the full blood count including platelet count and mean platelet volume (MPV). If abnormalities are identified in the platelet count, size, or distribution, then a blood smear can be examined to confirm these findings, and to further assess platelet granule content and morphology. This morphological assessment may identify other distinguishing features and sometimes is sufficient to make a diagnosis. Manual platelet counting still remains the gold standard in assessing platelet numbers but requires high levels of technical skill. It is time

consuming and most laboratories these days use automated analysers, optical counting methods or flow cytometry (64). This has improved their ability to distinguish large platelets from red cells and can sometimes provide more accurate counts.

1.2.1 Bleeding Time

The most simple of the platelet function test is the use of bleeding time. The Bleeding Time (BT) was originally described by Duke in 1910 (65) and further standardized by Ivy *et al*. in 1941(66). This test can be regarded as the oldest test of primary haemostasis investigating platelet function. The BT is an *in-vivo* test in which a small cut is made in the forearm of the patient, and the time taken for bleeding to stop is measured.

In the traditional Ivy method, a blood pressure cuff is placed on the upper arm and inflated. A stab wound is made on the underside of the forearm. The time from the stab wound until all bleeding has stopped is measured and is called the bleeding time. Every 30 seconds, filter paper or a paper towel is used to draw off the blood. The test is finished when bleeding has stopped completely.

In the Duke method, the wound is made in an ear lobe or a fingertip to cause bleeding. In the template technique, a disposable spring loaded

device is placed over the area to be stabbed and two incisions are made in the forearm using the template as a location guide.

The BT is subjective and is influenced by patient variables unrelated to haemostasis, such as age, gender, haematocrit, vascular pattern, skin thickness and skin temperature (67, 68). Although modern BT methods show improved standardization and are somewhat sensitive to both von Willebrand disease and platelet dysfunction, the BT is now rarely used in practice as it is non-specific, has low sensitivity, is invasive, and has poor reproducibility due to high rate of inter-operator variability.

1.2.2 Platelet function analyser (PFA-100)

The platelet function analyser **PFA-100® System** (Siemens Diagnostics) is a test to determine the thrombosis time automatically *ex vivo* based on the method first described by Kratzer and Born (69,70). This test is a microprocessor-controlled instrument (Figure 1.3) which emulates *in vitro* the platelet dependent phase of primary haemostasis, while delimiting the role of the rheological factors.

Figure 1.3: PFA-100 system. (Adapted from the web: http://usa.healthcare.siemens.com/hemostasis/systems/pfa-100)

Basically, the system monitors platelet interaction on collagen-ADP or collagen-epinephrine coated membranes. Samples of citrated whole blood are drawn under controlled flow conditions (through a capillary producing high shear forces: 4,000-5,000/s) through a 150 micrometre aperture cut within a membrane coated with either collagen and epinephrine or collagen and ADP. These agonists induce platelet adhesion, activation and aggregation leading to rapid occlusion of the aperture and cessation of blood flow termed the closure time. Thus, the haemostatic capacity of the platelets in the blood sample is

indicated by this closure time. The PFA-100 was designed as a screen to detect problems with primary haemostasis and in part to replace the bleeding time and in this respect, it is better standardized. It only requires small volumes of citrated venous blood [800µL] and so the test is useful for investigating platelet function in children. It can be used by non-skilled personnel and is both rapid and automated. On the other hand, it is dependent on platelet numbers, haematocrit, vWF, drugs, food and other acquired platelet disorders (for e.g. uremia, liver disease).

The PFA-100 has a high negative predictive value, in other words, if it gives a normal result then with some exceptions (e.g. Primary Secretion Defects, mild Type 1 VWD) primary haemostasis is intact and thus may obviate further screening of platelet function, while if the PFA-100 is abnormal then formal platelet aggregation testing will be required to establish the underlying cause. Thus at present, its use is mainly limited to research studies and clinical trials.

1.2.3 Light transmission aggregometry (LTA)

The concept of light aggregometry was invented in the 1960's (70) and is widely considered as the gold standard in platelet function testing. Despite this, there is still lack of international standardisation of this

technique. In this method, platelet aggregation in response to an agonist is detected in platelet-rich plasma (PRP) by turbidometry. In the Born aggregometer, citrated blood samples are centrifuged to prepare platelet rich plasma (PRP) and platelet poor plasma (PPP). To prepare PRP, a whole blood tube is centrifuged at 170-200 g for 10 minutes in a swing-out rotor at room temperature without application of the brake. Some laboratories dilute the PRP to a final platelet count of 200-300 x 109/l with autologous PPP (71). PRP is stirred in a cuvette at 37°C and the cuvette sits between a light source and a photocell. The turbidity of the suspension is constantly measured by recording transmission of a light beam directed through it, and it is recorded as a change in voltage on a chart recorder. When an agonist is added the platelets aggregate and absorb less light and so the light transmission increases progressively and this is detected by the photocell, producing an "aggregation trace" on the recorder (Figure1.4). The machine is calibrated for each sample so that the platelet suspension to be studied reads 5%-that is, minimum transmission-and the corresponding platelet poor plasma reads 95% (maximum transmission) (71).

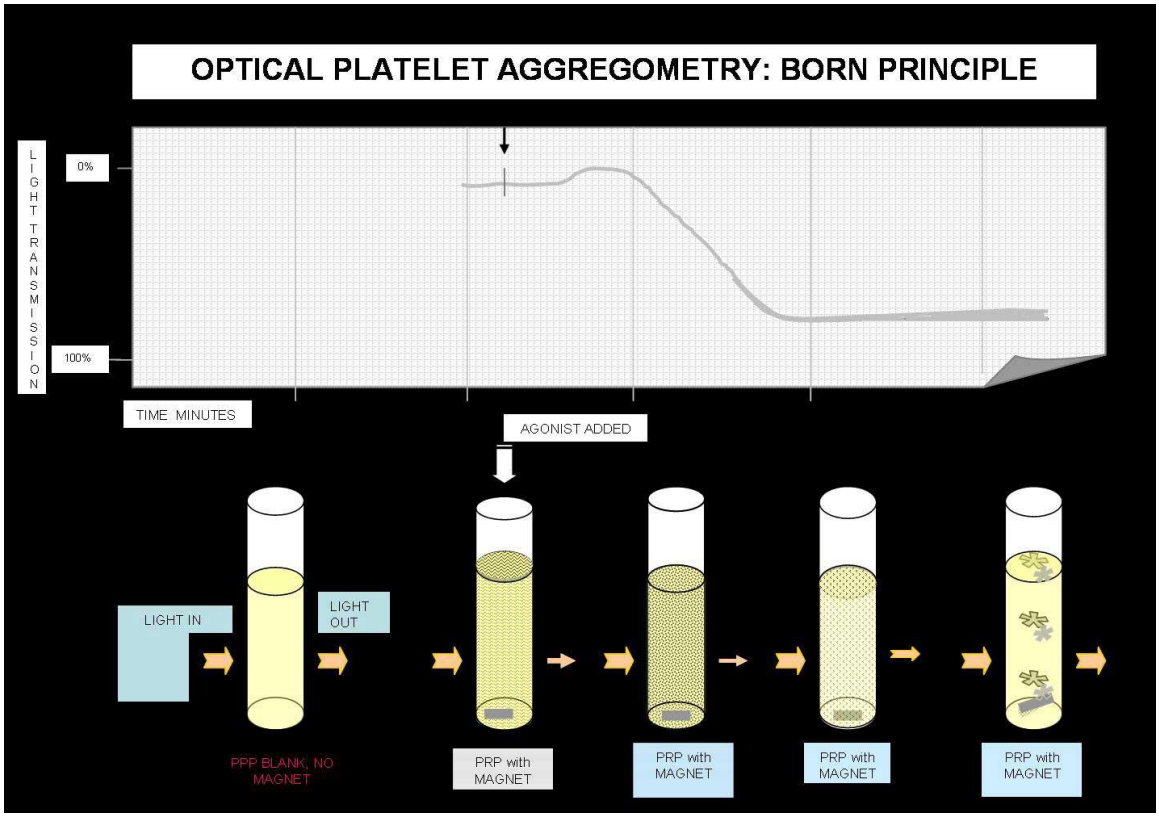


Figure 1.4 Legend: Depiction of Born principle of LTA: PRP is stirred in a cuvette at 37°C and the cuvette sits between a light source and a photocell. The turbidity of the suspension is constantly measured by recording transmission of a light beam directed through it, and it is recorded as a change in voltage on a chart recorder. When an agonist is added the platelets aggregate and absorb less light and so the light transmission increases progressively and this is detected by the photocell, producing an "aggregation trace" on the recorder.

(Adapted from: http://practical-haemostasis.com)

The fundamental advantage of LTA is that it measures, albeit in an *in vitro* system, a very important function of platelets: the kinetics of their aggregation in a glycoprotein IIb/IIIa dependent manner.

There are many variables, which can affect the LTA results, ranging from pre-analytical factors (for e.g. drugs, food, temperature, pH etc), sample collection and PRP preparation techniques, to the type and concentration of agonists used. Commonly used agonists in routine practice are ADP, collagen, ristocetin, arachidonic acid, adrenaline, and PAR-1-activating peptide (TRAP-6, SFLLRN). The panel of agonists can be extended. Other agonists are, for example, gamma thrombin or alpha thrombin, TRAP-4 (AYPGKF), thromboxane mimetic U46619 (stable analogue of the endoperoxide prostaglandin H2) calcium ionophore A23187, polymerized immunoglobulins, collagen-related peptide (CRP) misfolded proteins like AGE-proteins and oxLDL, alpha defensins, proteins from microorganisms like EAP and snake venoms like convulxin (72).

LTA overall is an expensive, time consuming, not readily reproducible test, lacking a standard protocol, which makes it unsuitable for widespread routine use in clinical practice.

1.2.4 Whole blood aggregometry

To overcome the technical and physiological issues associated with traditional aggregometry the impedance aggregometer was invented in 1980's (73) In this technique whole blood is stirred at 37°C and aggregation is detected by the accretion of platelets to the surface of

two fine, precious metal, wire electrodes, The impedance technique measures the progressive increase of electrical impedance between two electrodes as platelets adhere and aggregate to these electrodes in stirred whole blood. Some of the technical problems of whole blood impedance aggregation have been overcome by the development of disposable electrodes, standardised reagents and the availability of a 5 channel multiple electrode platelet aggregometer (Dynabyte, Munich, Germany) (74)

There is still lack of comparative data between whole blood and traditional aggregometry and thus its use is limited in practice. It also requires specialist skills, equipment and is a lab based test.

Currently, there are several commercially available instruments for measuring platelet aggregation based on the principles of light transmittance, impedance, luminescence and some in combination.

1.2.5 Flow cytometry

A flow cytometer operates as a cell counter, that uses argon ion laser as an excitation light source to additional detectors for fluorescence signal measurement. This allows gathering information on the size and internal complexity (forward scatter and side scatter, respectively) as well as markers, identified by staining with fluorescent probes. Before analysis, platelets are labelled with a fluorescently conjugated

monoclonal antibody. The cell suspension then passes through the flow chamber, through a focused laser beam that activates the fluorophore. The emitted fluorescence and scattered light characteristics can then be used to identify the cells of interest.

Flow cytometry can be used to yield definitive information regarding the phenotypic status of platelets. It allows the analysis of individual platelet functional capability and quantitative assessment of the physical and antigenic properties of platelets e.g. the expression of surface receptors, components of granules, bound ligands, and interaction of platelets with other platelets, neutrophils and monocytes thereby facilitating the diagnosis of inherited or acquired platelet disorders (e.g., Bernard-Soulier syndrome, Glanzmann thrombasthenia, storage pool disease), the pathological activation of platelets (e.g., in the setting of acute coronary syndromes, cerebrovascular ischemia, peripheral vascular disease, cardiopulmonary bypass), and changes in the ability of platelets to activate via specific stimuli (e.g., efficacy of antiplatelet therapies) (75).

Flow cytometry allows identification of platelet function defects even in patients with very low platelet count and therefore is the methodology of choice to determine whether a thrombopenic patient has in addition

a thrombocytopenia. It remains an expensive and technically demanding method and requires careful sample preparation. (76)

Measuring the phosphorylation state of vasodilator stimulated phosphoprotein (VASP) using flow cytometry is a completely P2Y12-receptor specific method for the evaluation of ADP-receptor inhibition. VASP is a second messenger in the signalling pathway of the P2Y12 receptor that is regulated by protein kinases and phosphatases according to the activity of the receptor.

Inactive/resting platelets possess high cAMP levels that induce phosphorylation of VASP by cAMP-dependent protein kinases (VASP-P: resting state). In case of P2Y12-receptor stimulation, the activity of the adenylate-cyclase enzyme decreases leading to dephosphorylation of VASP (VASP: active state). Therefore, the ratio of dephosphorylated and phosphorylated VASP is a selective measure of P2Y12-inhibition unaffected by use of glycoprotein IIb/IIIa inhibitors.

1.2.6 Platelet nucleotide assays

Platelets have two separate nucleotide pools within them. Sixty percent is stored within the dense granules and is not metabolically active and the remaining 40% constitutes the metabolic pool and

provides the platelet with energy for various activities. The ratio of ATP: ADP is therefore of fundamental importance as there are pronounced differences between the relative concentrations in the two pools. Any storage defects are associated with a decrease in the amount of stored and released ADP with an increased ratio of ATP: ADP. Thus the measurement of total or released nucleotides along with aggregometry provides additional information of platelet disorders regarding any storage pool disorders or degranulation defects.

The simplest assay of released platelet nucleotides can be performed in real time with a Lumi-Aggregometer (77). This is a modification of light transmission aggregometry, which measures ATP release from the dense granules. It is based on quantitative bioluminescent determination of ATP in which the ATP reacts with luciferin and luciferase [firefly extracts] resulting in light emission. Light is emitted when oxidation of adenyl-luciferin occurs.

For measurement of aggregation, the lumi-aggregometer uses an LED which emits light in the infrared range and changes in the transmission of light are detected by a phototransistor. For the measurement of luminescence resulting from ATP secretion, it uses a photomultiplier tube located at right angles to the light path of the LED.

Dense granules are normally easily visible on electron microscopy and thus can be directly measured. It is possible to measure the uptake

and release of radiolabelled serotonin into and from the platelets with standardised assays as serotonin is actively taken up and stored within the platelet dense granules. Flow cytometry can also be used to measure the mepacrine uptake and release from dense granules. (78).

1.2.7 VerifyNow

VerifyNow Rapid Platelet Function Analyser (Accumetrics, San Diego, CA) is a point of care instrument (Figure 1.5). This assay is based on light aggregometry. The principle of this assay is that fibrinogen-coated polystyrene micro-particles will agglutinate in whole blood in direct proportion to the degree of platelet activation and subsequently activated glycoprotein (GP) IIb/IIIa receptors (79).

The tube containing the whole blood sample is inserted into a disposable plastic cartridge containing a lyophilized preparation of human fibrinogen-coated beads, a platelet-activating agent, buffer and preservative. The whole blood is first heated to 37ºC before being automatically drawn into a sample channel containing the platelet agonist and fibrinogen-coated beads and is then mixed by the movement of a microprocessor-driven steel ball. The light absorbance of the sample is measured 16 times per second by an automated detector. As the platelets interact with the fibrinogen-coated beads,

resulting in agglutination, there is a progressive increase in light transmission. A patient's blood sample that exhibits inhibited platelet function produces low light transmittance; whereas a sample with normal platelet function produces high light transmittance. The rate of agglutination is quantified as the slope of the change of absorbance over a fixed time interval and measured in millivolts per 10 s. The device then automatically displays this result in agonist-specific units. This system measures the rate of platelet response but not the extent of aggregation response; thus, data obtained from this system are more similar to slope determinations in classic LTA (80).

Figure 1.5: VerifyNow Instrument (Accumetrics, San Diego, CA) {Adapted from: http://www.accumetrics.com/)

Three types of cartridges are available for use with VerifyNow: one for measuring the effects of aspirin, another for P2Y12 inhibitors and a third for GP IIb/IIIa inhibitors.

So far, clinically, there has been poor correlation between the results of LTA and VerifyNow in assessing antiplatelet drug response (81).

1.2.8 Viscoelastic point-of-care haemostatic assays (VHA)

The VHA gives a graphic presentation of clot formation and subsequent lysis. Thrombelastography (TEG) (figure 1.6) was first described in 1948 by H. Hartert (82) as a method to assess the viscoelastic properties of coagulation in whole blood under low shear conditions. Rotation Thromboelastometry (ROTEM®) is another technique (Figure1.7). These measure the physical properties of a forming clot by the use of an oscillating cup. Whole blood is incubated at 37°C in a heated cup. Within the cup is suspended a pin connected to a mechanical–electrical transducer that monitors the motion of the pin (a torsion wire in TEG and an optical detector in ROTEM). The cup and pin are oscillated relative to each other through an angle. The movement is initiated from either the cup (TEG) or the pin (ROTEM). As fibrin forms between the cup and pin, the transmitted rotation from the cup to pin (TEG) or the impedance of the rotation of the pin (ROTEM) are detected at the pin and a trace generated. The trace is divided into parts that each reflects different stages of the haemostatic

process (clotting time, kinetics, strength and lysis, with slightly different nomenclature for TEG and ROTEM) (83).

Figure 1.6: TEG® 5000 Thrombelastograph® Hemostasis Analyzer System. (Adapted from: http://www.haemonetics.com/Products/Devices/surgical)

Figure 1.7 ROTEM Instrument.

(Adapted from: https://www.rotem-usa.com/products)

Maximal haemostatic activity is measured by a kaolin-activated whole-blood sample treated with citrate. Heparin is used as an anticoagulant to eliminate thrombin activity in the sample. Reptilase and factor XIIIa (activator F) are used to generate a cross-linked fibrin clot to isolate the fibrin contribution to clot strength. The contribution of the ADP or TxA2 receptors to the clot formation is provided by the addition of ADP or AA. The effect of aspirin or thienopyridine therapy also can be estimated by comparing the unmodified TEG curve (i.e., kaolin-

activated whole-blood sample) with the AA or ADP-stimulated TEG curve (84).

1.2.9 Platelet works

It is also possible to monitor platelet aggregometry in whole blood by a simple platelet counting technique. The Plateletworks (Plateletworks aggregation kits and Ichor full blood counter, Helena Laboratories, Beaumont, TX) methodology involves using a cell counter to measure total platelet count in a whole blood sample and then to repeat the platelet count on a second sample that has been exposed to a known platelet agonist. After this the platelets aggregate and the platelet count decrease compared to the first sample. Aggregated or agglutinated platelets will not be counted as platelets in the second sample (85).

The difference in platelet counts between the two samples provides a measure of aggregation, whereas the ratio of the two counts provides a measure of percentage inhibition.

1.2.10 Global Thrombosis Test

The Global Thrombosis Test (GTT, Montrose diagnostics, UK. Figure 1.8) is the first clinically available, comprehensive, point-of care test to simultaneously measure thrombotic occlusion time (OT), coagulation,

and spontaneous endogenous thrombolytic activity. The apparatus is pre-warmed to 37 degrees before use for testing. In this test the platelets are activated by high shear stress, without any other agonists. The collected blood is passed through a specially designed plastic conical tube containing narrow gaps (created by the gaps between the inside of the tube and two ceramic ball bearings inside). As blood passes through these narrow gaps, platelets are exposed to high stress conditions, similar those shear stresses that exist in a stenosed coronary artery. This causes platelets to be activated. The flow of the blood is monitored downstream with a light source and used to measure the arrest of flow suggesting total occlusion of the lumen and the time to restart flow due to spontaneous thrombolysis of the platelet plug (86). This test uses native, non-anti-coagulated whole blood and thus overcomes the shortcomings of many other tests. Although the initial activating stimulus is high shear stress, in the development of occlusive thrombi all physiologically-important platelet agonists (TXA2, ADP, thrombin) are closely involved. GTT only requires One 4.0 ml blood sample can be done by bed side. There is no calibration or internal standard required.

Figure 1.8: GTT instrument and tube.

(Adapted from http://www.nutr.kobegakuin.ac.jp/~seiri/english/images/GTT)

1.3. Limitations of platelet function tests

Despite the availability of a multitude of tests, there has been no single test that can adequately address all the issues with respect to platelet related disorders. Although, light transmission platelet aggregometry is regarded as the gold standard of platelet function test, it is relatively non-physiological because separated platelets are usually stirred under low shear conditions during the test and only form aggregates after the addition of agonists, conditions that do not

accurately mimic platelet adhesion, activation and aggregation upon endothelial injury. Conditions which impair light transmittance, for e.g. lipaemia and haemolysis will significantly affect LTA results. Conventional LTA using a full panel of agonists requires both high blood volumes and expertise to carry out and interpret results and thus makes it an impractical test for routine clinical use 24 hours a day, 7 days a week.

Furthermore, currently, all platelet function tests are performed on citrated PRP or whole blood. Citrate anticoagulation interferes with ionised calcium. The presence of calcium is essential for platelet aggregation and release of dense granular nucleotides and in a citrated sample all extracellular calcium gets bound with ethylenediamine-tetraacetic acid, thus irreversibly hampering platelet aggregation (87). Plasma Ca^{2+} is not constant, but varies with haematocrit between individuals, with exercise, and in various disease states such as hypertension, asthma, and diabetes. Despite the use of a fixed citrate:blood ratio, therefore, the actual Ca^{2+} concentration in citrated blood will vary considerably. Citrate reduces the plasma Ca^{2+} concentration from 0.94–1.33 mM to 40–50 μM. Platelet aggregation is optimum at levels of 100 μM Ca^{2+}, and at levels below 10 μM, platelet aggregation does not occur. Accordingly, a slight alteration in the circulating plasma Ca^{2+} level will manifest in markedly enhanced or

reduced agonist-induced platelet aggregation and response to antiplatelet agents in blood. In citrated blood, thrombin is not generated, and Ca2+ levels are significantly reduced to 40–50 μM (below the threshold level of 250 μM required to generate thrombin), resulting in suboptimum platelet aggregation (88)

The citrated sample is tested between 30 min and 4hour of collection assuming constant platelet behaviour but the responsiveness of platelets to aggregating stimuli changes with time (88). If the platelet function test is performed within minutes of blood draw, aggregation in response to almost all stimuli is minimal. Maximum aggregation is demonstrated only about 1 hour after collection of blood. The time taken for platelets to become responsive can be reduced by increasing the concentration of some agonists but this may require large concentrations of the agonists due to inter-individual variations and reduce the sensitivity of the test. This makes it impossible to determine the optimal storage time of the sample (88). Other factors like the procedure of collection of blood and agonists used may also interfere in the accurate assessment of platelet aggregation. Most of the platelet function tests described above are single agonist specific, in other words, they only measure response of platelets to one particular agonist for e.g. ADP, epinephrine, arachidonic acid or collagen. The effect of shear stress is not measured in these tests.

Also, overall thrombolytic status is difficult to measure using these platelet function tests as most of these tests do not measure the response to all fibrinolytic markers. Thus none of the test provides an assessment of global thrombotic status of an individual patient.

GTT has an advantage as it is performed on native blood within 15 seconds after collection; involves shear stress, and is able to assess the effect of thrombin on thrombosis. It also assesses endogenous thrombolytic activity and hence seems to be a more physiological test than any other tests available currently albeit with limited clinical data.

1.4 Correlation of PFT with clinical outcome

Clinical correlation between measured platelet function and cardiovascular outcome has been poor despite use of different tests (89).

Recently, in a multi-centre randomised French study, 2440 patients with stable angina or non-ST-elevation acute coronary syndrome undergoing PCI were randomised to either conventional antiplatelet strategy or to a monitoring-guided strategy for drug-eluting stent implantation with drug adjustment in patients with poor antiplatelet response. For patients in the monitoring group, the VerifyNow P2Y12 and aspirin point-of-care assays were used in the catheterization laboratory before stent implantation and in the outpatient clinic 2 to 4

weeks later. The primary end point was the composite of death, myocardial infarction, stent thrombosis, stroke, or urgent revascularization 1 year after stent implantation. In the monitoring group, high platelet reactivity in patients taking clopidogrel (34.5% of patients) or aspirin (7.6%) led to the administration of an additional bolus of clopidogrel, prasugrel, or aspirin along with glycoprotein IIb/IIIa inhibitors during the procedure. The primary end point occurred in 34.6% of the patients in the monitoring group, as compared with 31.1% of those in the conventional-treatment group (hazard ratio (HR), 1.13; 95% confidence interval [CI], 0.98 to 1.29; P=0.10). Interestingly, the main secondary end point, stent thrombosis or any urgent revascularization, occurred in 4.9% of the patients in the monitoring group and 4.6% of those in the conventional-treatment group (HR 1.06;95%CI, 0.74 to 1.52; p=0.77). The rate of major bleeding events did not differ significantly between groups. Thus the authors concluded that there were no significant improvements in clinical outcomes with platelet-function monitoring and treatment adjustment for coronary stenting, as compared with standard antiplatelet therapy without monitoring (90).

Updated American and European practice guidelines have issued a Class IIb recommendation for platelet function testing to facilitate the

choice of antiplatelet agent in selected high-risk patients treated with PCI, although routine testing is not recommended (Class III) (91).

The strategy of monitoring platelet function to yield better ischemic and safety outcomes might be still limited by at least one factor: what is the optimal platelet function test? Thus, the quest for an ideal platelet function test, which will not only provide quantitative data regarding platelet functionality but will also reflect on clinical outcomes and aid decision making in individual patients is still on going.

Chapter 2: Hypothesis and Aims

2.1 Hypothesis:

Chronic renal failure patients despite being on RRT (dialysis or having undergone a renal transplant) remain at increased risk of acute thrombotic events. The reason for this is unclear, but may be related to either increased platelet reactivity (tendency to activate and aggregate), impaired endogenous thrombolytic activity or both. For the purpose of this study, we hypothesised that there are differences in either the aggregatibility of the platelets or the tendency of spontaneous thrombolysis of a formed platelet rich thrombus in ESRD patients, which may account for their increased thrombotic events, over and above traditional risk factors.

2.2 Aims:

The aims of this project were as follows:

1) To characterize thrombotic status, using the Global Thrombosis Test (Montrose diagnostics, London, U.K) in ESRD patients

2) To compare the thrombotic status of the ESRD patients with that of normal healthy volunteers and patients with known coronary artery disease but no renal disease.

3) To investigate whether a platelet function test of overall thrombotic status could identify those patients who despite RRT, remain pro-thrombotic and/or have impaired endogenous thrombolysis.

4) To observe whether any variation of platelet reactivity in renal disease patients is related to an increase in acute thrombotic events.

5) To identify whether there are any variations in platelet function within patients on different modes of RRT (HD vs. peritoneal dialysis (PD) vs. renal transplant recipients)

6) To identify whether certain clinical subgroups are more likely to be prone to acute thrombotic events (for e.g. women, Asians, diabetics).

Chapter 3: Methods

3.1 Study population

The main study group was of the patients with ESRD undergoing HD for at least 3 months under supervision of the East and North Hertfordshire NHS Trust renal department. These patients were receiving treatment by regular HD at the Lister Hospital, Stevenage, or its satellite units at St Albans City Hospital, St. Albans, and Luton and Dunstable Hospital, Luton. Further subgroups included patients with renal transplant under the care of Lister Hospital and patients on PD supervised by the Lister Hospital. The subgroups will be discussed later in separate chapters.

3.2 Inclusion criteria:

All patients aged 18-90 years and who met the criteria for ESRD on RRT as described above, who were able to consent and did not have any exclusion criteria as described below were approached for participation in the study.

3.3 Exclusion Criteria:

The following exclusion criteria were applied to this study**:**

- Inability to consent
- Current participation in another interventional study
- >90 years and <18 years

- ST-segment elevation myocardial infarction within 2 weeks
- Cardiogenic shock
- Inter-current illness, such as pneumonia, sepsis, ACS or heart failure in last 3 months
- Malignancy (active)
- Bleeding diathesis
- When complete follow up over 1 year period was unlikely (e.g patients planned for live-donor transplant or planned to move out of the area.
- Other disease (non –cardiac conditions) shortening life-expectancy to less than 12 months
- Blood dyscrasia (platelets <70, Hb <8g/dl, INR>1.4, APTT>x2 ULN, leukocyte count <3.5x 109/l, neutrophils count <1 x 109/l)
- Warfarin or other anticoagulant treatment
- Thrombolysis or glycoprotein IIb/IIIa inhibitor prior to sampling (within last 7 days).

3.4 Funding and Ethics:

The funding for the project was provided by the cardiovascular research department of the East and North Hertfordshire NHS Trust. The study protocol was then submitted to the local ethics committee (Essex and Hertfordshire research network). The ethics committee

approved the study protocol and documents after an application form submission process followed by an interview of the researchers by a specialist panel. The letter of approval is available to view in the appendix section.

The protocol was approved by the research and development department of the trust after an application and review process.

3.5. Patient selection and consenting

3.5.1 Haemodialysis patients

A list of all the patients undergoing HD was obtained on weekly basis from the schedule available in each individual unit and patient notes were reviewed to screen eligible patients.

The patients were approached by me during their attendance to discuss the study on a face-to face basis individually. Explanation was provided that this is purely a research study and will not affect their clinical outcome or management in any way. All patients approached were informed that participation is entirely voluntary and would not benefit them directly and that they would be free to withdraw from the study at any time and it would not impact on their current or future clinical care. They were also made aware of the follow up procedure and need for repeat contact as part of the study. A patient information sheet (appendix), which was approved by the local ethics committee,

was provided to each patient on individual basis and at least 48 hours were given before re-approaching patients regarding their decision about participation. If the patients decided to participate in the study then a full informed, written consent was obtained in all cases. A consent form which was approved by the local ethics committee was used (appendix).

3.5.2 Peritoneal dialysis and Renal transplant recipients

Patients on PD under supervision of the Lister hospital and all renal transplant recipients were approached for participation in the study on the day of their out-patient appointment at the Lister hospital. They were provided information in same manner as described above for the HD patients. They were re-approached for consent and sampling only on the day of their next clinic appointment.

3.6 Sample collection

Most patients in all the units have a fixed time table which is generated beforehand and they attend the dialysis units at a pre-fixed times and days of the week with flexible arrangements in place, by all the units, to accommodate any patient requests for time or day alteration due to personal or clinical reasons. As all the patients were approached to discuss the study and provided with leaflets beforehand,

the consent for participation was obtained in advance to the sampling date. The sampling time and date was then agreed with the patient according to their time slot for dialysis. In all units there were four slots available to patients – early morning (commencing at 07:00 a.m.), mid-day (12:00 p.m.), early (15:00 p.m.) and late evening (18:30 p.m.).

I use to arrive at the renal unit approximately 30 minutes before the scheduled patient dialysis time to get the GTT machine ready (pre-heat according to the GTT protocol as discussed in chapter 1 section 2.7.4) and also to set up the machine and identify a suitable place to sample the patient without causing interference with their own or any other patient's treatment attending the dialysis unit at the time. The majority of time, the machine was placed at the bedside of the patient but if this was not possible (usually when two patients' beds were very close making it difficult to move freely or if an electric plug was not available near the bed for the GTT machine) then an appropriate area within the dialysis unit was used to place the machine and patient sample was obtained there. This usually was the area where patients are weighed prior to the start of HD on the day. Depending upon the number of GTT machines being available on the day of the sampling, either 2 or 4 patients were sampled just before any one dialysis time slot. As each machine has four ports, it was only possible to sample 2

patients using one machine in order to have two samples from each patient (one as a control sample to avoid any spurious results and minimise operator related errors). As the GTT sampling process is very operator dependant, it was a learning curve for me in the first few patients and I used up-to four samples in the initial 20 patients to minimise any error and develop a consistent technique although only valid readings were used from these patients and the non-valid readings discarded and not used in subsequent analysis.

Whole blood was used as part of the protocol to assess the global thrombotic status in the study population using GTT. Nine mL venous blood was obtained from a peripheral (non-fistula) vein from each patient and tested immediately after withdrawal. In all the patients the samples were collected by applying the tourniquet for very short duration and not very tight to avoid any spurious results caused by venous stasis. In HD patients, blood was obtained immediately prior to dialysis, before the routine low-molecular weight heparin was administered according to local protocol. Low-molecular weight heparin is used in the renal unit at the East and North Herts NHS Trust, as a single intravenous bolus immediately pre-dialysis. This allowed between forty eight to seventy two hours duration between low molecular weight heparin administration and GTT sample collection. The timing of the sample collection was according to the individual

patient's dialysis schedule start time and to standardise sample collection approximately 48 -72 hours post dialysis in all patients. This meant although all the patients on HD were sampled approximately after a similar gap post the previous dialysis session but sample collection was at different hours of the day according to individual dialysis time schedule.

Blood was taken using an 18-gauge butterfly cannula using a three - syringe technique. Three 5 ml syringes were used for blood collection. The first 3ml was either used as a control sample or discarded if there was any suspicion of haemolysis or air bubble in the butterfly cannula during venepuncture, the next 3-5 mL was used for routine tests (blood count, biochemistry, bicarbonate, and bone-profile) and the next 3 mL for global thrombotic status assessment. Where the quantity of blood obtained was not sufficient the procedure was abandoned and patient were re-sampled on a different day, if they agreed to it. Where the patients had had some routine tests within the last 1 week of sampling then the tests were not repeated and the available results were used. Blood was aspirated into a standard polypropylene syringe, which was directly and immediately inserted into the fitting in the GTT instrument (within 15 s of withdrawal). To avoid any sampling delay and to minimise any sample transit time the GTT instrument was positioned next to the patient's bed where

possible or in very close proximity. As the GTT instrument can test more than 1 patient sample simultaneously, the machine was placed in between the patient bays to minimise transit time. When this was not possible or the patients were ambulant, then the test was conducted in a dedicated room within the dialysis unit before the patients were put on dialysis, and the patient brought to that room for blood taking. The syringes were pre-labelled to avoid mixing up of samples.

Coefficient of variation was assessed by testing 10 healthy volunteers twice, at 48 hours interval and also by testing 10 HD patients twice at 48 hours interval. The coefficient of variation for OT was 8 % in normal volunteers and 6 % in HD patients.

To assess the effect of antiplatelet therapy on thrombotic status of normal healthy volunteers a small subgroup (20 healthy volunteers) was tested with GTT pre and post antiplatelet drugs. Volunteers were tested for GTT and then given a 300mg dose of aspirin and then GTT repeated few hours later. This same group, after a month of washout phase, was assessed again before and 12 hour after a 300mg loading dose of clopidogrel. This was done taking into account the half-lives of Aspirin and clopidogrel. Half-life of Aspirin varies according to the dose and at lower dose it is short, approximately 2 hours, but at higher doses it varies between 15- 30 hours. Clopidogrel has a half-life of about 6 hours.

3.7 Assessment of thrombotic and thrombolytic status

3.7.1 Global thrombosis test

Thrombotic status and endogenous thrombolytic activity were assessed using the GTT (Montrose Diagnostics Ltd, UK). This is a novel, point-of-care assay that employs non-anticoagulated blood. The GTT instrument is portable and easy to carry around (Figure 3.1). It is powered using an AC adaptor which can be plugged in any standard UK electrical socket.

Figure 3.1: GTT instrument.

The instrument has 4 channels to take up to four test tubes and thus four patient samples simultaneously. Once the machine is plugged into the electric mains it starts warming up and the LED indicator lights starts flashing. After the instrument is warmed up to 37 degrees

centigrade the LED indicator lights go off and the display panel shows "GTT ready" message. Any of the four channels can be used for sample analysis by choosing the channel number from the key pad on the instrument under the display screen. Once the sample is inserted in to the test tube inside a channel, the start button can be pressed on the key pad and the LED indicator light turns green. The LED indicator light of the channel in use remains green during sample analysis and turns amber once occlusion time reading is available (described below) and finally red once analysis is complete.

The instrument measures the time taken to create shear-induced thrombi under pathophysiological conditions (discussed previously in chapter 1) and in the second phase of the test, measures the time to achieve spontaneous lysis of thrombi created during the first phase. The principle of the GTT described below and is shown in Figures 3.2 and 3.3.

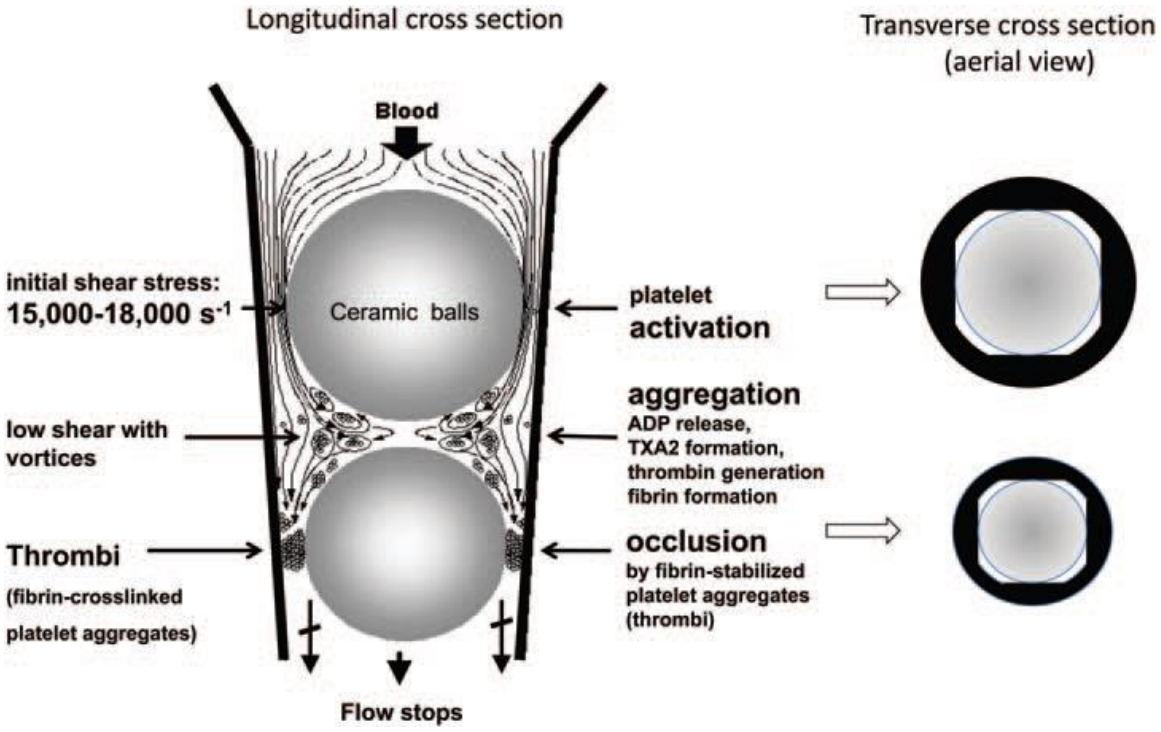


FIGURE 3.2: Mechanism of GTT

(Adapted from www.globalthrombosis.com)

Blood is introduced into a plastic tube in which two metal balls are situated in the conical part of the tube. The blood flows through this conical tube, which has got an outer cylindrical glass test tube for collection of downstream flow. There are four narrow gaps between the inner plastic surface and the balls (Figure 3.2). When blood flows through these gaps adjacent to the upper ball, the resulting high initial shear stress (180 dynes/cm^2) causes activation of platelets. In the space between the balls, due to the turbulent flow and low shear, the activated platelets aggregate. Thrombin is generated, which accelerates the formation of these aggregates and stabilizes them

through fibrin. When these stable thrombi reach the gaps around the lower ball, they gradually occlude these gaps, reducing the flow rate and finally arresting flow. The instrument measures the time (d) between consecutive blood drops. At the start of the test, flow is rapid and hence (*d*) is small. Subsequently, the flow rate decreases and hence (*d*) increases. When, the actual $d \geq 15$ sec, the time (first reading marked T1 on the instrument display panel) is displayed as Occlusion Time (OT sec). Subsequently the flow completely ceases. There is also a preset "thrombus stabilization time" following OT (200 sec), during which the sensors are inactive. This time is to allow stabilization of the formed thrombi, lasting occlusion and ignores small re-bleeds. Eventually, due to endogenous thrombolysis, flow is (partially) restored as indicated by detection of the first blood drop after OT (marked T2 on the instrument display panel). Lysis Time (LT sec) is calculated automatically by the instrument using the formula, LT= T2 – (T1+200).

The restart of blood flow following occlusion is due to spontaneous thrombolysis (lysis time, LT; seconds). If lysis does not occur until 6000 s following OT (LT cut-off time), 'no lysis' is recorded.

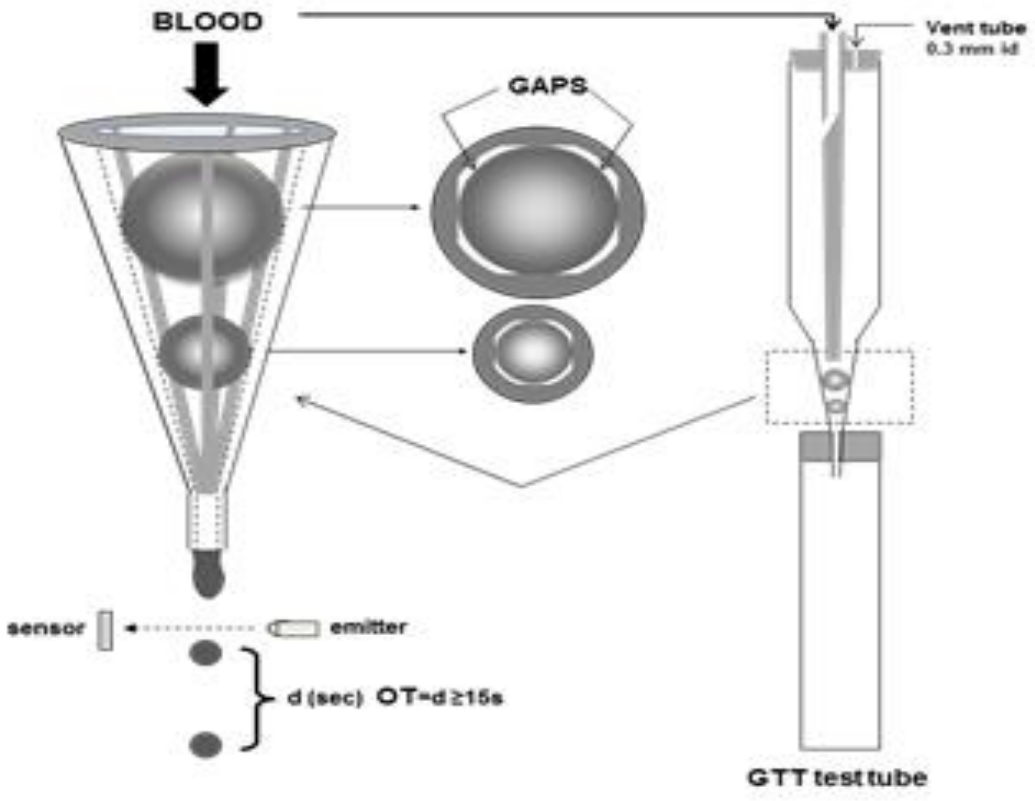


Figure 3.3: Principle of GTT (Explanation in text)

(Adapted from www.globalthrombosis.com)

The GTT assesses thrombus formation under pathophysiological conditions, since (i) whole blood is used which is non-anticoagulated, with physiological calcium-ion concentration, (ii) similar to pathological conditions, platelet-rich thrombus formation is initiated by high shear forces, with release of soluble agonists (TXA2, ADP, thrombin) playing only a secondary role, and (iii) thrombin generation from shear-activated platelets (procoagulant activity of platelets) plays a major role in the formation and stabilization of thrombi (92). As soluble

agonists are involved in the process of thrombus generation there is no need for several tests with different platelet agonists.

There was a small sub-study conducted prior to starting recruitment for the main study population (HD patients). This study which is discussed in detail in Chapter Five assessed consecutive GTT results from 2 locations in 20 patients. In each patient there were two samples obtained, one from a peripheral vein and the second from the fistula site at the time of HD. Both samples were used to assess thrombotic status by using GTT machine as described previously. The comparison showed variation in the OT and LT results obtained from the two sites and thus to maintain uniformity and prevent any error it was decided to use the peripheral venous sample to obtain GTT results for the planned main study (HD) population.

3.8 Data collection and follow-up

3.8.1 Data Collection

Baseline demographics, including dialysis vintage, were collected from the central electronic data base of all the RRT patients under the renal unit of the E&N Hertfordshire Trust. I received training in the use and interrogation of the database by the dedicated staff responsible for the running and maintenance of the software. The urea kinetic adequacy

parameters relating to the previous month's routine testing were also collected. Past medical history and any relevant clinical information were also identified from the data base or previous clinic letters.

This included the aetiology of renal disease where available, all past and present medical conditions, full drug history and history about previous and current renal replacement modality. The study patients were given a unique study reference number and all the data was kept anonymous to prevent exposure of any personal and demographic details. All further analysis and data interpretation was done using the patient's study number and no patient-identifiable information was used. All the data was collected on a pre-designed data collection form, which had been approved by the ethics committee (appendix).

Post collection, data was transferred onto Microsoft Excel software for keeping record and also for analysis purposes. All paper data was anonymised and the original data collection forms are all kept locked in the cardiology research office at the Queen Elizabeth 2 Hospital, Welwyn Garden City. The patient consent forms are also kept in a similar fashion in a dedicated folder in the cardiology research office.

A letter was sent to the general practitioner of the participating patients after prior approval from the patients. This letter format was also approved by the local ethics committee. This is available in the appendix section.

3.8.2 Follow-up

Follow-up was performed at 30 days and then at 3-monthly intervals for up-to a year in all patients. Some patients were followed up even after 1 year depending on time of recruitment into the study and patient willingness. The data beyond 1 year was not used in analysis as it was only available in small proportion of patients. Where possible the follow-up was done in the dialysis unit by a face to face interview with the participating patients. The proforma used is available in the appendix section. Where it was not possible to visit the patient during dialysis session, then a telephone contact was made with prior permission from the patients, which was obtained at the time of consenting.

3.9 Study endpoints

The primary endpoint of the study was the occurrence of major adverse cardiovascular events (MACCE) defined as the composite of cardiovascular death, non-fatal myocardial infarction (MI), or

cerebrovascular event. The secondary endpoint was the occurrence of peripheral vascular thrombosis including acute ischaemic limb and arterio-venous (AV) fistula thrombosis.

3.9.1 Cardiovascular events

New cardiovascular events were diagnosed in the presence of following two clinical events:

(I) Cardiovascular death, defined as death from MI based on the Universal Definition of Myocardial Infarction (93) This has been defined as sudden, unexpected cardiac death, involving cardiac arrest, often with symptoms suggestive of myocardial ischaemia, and accompanied by presumably new ST elevation, or new left bundle branch block, and/or evidence of fresh thrombus by coronary angiography and/or at autopsy, but death occurring before blood samples could be obtained, or at a time before the appearance of cardiac biomarkers in the blood), significant arrhythmia, or refractory congestive heart failure, or death attributed to cardiovascular cause at post-mortem; confirmed from death certificates as well as medical records and observers' accounts; sudden death was included as a cardiovascular event.

(II) Non-fatal MI, defined according to the Universal Definition of Myocardial Infarction as a rise and/ or fall of cardiac troponin with at least one value above the 99th percentile of the upper reference limit

together with evidence of myocardial ischemia with at least one of the following: symptoms of ischemia; ECG changes indicative of new ischemia (new ST-T changes or new left bundle branch block); development of pathological Q waves in the ECG; or imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

3.9.2 Cerebrovascular events

New-onset cerebrovascular event was suspected with recent onset of neurological symptoms or signs, e.g. aphasia, focal deficits, or unilateral paresis, thought to be vascular in origin and confirmed by computerized tomography (CT) or MRI brain. This included events with and without spontaneous clinical resolution, and thus included both stroke and transient ischaemic events.

3.9.3 Cause of death

Deaths were classified based on data obtained from post-mortem, from hospital medical records, or from the general practitioner, regarding the patient's last illness, according to WHO criteria based on ICD 10 (2010).

3.9.4 Secondary Endpoints

Peripheral thrombotic events were defined as occurrence of acute ischaemic limb(s) or AV fistula thrombosis on clinical examination and confirmed by contrast angiography and/or duplex ultrasound.

AV fistula thrombosis was included since this bears many similarities to arterial thrombosis. It occurs in the presence of arterial blood, under systolic pressure and pulsatile flow. In 80% of cases, it is associated with a significant stenosis, at or close to the AV anastomosis, predominantly due to intimal hyperplasia and associated with inflammation (94, 95). Abnormal haemodynamic shear stress is the most important upstream factor responsible for AV fistula failure. High shear rates upstream of a stenosed AV fistula predispose to platelet thrombus formation (rather than erythrocyte- and fibrin-rich thrombi formed in low shear, venous settings) and this is supported by histological findings.

3.10 Study end point data collection.

Once a clinical event was identified in a participating patient, further details were sought to confirm achievement of study end point. This was done where possible without checking the results of the GTT test in the patient to minimise any bias.

Clinical event was confirmed by details of individual event obtained from the medical records and analysis of laboratory results. If the clinical event was cardiovascular the information was also confirmed from any cardiological intervention or investigation records. In cerebrovascular events the results of any form of brain imaging or specialist neurological input were sought to confirm an event. In rare cases of events happening out of the catchment area of the East and North Hertfordshire NHS Trust, the details were obtained from patient's GP, discharge summaries from other hospital and relevant documents (imaging or cardiac catheterisation reports) were obtained via fax or post from the involved hospital.

In case of death as an event, the details were obtained from the medical records, death certificate, post mortem report, coroner's office or GP surgery.

All events were adjudicated by the principal investigator and me before being called as a study end point. All the data obtained was uploaded on to an excel document for analysis and recording purposes.

3.11 Statistical analysis

The normal range for OT and LT was established from healthy volunteers. The required sample size for the study was calculated based on the Cox Proportional hazards (PH) prognostic model. On the

basis of earlier work (96) in patients acute coronary syndrome and normal renal function the unadjusted Hazard ratio (HR) for LT dichotomized by 3000 seconds was 2.25 (95% CI= 1.34–4.7).

Based on the assumption that around 14% MACCE events per year will be observed in the ESRD cohort (15,17,35) a sample size of 200 was predicted to yield 30 events per year and the final Cox PH model with four covariates would provide 5% significance and 80% power (97,98). Given 200 patients in the study group and arbitrary selected sample size of 100 healthy volunteers, the difference in LT (OT) between these groups is classified on the moderate (semi-large) effect size level. The Cox PH model was used to assess sample size and Mann–Whitney U test used to compare patients and healthy volunteers, with 5 % significance and greater than 90 % power.

Unpaired t-test was used for the normally distributed variables and Mann–Whitney U test was used for non-normally distributed variables. Two-sided tests were used. Dichotomous variables were compared by x^2 test or Fisher's exact test, as appropriate. The uni-variate linear regression model was used to assess the relationship between a continuous variable (dependent) given a dichotomized variable (independent). Where necessary, log transformations were applied. Correlations were analysed using Spearman's rank test.

Ability of OT or LT to discriminate between patients with and without MACCE was evaluated by receiver operating characteristic (ROC) curve analysis. The optimal cut-off was determined by the value providing the greatest sum of sensitivity and specificity. The LT level significantly discriminated between patients with and without major adverse cardiovascular events (MACE) with an area under the curve of 0.67 (95% confidence interval: 0.55 to 0.69; $p < 0.05$). An LT >3,000 s was identified as the optimal cut point to predict MACE outcome, with sensitivity of 60% and specificity of 80%. Suggesting the inclusion of LT > 3000s as a risk factor can correctly identify the patients with MACCE in 60% of patients (True positive- Sensitivity) and can correctly identify 80% of patients with no MACCE (True negative- Specificity)

Kaplan–Meier estimates with log rank tests were used to compare survival curves. Uni-variate Cox proportional hazard regression was performed on LT divided up into groups of 1000 seconds to investigate the relationship between LT and MACCE, and to identify risk factors from which a multivariate Cox proportional hazard prognostic model was proposed. The hazard proportionality assumption was evaluated in the Cox model with scaled Schoenfeld residuals. The test was carried out for the uni-variate model including each of the patient variables, and its multivariate versions: baseline (age, sex, haematocrit) and

extended (age, sex, haematocrit, LT≥3000). In both setups, the hazard proportionality assumption was not rejected at p value = 0.05 significance.

To assess the added predictive ability of LT≥3000 seconds for MACCE, net reclassification improvement analysis (99) was performed. For chosen risk cut-offs, models do not recognize patients within the low-risk group (5 %), among patients with and without events.

The effect of interventions (heparin, aspirin, clopidogrel, and dialysis) on thrombotic status was evaluated with Wilcoxon signed rank test. All tests were two sided and significance was defined as p value < 0.05. Analyses were performed with R project for statistical computing software (R Foundation for Statistical Computing, Vienna, Austria).

Chapter 4:

Pilot study: Fistula Vs. Venous sample

4.1 Background

In ESRD patients, vascular access is important. It is traditional in many renal units to check the patients' biochemical parameters pre and post HD. The samples are normally taken from the fistula needles for convenience and to protect peripheral veins for potential fistula formation in the future. There is no consensus in the literature about the reliability of platelet function studies in samples obtained from sites other than peripheral veins.

4.2 Aim

Blood sampling and analysis of thrombotic status was a key feature of my research study. With this background in mind, a small pilot study was done to compare the thrombotic status results in 25 patients with samples obtained either from a peripheral vein with minimal tourniquet time or from the arterio-venous fistula.

4.3 Methods

The fistula samples were obtained before the start of dialysis at the time of needling of fistula. Simultaneous peripheral venepuncture was performed on the ante-cubital vein of the opposite limb. Samples from both sites were used for thrombotic status analysis using global

thrombosis test as explained previously. The peripheral venous sample was obtained from the non-fistula limb using the ante-cubital vein, where possible, and the same technique as described in the methods chapter for the main study. Where possible the tourniquet was avoided for the peripheral vein sampling and when used the duration was kept to minimum. The 18- gauge butterfly needle was used for the peripheral venous sample. All the fistula samples were collected without the use of any tourniquet and using the standard single needle procedure as per the dialysis unit protocol. For most fistula samples this was also 18 gauge needle but with no butterfly extension tubing. The fistula sample was taken by the dialysis specialist nurse while handling the fistula prior to the commencement of the dialysis and prior to any heparin administration. No extra punctures were done to the fistula for the purpose of this study.

4.4 Results

The median OT in the sample obtained from the fistula site was 487 ± 108 seconds. In the same patients the median OT from the peripheral venepuncture site was 408 ± 131 seconds (p=ns, OT values with standard deviation).

The OT values were not correlated as confirmed by Pearson correlation coefficient of 0.28.

The median LT value obtained from the fistula sample was 1049 seconds compared to 1167 seconds from the peripheral venepuncture. Again the Pearson r value did not show any correlation with a coefficient of -0.18. This suggested a wide variation in results in the samples obtained from fistula compared to the peripheral venous sample.

The data is shown in table 5.1.

Patient Number	OT- Fistula	OT-Venous	LT- Venous	LT–Fistula
1	364.7	408.3	1708	937
2	373.1	536.5	1119	826
3	471.8	577.7	1335	1320
4	477	607.7	1149	1746
5	603	468	1099	865
6	323.6	362.7	1473	1639
7	547.7	341.9	806	655
8	601.6	322	822	759
9	562	421.7	2243	2992
10	694.8	518.8	1265	1464
11	374.1	325.5	1313	916
12	487.8	348	6000	1028
13	618	566.6	1036	919
14	670	569.2	1660	1581
15	487.6	439.3	1124	2874

16	469.8	432.3	977	1865
17	356.1	283.2	1049	1422
18	439.8	103.6	529	1049
19	632	401	925	489
20	445.3	375.6	1805	1453
21	358	376.5	1705	742
22	451.4	637.2	6000	660
23	506.9	148.3	1129	2259
24	523.1	400.2	1167	1627
25	647	488.9	6000	800

Table 4.1- Table showing comparison of OT and LT values obtained from peripheral vein and fistula

4.5 Discussion

There are few studies comparing blood results obtained from a peripheral venepuncture and samples obtained from central catheters in dialysis patients. One study compared three different methods of blood sampling for INR values in HD patient (100). The INR samples were obtained from a peripheral venepuncture site, the central venous line, and the arterial bloodline sample port. The peripheral INR sample was used as the gold standard. The sample included 33 HD patients with patent dialysis central venous lines. Blood samples were drawn with a standardized protocol during one HD session from each of the three sites. The Pearson correlation coefficient for the peripheral

sample versus the central venous catheter line sample was 0.97 ($p < 0.001$) and was 0.99 ($p < 0.001$) between the peripheral sample and the arterial bloodline sample. These results suggested that both alternative methods are suitable for taking INR samples in HD patients. The purpose of this study was to ensure that samples for INR can reliably be taken from the central venous catheter since it is heparinized between dialysis sessions. The authors here again were faced by the same question as I was for my study regarding choosing the appropriate site for blood sampling in HD patients. In my study the turbulent flow in the fistula can alter platelet function, in theory, thus choosing the site for sampling blood was a vital part of my study. This has been suggested in other studies of similar design but there are not many studies comparing samples obtained from peripheral venepuncture to fistula samples.

In 1983, David et al (101) compared the samples taken from peripheral vein to the ones obtained via the arterio-venous fistula. When a single-needle system is used, the blood is obtained through the arterial limb of the Y cannula, thus avoiding an extra venepuncture. The pre dialysis sample is taken immediately after the cannula is inserted and the post dialysis sample is taken after the blood lines have been disconnected and the venous limb clamped. In this study they compared samples taken from the arterio-venous fistula and

those taken simultaneously from a peripheral vein in the opposite arm. The authors, in this study, concluded that post-dialysis concentrations should be determined from peripheral vein samples only based on the differences found in the results. This study addressed the question of whether sampling blood for urea measurement would give similar results when blood is drawn from the fistula or from a peripheral vein. Any differences will relate to the timing of the samples since urea is distributed in 2 pools which take some time to equilibrate. Hence sampling from the fistula (blood form central circulation) immediately post dialysis will show a lower urea concentration than that taken peripherally, which is equilibrated. The reasons for the differences seen in my study may be not related to the observations here, but nevertheless raise the question about using the appropriate site for obtaining samples in HD patients.

In a very small but interesting study Twardowski et al (102) measured platelet counts of 20 patients under regular dialysis with an arterio-venous fistula in one forearm, from blood samples taken simultaneously from the ante-cubital vein, the arterialized vein near the site of anastomosis, the femoral vein, and the femoral artery. Samples of blood from the fistula and from the cubital vein of the opposite limb were taken with and without tourniquet application (causing stasis). The highest platelet counts were found in blood taken

from the cubital vein without stasis. Platelet counts were identical in femoral artery and vein, but were 4.8 and 4.9%, respectively, lower than the cubital vein values. Platelet counts from fistula blood taken without stasis were 13.7% lower than in the cubital vein. Platelet counts were lower when samples of blood were taken with stasis: 11.2% in the cubital vein, 7.4% in fistula blood. These are important findings, especially when studying platelet functions in dialysis patients. It has been shown in other studies that shear forces at physiological level (1,000–10,000 s^{-1}) can form small transient platelet aggregates by development of membrane tethers (103*)* whereas at higher shear rates (>10,000 s-1), which are considered pathological (similar to shear rates found in a stenosed vessel) large rolling aggregates can develop independently of integrin IIb 3 and platelet activation (85). There have been some recent rheological studies showing that platelet and vWF modulation by hydrodynamic force is a mechanism for activation-independent aggregation that may contribute towards thrombotic arterial occlusion (104). This is unlike the findings from older studies which showed that shear stress in excess of 60 dyn/cm^2 can induce vWF binding to GP Ibα, (105) resulting in platelet aggregation, but only if activation can occur and αIIbβ3 is fully functional (106). Thus as both the fistula flow and tourniquet application causes increased shear rates, it can cause the altered

absolute platelet count measurement from samples taken from the fistula or after tourniquet application, due to platelet clumping. The varying shear forces acting on the blood flowing through a fistula and after a tourniquet application can result in platelet activation independent aggregation as described above and thus potentially altering global thrombotic status assessment in such situations. It cannot be predicted what exact effects will be observed based on the current knowledge as the effects vary with differing shear stress rates. As blood sampling was a key feature of my research study it was very important to avoid any discrepancy in the results obtained due to sample site choice.

4.6 Conclusion

Although this was a very small sample size, there was clear lack of correlation in the results obtained from two different sites in this sub-study. This could merely be due to lack of adequate numbers or could be due to the fact that two operators were involved in obtaining individual samples and thus there can be operator variability in handling the sample. Also as the peripheral venous sample were obtained after tourniquet application it can contribute towards shear induced transient platelet aggregation as described in the literature and thus affecting the results of the GTT. It is also possible that the

different needles used to obtain sample from the two sites contributed in the observed results, as the shear forces acting transiently will alter platelet functionality differently at variable shear levels.

This study was done before commencing recruitment for the main study and my technique was on a learning curve phase and thus there is a possibility that the variation in results could have been partly contributed by the lack of consistency in sample handling. The findings of this small sub-study cannot be generalised to all patients with fistula but due to the differences observed in the GTT values obtained via the fistula compared to a peripheral vein, it was concluded that fistula blood sample cannot be reliably used to analyse thrombotic state of HD patients for the purposes of main study. Thus the peripheral venepuncture would be used as the default method as described earlier (Chapter2).

Chapter 5:
Results

5.1 Introduction

In this section I will discuss the demographics and the results of the main group (HD patients) of the study population. The main population of this study were the patients undergoing HD under supervision of the Nephrology department of the East and North Hertfordshire NHS Trust at their three dialysis units. The main unit is based at the Lister hospital in Stevenage and the other two satellite units are based at the St. Albans City Hospital and the Luton and Dunstable Hospital. The patients were approached and given information and consented according to the protocol previously described in chapter 3. The ethics approval was obtained as previously described.

A control group comprising of 100 healthy volunteers was also tested using the GTT. This group was not taking any regular medications and was matched proportionally for sex and race. The average age in this group was 38 ± 11 years. There were 55 males and 45 females in this group. Subjects were recruited, through advertisement and direct approach, from among hospital staff and from relatives and carers of patients attending the outpatient department.

In total two hundred and sixteen (n=216) HD patients were recruited into the study from across the three dialysis units. The majority were receiving treatment at the Lister hospital renal unit. All the inclusion and exclusion criteria were met as described in the methodology

chapter previously (chapter 3). All patients had been on established HD for at least three months.

Blood samples for GTT were obtained from all the participants on the day of their dialysis appointment, prior to commencing the dialysis treatment for the day.

5.2 Study population demographics

The average age of the study participants was 64 years, with age range between eighteen and ninety years. Out of the two hundred and sixteen participants one hundred and thirty eight were males (64 %). The majority of the participants were Caucasian (84 %) with 16 % non-Caucasians of which 64 % were of south-east Asian origin and remainder Afro-Caribbean. Majority of the participants (70 %) had hypertension. Approximately, just under a third of patients had diabetes (28.7 %) and history of known coronary artery disease (28.7 %). A total of 11 % had a documented history of myocardial infarction previously.

About 62 % of the participants were on an antiplatelet agent with majority (51 %) taking aspirin and around 11 % taking clopidogrel.

The other major drug groups in use in the study population included proton pump inhibitors (55 %), statins (44 %) and diuretics (41 %). Almost the entire group was on erythropoietin (94 %).

Apart from the patient details, medical history, list of medications, blood test results (biochemistry, dialysis parameters) pre and post dialysis weight and blood pressure readings were also recorded from the day of the test. The proforma used to collect the data is shown in the appendix. All the data was transferred from paper format to electronic, anonymised format using the Microsoft excel software.

The detailed characteristics of the study population are shown in Table 5.1.

Table 5.1: Study Population demographics:

Baseline Characteristics	
Age, mean (range)	64.4±14.8
Male Gender	138 (63.9%)
Hypertension	151(69.9%)
Hyperlipidemia	43 (19.9%)
Diabetes	62 (28.7%)
Prior CAD	62 (28.7%)
Prior CVA	37(17.1%)
Prior MI	25 (11%)
LY Dysfunction	32/152 (21.1%)
BMI	26±6
Drug History	
Aspirin	111 (51.4%)
Clopidogrel	24 (11.1%)
Ace-I	73 (33.8%)
Statins	105 (48.6%)
Calcium Channel Blockers	65(30.1%)
Proton pump Inhibitors	118 (54.6%)
Diuretics	89 (41.2%)
Erythropoietin	203 (94%)
Laboratory Results	
Haemoglobin (g/dl)	11.0±1.2
Haematocrit	32.8±3.5
Platelet Counts($x10^9$)	227±75.4
Urea (mmol/l)	20.5±6.8
Creatinine (µmol/l)	732.1±250
Sodium (mmol/l)	136.5±3.4
Potassium (mmol/l)	5.0±0.8
Calcium(mmol/l)	2.4±0.2

Phosphate (mmol/l)	1.6±0.5
C-Reactive Protein (mg/l)	23.7.0±18
Albumin (g/l)	36.0±4.4
Bicarbonate (mmol/l)	23.6±3.1
Total Cholesterol (mmol/l)	4.1±1.2
LDL/HDL Ratio	3.5±1.1
Fibrinogen (g/l)	5.7±2.2
Parathormone(pg/ml)	52.3±51
Dialysis Parameters	
Duration Of HD(minutes)	187.9±34.9
Kt/V	1.3±0.3
Dialysis Vintage (months)	6.3±1
KRU(ml/min/m^2)	1.4±1.7

Table5.1: Baseline demographics of the main study population. n=Number, CAD= Coronary artery disease, PVD=Peripheral vascular disease, CVA=Cerebrovascular accident, MI=Myocardial Infarction, LV=Left ventricle, ACE-Inhibitor= Angiotensin converting enzyme inhibitor, PPI= Proton pump inhibitor

5.3 Study Methods

The main study protocol was designed to assess the thrombotic status by using GTT in the HD population and then comparing this to the thrombotic status of normal healthy volunteers. The methods used to obtain samples are described in detail in Chapter 3. This was followed by analysing for any influence of patient demographic profile, medical history, medications and dialysis parameters on the results obtained.

The patients were then followed up for study end points, as described in Chapter 3 and the events statistically analysed for any possible correlation with the results of the thrombosis test GTT. Statistical methods used in the study have been defined previously in the methodology chapter (Chapter 3, section 3.11).

5.4 Assessment of thrombotic status in HD patients and controls.

As described in Chapter 3 the sample obtained for GTT analysis provides two results, the occlusion time (OT) and the lysis time (LT).

216 HD patients were sampled to assess the GTT along with the pool of 100 normal volunteers. All OT values in this thesis are quoted with the standard deviation.

The median OT observed in the HD patients was 491± 177 seconds. This is the time taken for forming the occlusive thrombus. In the volunteer group the OT was 378 ± 96 seconds. The OT in the study group (HD patients) was significantly prolonged (p= 0.001). Thus HD patients make thrombus less quickly than normal volunteers. The coefficient of variation for OT was 8 % in normal volunteers and 6 % in HD patients, similar to earlier studies using GTT (96).

The LT in the study population was prolonged compared to the healthy volunteer group. The median LT was 1820 seconds in the study

population compared to 1053 seconds in the volunteers group ($p < 0.001$.) Thus HD patients are less efficient at dissolving a thrombus, once formed, than normal volunteers. The coefficient of variation for LT was 10 % in normal healthy volunteers and 5 % in HD patients.

The relation between OT and LT in study population compared to the control group is shown in figure 5.1.

Figure 5.1: Relationship between OT and LT in the HD group and in healthy volunteers

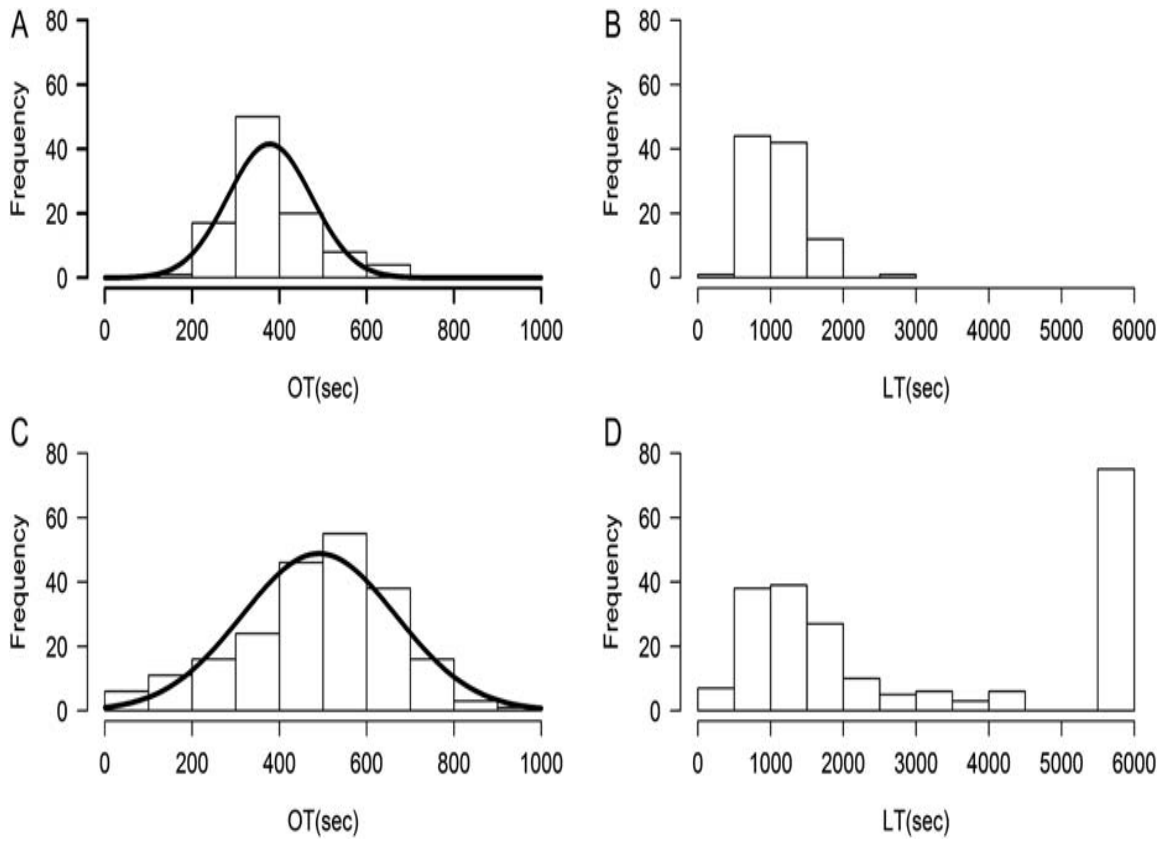


Figure 5.1 Legend: Distribution of (A) occlusion time and (B) lysis time of healthy volunteers and (C) occlusion time and (D) lysis time in HD patients. Y-axis shows number of subjects.

There was no correlation between OT and LT (Spearman's rank correlation coefficient = 0.15).

The OT both in the study population and the healthy volunteer group followed a Gaussian (normal) distribution. The LT on the other hand did not follow the Gaussian distribution in either group and in the HD patients had a very late second peak, which was not present in the control population.

In the control group none of the subjects had LT≥3000 seconds compared with 41.7 % of the study patients. Remarkably, over a third (34 %) of the study population group demonstrated markedly impaired thrombolytic status with LT ≥ 6000 seconds (second peak).

To assess the effect of HD on thrombotic status, a subgroup of 20 patients was tested immediately before starting dialysis and then retested 2 hours after finishing dialysis.

OT was similar in this group both pre and post dialysis with OT value of 570 ± 138 seconds pre dialysis and 545 ± 126 seconds post dialysis ($p = 0.368$). LT in this sub group was also similar with median pre dialysis reading of 1725 seconds compared to post dialysis LT of 1665 seconds (p value= 0.753). This suggests that the thrombotic status was not affected by HD.

5.5 Covariate analysis: influence of demographic profile, medical co-morbidities and medications on thrombotic status of HD patients.

All patient characteristics and variables including demographics, past medical history and baseline blood results were interrogated for effects on OT and LT.

Patients who were taking clopidogrel exhibited longer (less thrombotic) OT than those not taking clopidogrel. In patients on clopidogrel the OT was 564±179 seconds compared to 482±175 seconds in the non-clopidogrel takers, giving a p value of < 0.021. This would be as expected with an anti-platelet drug. Thus, provided that patients are responsive to clopidogrel they should have prolonged OT or a less thrombotic tendency. Occlusion time was also directly correlated with serum sodium and urea level. OT was also correlated with male gender with prolonged OT in male patients compared to the females. This is a surprise finding as the thrombotic events are generally more common in males; one would have expected to see a prolonged OT in females thus offering them some protection from pro-thrombotic events. Although the OT was prolonged in males it did not have any effect on the study end points and the thrombotic events were not common in either gender. This may be due to selection bias as the majority of the patients in this study were males (68%- Table 5.1).

In the control group of healthy volunteers aspirin significantly prolonged OT. The OT pre Aspirin dose was 356 ± 54 seconds compared to 530 ± 99 seconds post dose of Aspirin (p = 0.0001).The use of aspirin did not affect LT, with LT of 1043 seconds in the pre aspirin phase and vs. 1049 seconds post the dose(p value =0.741). Use of clopidogrel also increased OT in the control group with OT 365 ± 54 seconds pre clopidogrel dose and 569 ± 84 seconds post dose (p value = 0.001. Again similar to aspirin, clopidogrel usage did not affect LT (with LT 1043 seconds pre-dose and 1067 seconds post-dose, p = 0.731). The prolongation of OT in response to clopidogrel was marginally more than compared to the similar response seen secondary to aspirin (as given above, p = 0.02).

In the study group lysis time was not affected by age, gender, race, co-morbidities like hypertension, diabetes, peripheral vascular disease, and history of MI or dyslipidaemia. LT ≥ 3000 seconds was significantly associated with history of prior coronary artery disease, with patients with CAD demonstrating prolonged lysis time (p=0.042)
Both of the antiplatelet drugs (aspirin and clopidogrel) did not have any effect on the lysis time but LT was significantly prolonged in patients using proton pump inhibitors (p<0.001). Although the number of patients this affects is very small, we can postulate that this may

reflect a possible effect of proton pump inhibitors inhibiting the CYP2C19 iso-enzyme, thereby reducing the ability of clopidogrel in inhibiting platelet aggregation.

The global thrombotic status of the HD patients was not affected by the low-molecular weight heparin which was administered during the previous session of dialysis. GTT samples were taken before administration of the low- molecular weight heparin as described in the methods chapter. This allowed a gap of at least 48 hours between heparin administration and GTT sampling. Furthermore, to make sure that the administration of LMWH (48 hours earlier) did not interfere with the results anti-FXa level were checked and were undetectable (0.0 iu/mL) in all samples checked.

5.6 Study End points

All the study participants were followed up from the day of blood sample collection as described previously. The follow up was done to look for occurrence of any primary and secondary end points (end points described in detail previously in chapter 3). Briefly, the primary endpoint of the study was the occurrence of major adverse cardiovascular and cerebrovascular events (MACCE) defined as the composite of cardiovascular death, non-fatal MI, or cerebrovascular event. The secondary endpoint was the occurrence of peripheral

vascular thrombosis including acute ischaemic limb and AV fistula thrombosis.

Follow-up was available in all patients. The minimum duration of available follow-up data was for 365 days. The healthy volunteers were not followed up.

There were 12 non-cardiovascular deaths in the study group. Four of these were attributable to dialysis withdrawal. These patients died either in a hospice or at home after general health decline and cessation of RRT on clinical and personal grounds. Three patients in the group died due to bleeding, one patient had hematemesis secondary to chronic liver disease and history of hepatitis and two died of unspecified gastrointestinal haemorrhage. Two patients died due to sepsis (one of these developed it post knee surgery and the second one developed sepsis secondary to abscess in scrotum related to epididymoorchitis) and one due to pneumonia. One patient developed entero-colitis due to Clostridium difficile and subsequently died. One patient was diagnosed with a malignant neoplasm of the colon and died during the follow up period.

In the remaining study population a total of 40 study end point events were observed, out of which 23 were MACCE events and 17 peripheral thrombotic events. (The detailed description individual components of

MACCE and classification of peripheral thrombotic is given previously in the chapter 3 titled: Methods.) Out of the 17 peripheral thrombotic events 15 were AV-fistula thrombosis (14 in natural fistulae, 1 in an AV graft) and two patients had acute ischaemic limbs.

5.7 Survival analysis: Determination of effect of thrombotic status.

Survival analysis was performed to look for any relationship between thrombotic status and primary end points of the study (MACCE). This demonstrated no relationship between OT and MACCE, with hazard ratio of 1 (95 %% CI 0.9976 to 1.002, p= 0.969). On the other hand this analysis revealed a strong relationship between LT and MACCE. The optimal cut off point correlating with MACCE was LT ≥ 2940 seconds. This was rounded to 3000 seconds for clinical ease and calculation purposes. LT ≥3000 seconds was associated with a significantly higher risk of MACCE overall, giving a Hazard Ratio of 4.25 (95 %% CI 1.57 to 11.46, p = 0.004). This is depicted in figure 5.2.

Figure 5.2: Incidence of events based on dichotomised Lysis Time.

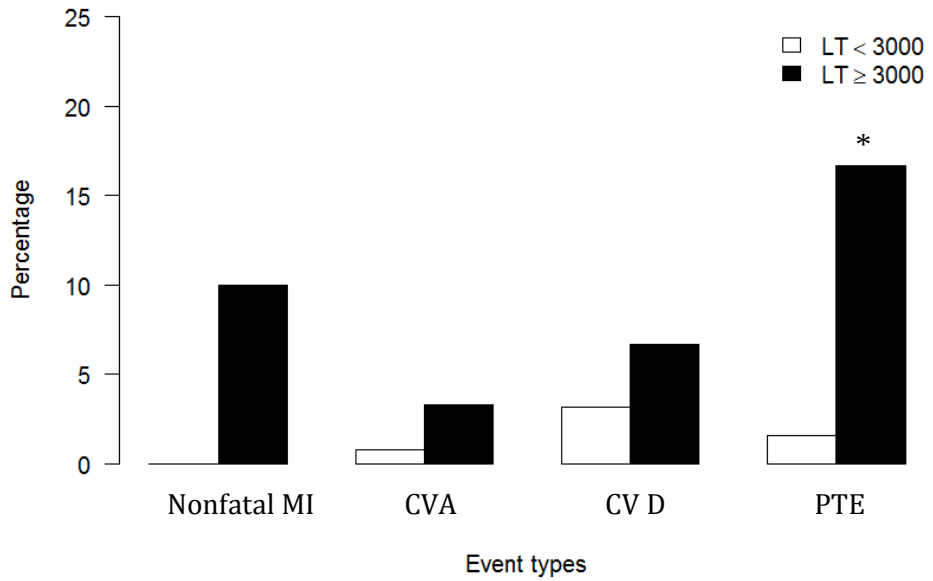


Figure 5.2 Legend: LT= Lysis time, MI= Myocardial Infarction, CVA=Cerebro-vascular event, CVD=Cardiovascular death, PTE=Peripheral thromboembolic events.

The individual events were also analysed for any correlation between LT and events. Nonfatal myocardial infarction or cerebrovascular events were strongly related to LT with a hazard ratio of 14.28, 95 % CI of 1.86– 109.9 and p= 0.01. Peripheral thrombotic events, when assessed separately, also were strongly related to LT. The hazard ratio of having a peripheral vascular event with LT ≥ 3000 seconds was 9.08, with 95 % CI between 2.08–39.75 and p = 0.003.

The break-down of MACE events and hazard ratios based on LT are summarised in table 5.2.

Table 5.2: Breakdown of MACE events and hazard ratio (HR) based on LT.

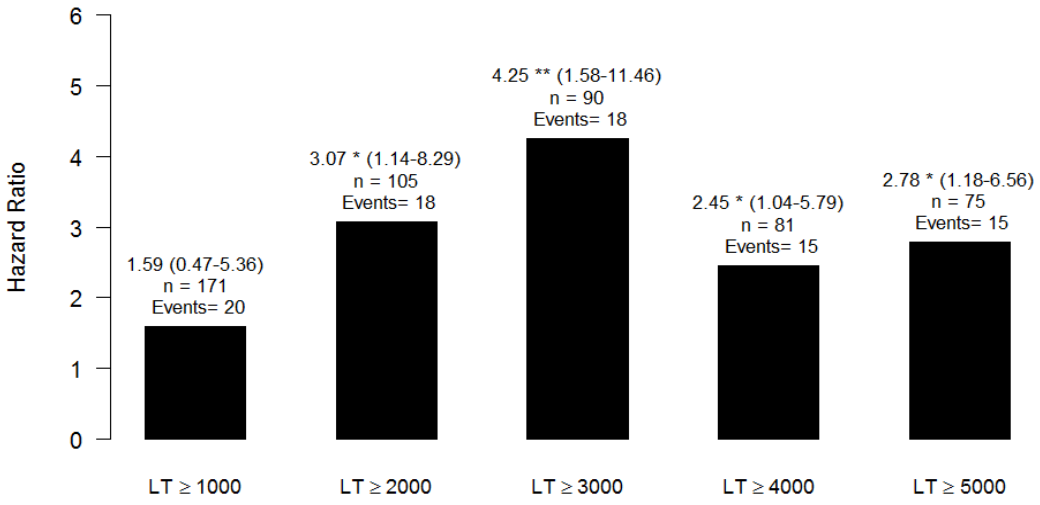
	Overall n=216	LT<3000 n=126	LT≥3000 n=90	HR(CI)	*p*-value
CV death, MI, stroke	23 (10.6)	5 (4.0)	18 (20)	4.25 (1.58-11.46)	0.004
MI and stroke	13 (6.0)	1 (0.8)	12 (13.3)	14.28 (1.86-109.9)	0.011
CV death	10 (4.6)	4 (3.2)	6 (6.7)	1.75 (0.49-6.22)	0.22
MI	9 (4.2)	0 (0.0)	9 (10.0)	-	-
Stroke	4 (1.9)	1 (0.8)	3 (3.3)	3.57 (0.37-34.42)	0.18
PTE	17 (7.9)	2 (1.6)	15 (16.7)	9.08 (2.08-39.75)	0.003

Table 5.2 Legend: CV death = cardiovascular death, MI = Myocardial infarction, PTE= peripheral thromboembolic events. HR=Hazard ratio, CI= confidence interval

All non-fatal myocardial infarctions and 15 out of the 17 peripheral thrombotic events occurred in those with LT ≥ 3000 seconds. Non-

cardiac death was not related to LT. As LT increased, hazard ratio for having an event increased up to LT ≥ 3000 seconds (Figure 5.3 and accompanying table 5.3). But there was no additional risk of having an event beyond the LT of 4000 seconds, possibly because a large number of study participants had severely impaired LT (LT≥6000 s, Figure 5.3 D).

Figure 5.3: Hazard ratio for Events based on LT divided into 1000 second intervals



*= Hazard ratio with confidence intervals in brackets.

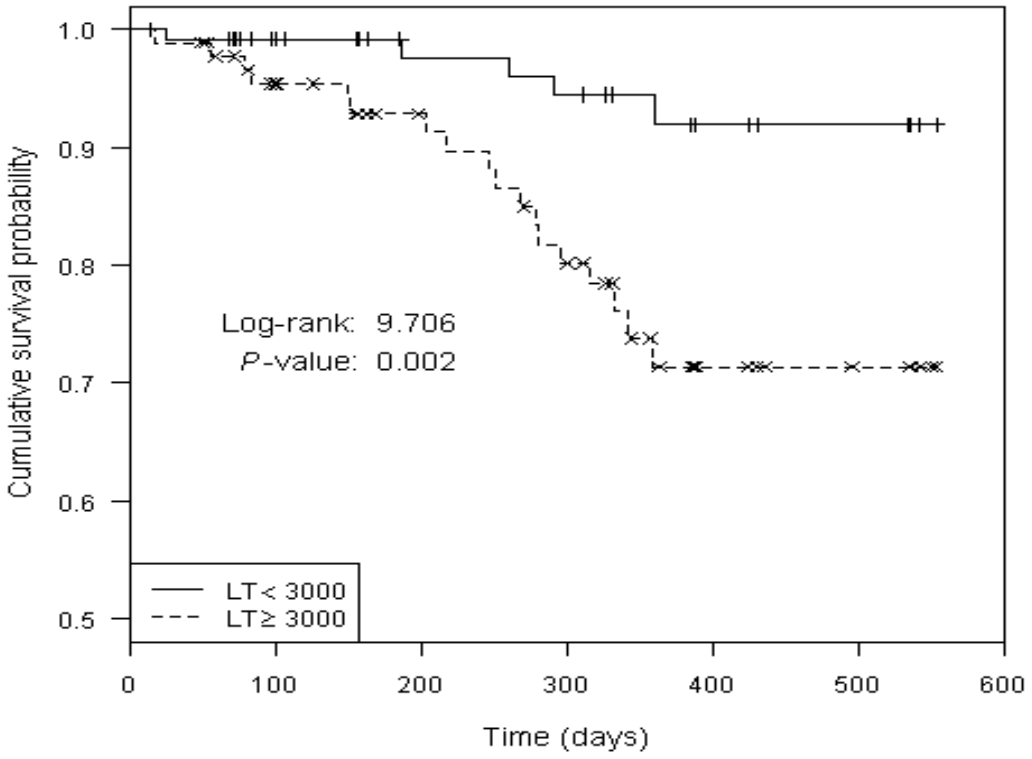
5.3: Hazard ratio based on LT

	HR	CI 95% Lower limit	CI 95% Upper limit	p value
LT ≥ 1000	1.59	0.47	5.36	0.075
LT ≥ 2000	3.07	1.14	8.29	0.001
LT ≥ 3000	4.25	1.58	11.46	0.000
LT ≥ 4000	2.45	1.04	5.79	0.003
LT ≥ 5000	2.78	1.18	6.56	0.005

Legend: LT =Lysis time, HR=Hazard Ratio, CI=Confidence interval

LT is therefore associated with an increased risk of having thrombotic events (events defined as described in the study methods previously) and prolonged LT is representative of impaired endogenous thrombolytic status. Thus it is logical to conclude that impaired endogenous thrombolytic status (represented by prolonged LT) was a very strong predictor of MACCE. This is shown by the Kaplan-Meir survival analysis estimates to look at the probability of event free survival in the study population based on the observed lysis time. Figure 5.4 shows the Kaplan-Meir curves.

Figure 5.4:



Legend: Kaplan–Meier curves showing probability of event-free survival in end-stage renal disease based on lysis time. Thrombolytic status was strongly predictive of major adverse cardiovascular events.

A uni-variate analysis was performed using all the patient characteristics and medication history as summarised in table 5.1 to look for any relation between these variables and the occurrence of MACCE. This model showed that only the following variables were related to MACCE: serum calcium (p value = 0.038), (log) CRP (p value = 0.04), and haematocrit (p value = 0.013). There was a

negative relation between use of diuretic treatment and MACCE (p value = 0.044).

Regression analysis was performed using a back-step model selection procedure, the following three variables were then entered into the baseline multivariate Cox proportional hazard model: haematocrit (HR = 0.87, 95% CI = 0.78–0.97, P = 0.015), and two traditional risk factors, namely age (HR = 0.999, 95% CI = 0.97–1.03, P = 0.944) and male sex (HR = 1.24, 95% CI = 0.5–3.03, P =0.645).

None of these basic covariates were correlated either with LT or its dichotomized version, which would have increased the standard error of the hazard ratio in the Cox proportional hazard model.

Multivariate Cox proportional hazard analysis including the baseline covariates showed that LT ≥ 3000 seconds remained strongly associated with MACCE after adjustment for the baseline risk factors (HR = 4.37, 95% CI = 1.58–12.12, P = 0.005). Table 5.4 shows the Hazard ratio obtained after adjustment for haematocrit, age and sex to LT ≥ 3000 seconds.

Table 5.4: Table showing adjusted hazard ratio based on LT. Adjusted for haematocrit, age and sex.

	Overall n=216 n (%)	HR(CI)	*p*-value	HR(CI) Adjusted	*p*-value
CVD, nonfatal MI, CVA	23(10.6)	4.25 (1.58-11.46)	0.004	4.37 (1.58-12.12)	0.005
Nonfatal MI and CVA	13(6.0)	14.28 (1.86-109.90)	0.011	15.76 (2.00-124.06)	0.009
CVD	10(4.6)	1.75 (0.49-6.22)	NS	1.64 (0.44-6.15)	NS
Nonfatal MI	9(4.2)	-	-		
CVA	4(1.9)	3.57 (0.37-34.42)	NS	4.89 (0.46-51.4)	NS
PTE	17(7.9)	9.08 (2.08-39.75)	0.003	10.81 (2,45-47.68)	0.002

Legend: CV death = cardiovascular death, MI = Myocardial infarction, CVA = cerebro-vascular accident, PTE= peripheral thromboembolic events. HR=Hazard ratio, CI= confidence interval

This baseline model (age, sex, haematocrit) was then extended by including LT≥3000 seconds to give the final predictive model. After this, of all the baseline model covariates, only haematocrit remained significant in the extended model adjusted for age, sex, and LT (HR = 0.89, 95% CI= 0.79–0.99).

Receiver operating characteristic (ROC) curve analysis indicates an improvement in the area under the curve with the extended model with borderline significance (DeLong test Likelihood ratio test p value = 0.058) indicating the usefulness of net reclassification improvement analysis, as ROC may not be sensitive enough to detect the new marker improvement (107).

Figure 5.5: Receiver operating curves for (A) occlusion time and (B) lysis time and (C) receiver operating curve analysis showing improvement of the prognostic model by adding LT ≥ 3000 s. Lysis time significantly discriminated between patients with and without major adverse cardiovascular events.

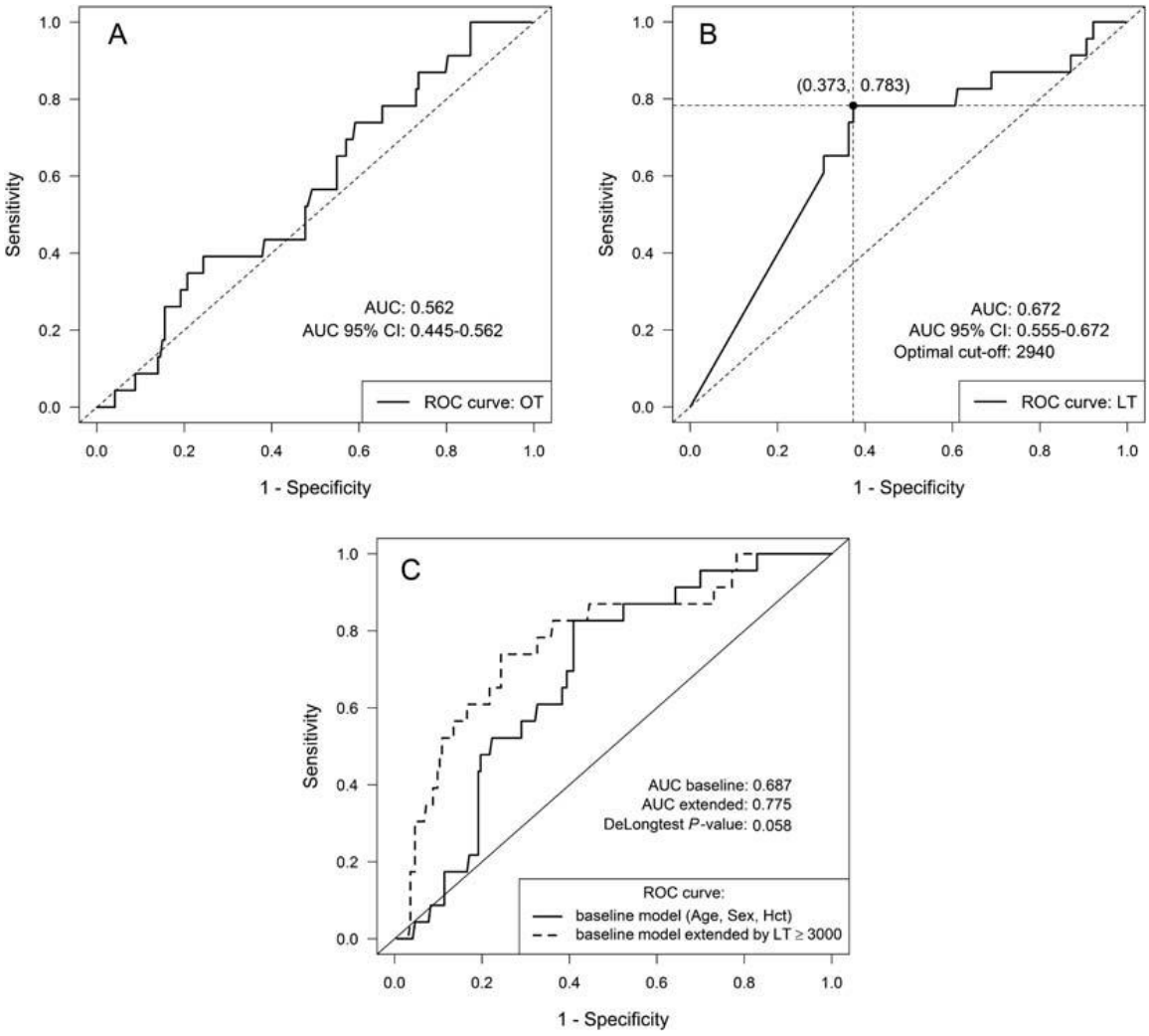


Figure Legend: AUC= area under the curve.

5.8 Reclassification

Reclassification analysis was performed on the previously mentioned baseline model with respect to the extended model (which included LT). This showed that adding LT to the baseline risk factor model improves risk stratification of patients with ESRD in terms of cardiovascular risk. Inclusion of LT≥3000 seconds in the model containing three baseline predictors (haematocrit, age, sex) significantly added to the model effectiveness (net reclassification improvement = 0.61, p value < , 0.001) leading to improvement in reclassification mainly of non-event patients.

The analysis is done with respect to the arbitrary 5 and 20 % cut off points of chance of having an event (risk cut-off levels). This divides the patients in to three risk categories, low (less than 5 %), medium (between 5 and 20 %) and high risk (more than 20 %). For chosen risk cut-offs, models do not recognise patients within the low-risk group (<5%), among the patients with and without event.

The extension of the baseline model leads to the improvement in reclassification of the group of patients with no events, explained in figure 5.6 and accompanying table 5.5. The model extended by LT>3000 reclassifies 62 (57.9 %) of the high-risk group (>20 %)

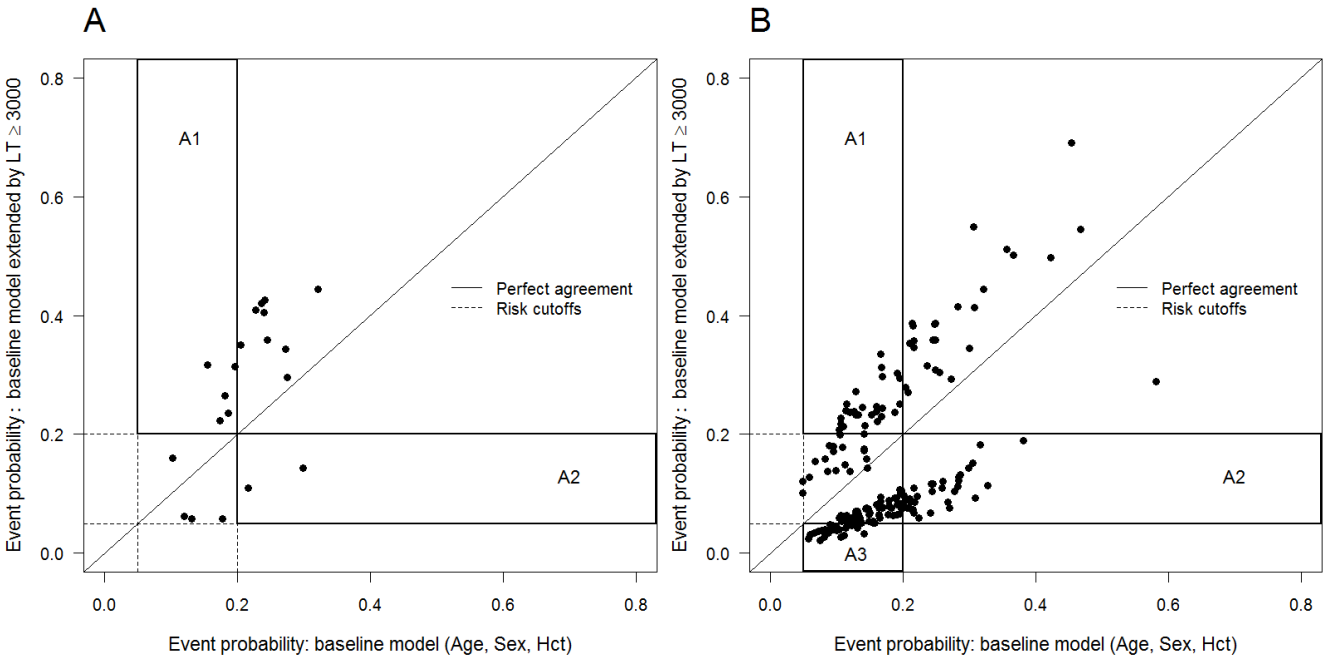
patients to the mid-risk group (> 5 % and <20 %), and 3 (4.4 %) from the mid to the low-risk group.

Both models roughly agree about the patient group with events within the high risk group (87.5%). The extended model reclassifies 5 of them (62.5%) from the medium up to high risk group.

Optimal risk cut-offs

The results obtained, however, depend on arbitrarily chosen risk cut-off levels. The net-reclassification analysis applied backwards allows finding the cut-off which provides the optimal (smallest p-value) reclassification. The new risk cut-offs are 13 and 43%, with NRI= 0.61 (p value < 0.001). Figure 5.6 shows the reclassification done with respect to these optimal points.

FIGURE 5.6: RECLASSIFICATION PLOT FOR BASELINE AND EXTENDED MODEL



Legend: Reclassification plot for baseline and extended models. The risk cut-offs are arbitrary values of 5 and 20% (dotted lines). A= patients with events and B= patients with no events. Extending the baseline prognostic model (age, sex, haematocrit) by inclusion of LT≥3000s helps reclassify patients without events (see text).

Figure A:

A1: region where the extended model correctly reclassifies the patients with event with respect to the baseline model from the medium to high-risk group.

A2: region where the extended model correctly reclassifies the patients with no events with respect to the baseline model from the high to medium risk group.

Figure B

A1: region where the extended model wrongly reclassifies the non-event patients with respect to the baseline model from the medium to high-risk group.

A2: region where the extended model correctly reclassifies the non-event patients with respect to the baseline model from the high- to medium risk group.

A3: region where the extended model correctly reclassifies the non-event patients with respect to the baseline model from the high- to mid-risk group.

Table5.5. Accompanying table to Figure 5.6 showing that the extended prognostic model including LT≥3000s helps reclassify the patients without MACE events from high to medium risk group, and from medium to low risk group.

NO EVENT					
		Extended model			
	Risk groups	Low	Medium	High	Total
Baseline model	Low	0	2(100%)	0	2
	Medium	29(22%)	76(58%)	27(20%)	132
	High	0	33(56%)	26(44%)	59
	Total	29	111	53	193
EVENT					
Baseline model	Low	0	0	0	0
	Medium	0	4(40%)	6(60%)	10
	High	0	2(15%)	11(85%)	13
	Total	0	6	17	23

Chapter 6:

Sub-study: Peritoneal Vs. Haemodialysis

6.1 Background

ESRD has been associated with profound clinical effects ranging from thrombosis to bleeding complications. RRT can be provided by different modalities, including HD, PD and renal transplantation. There is no consensus regarding the physiological or clinical outcome differences seen in these people based on the modality of RRT received. There is evidence suggesting that overall patient survival is similar for peritoneal and HD patients but that important differences do exist within select subgroups, particularly those subgroups defined by age and the presence or absence of diabetes. (108). An earlier study (108) compared 6 registry data based studies and 3 prospective cohort studies conducted in USA, Canada, Denmark and Netherlands. The authors also compared these studies to the US medi-care data. Differences in results between these 9 studies can be attributed to the degree of case-mix adjustment carried out and to the use of different subgroups when comparing mortality between HD and PD. After accounting for these differences, the authors found a remarkable degree of synergism in results between the registry studies and, to a lesser degree, the prospective cohort studies. PD was generally found to be associated with equal or better survival among non-diabetic patients and younger diabetic patients in all four countries. However, among older diabetic patients, results varied by country.

The Canadian and Danish registries showed no difference in survival between PD and HD among older diabetics while in the US, HD was associated with better survival for diabetics aged 45 and older. In another registry, the investigators compared mortality between patients treated with PD and HD (including home HD) using data from over 25000 patients in the Australia and New Zealand Dialysis and Transplant Registry. Overall mortality rates were significantly lower during the 90 to 365 day period among those on PD at day 90 (HR 0.89; 95% CI 0.81 to 0.99; $P < 0.001$). This effect, however, varied with the presence of comorbidities: younger patients without comorbidities had a mortality advantage with PD treatment, but other groups did not. After 12 months, the use of PD at day 90 was associated with significantly increased mortality (HR 1.33; 95% CI 1.24 to 1.42; $P < 0.001$). These data suggest that the effect of dialysis modality on survival for an individual depends on time, age, and presence of comorbidities. Treatment with PD may be advantageous initially but may be associated with higher mortality after 12 months.

These findings from the registry could be attributable to confounding factors which persist despite statistical corrections. For example, it has been postulated that acutely ill patients who start dialysis urgently are at high risk of death and they are treated predominantly with HD. This could induce selection bias in the comparison of mortality between HD

and PD patients. Couchoud et al (109), found a significant difference in mortality between "unplanned" and "planned" HD starts and suggested that comparing PD and HD after removing unplanned HD starts would likely provide a more balanced estimate of the effect of modality choice on survival in elderly patients. Similarly it has been suggested that other techniques involved in providing dialysis are frequently not taken into account when doing such comparisons (110). In many survival comparisons, the type of vascular access used for HD is not included in the analyses. For instance Perl et al. (111) recently showed that type of vascular access plays an important role in the relationship between dialysis modality and mortality. They found in this registry based, Canadian cohort, observational study that starting HD with a central venous catheter was associated with higher early mortality risk when compared to patients starting dialysis with a functional AV fistula or AV graft. The 1 year mortality in the graft/fistula group was similar to the PD patients. These relationships persisted over a 5-year follow-up period. As this study was a non-randomized observational study, so there are many confounding factors that can account for the results.

Nevertheless, despite the possible bias and lack of any randomized trail data, the differences in mortality based on dialysis modality are variable and three factors, namely DM, age, and co-morbidity, have repeatedly been found to modify the effect of treatment modality on

patient outcomes. Historically, it has been felt that the slower loss of residual renal function and urine output in PD patients may provide an early survival advantage and that the loss of ultrafiltration capacity may complicate volume control in PD patients, leading to an increased risk of death with time on therapy (112). Whether these findings are due to increased cardiovascular or thrombotic complications secondary to change in global thrombotic status is unknown.

There have been small studies in the past comparing the platelet function and thrombotic status in patients on PD to HD patients but there is still lack of consensus on this issue. In a small study (113) 37 normal healthy subjects , 18 patients with mild chronic renal failure, 15 patients with advanced renal failure, 18 HD patients and 11 PD patients were included and the expression of platelet surface receptors GPIb (the receptor for vWF) and GPIIb/IIIa (the receptor for fibrinogen) was investigated with monoclonal antibodies CD42 and CD41 flow cytometry. The authors found a negative correlation between serum creatinine and the expression of glycoprotein GPIb. The defect was not corrected by HD and/or PD. On the other hand HD and PD had different impact on the expression of GPIIb/IIIa glycoprotein with PD seemingly favourably restoring normal values of the expression of this membrane protein. Theoretically this data could be correlated to the better biocompatibility of the PD and to more

favorable clinical outcomes in terms of accelerated atherosclerosis and athero-thrombotic complications in patients with ESRD.

6.2 Aim

The aim of this sub-study was to establish any differences in global thrombotic status of ESRD patients based on the dialysis modality used for RRT.

6.3 Methods

Patients on PD under supervision of the Lister Hospital were approached for participation in the study on the day of their out-patient appointment at the hospital. They were provided information in same manner as described in the chapter 2 for the HD patients. They were re-approached for consent and sampling on the day of their next clinic appointment. Blood samples were collected for thrombotic status analysis from peripheral veins as described in chapter 2.

Twelve peritoneal patients were enrolled in this sub-study. The majority of the patients in the unit at the time of the study were on HD and only around 30 were on PD. All of them were approached for participation but only 12 agreed. The blood samples for GTT were collected using the same technique as described earlier for the main study population. Patients were approached at the time of their clinic

appointment and study was discussed and relevant paper work, including the patient information sheet (appendix) and consent form provided. These patients were then re-approached at the time of their next clinic follow up appointment and, if they agreed, were recruited into the study. All patients had the sample taken in the morning at the time of their outpatient clinic appointment.

The 12 PD patients were compared to 216 HD patients. Blood samples were tested with the GTT to obtain the OT and LT values as described in the methods Chapter 3 earlier. Unlike the HD patients the PD patients were not followed up for any clinical events and the role of the study was just to compare baseline thrombotic status of patients receiving different dialysis modalities.

6.4 Results

All patients had been on PD for at least 3 months with average duration being 22 months. The PD patients were younger compared to the HD patients with the average age in PD patients being 52 ± 15 years compared to 64 ± 14 years amongst HD patients. The OT in patients receiving PD was 419 ± 134 seconds and the median LT was 2561 seconds. The thrombotic status in the PD patients in this subgroup study did not differ significantly from patients on HD (OT

419±134 seconds vs. 491±177 seconds, p value= 0.672 and LT 2561 vs. 1820 seconds, p value= 0.574).

6.5 Discussion

The pathogenesis of bleeding in uremic patents is considered multifactorial. It has been attributed to platelet dysfunction particularly platelet-platelet and platelet-vessel wall interactions. RRT has helped reduce bleeding related complications, but the risk persists. Abnormalities of blood coagulation and fibrinolysis predispose uremic patients to a hypercoagulable state carrying the risk of cardiovascular thrombotic events and thrombotic complications such as thrombosis of the vascular access. There are differences in the measurement of various haemostatic parameters in patients with ESRD concerning treatment with either HD or continuous ambulatory PD. There have been small studies in the past showing significant differences between totally aggregated platelets, reversibly and irreversibly aggregated platelets, the percentage of large platelets ($p<0.0001$) and the average number of platelets per aggregate ($p<0.001$) in dialysis patients compared with control persons. However, no differences have been found between HD and PD groups, or the duration of dialysis treatment (114).

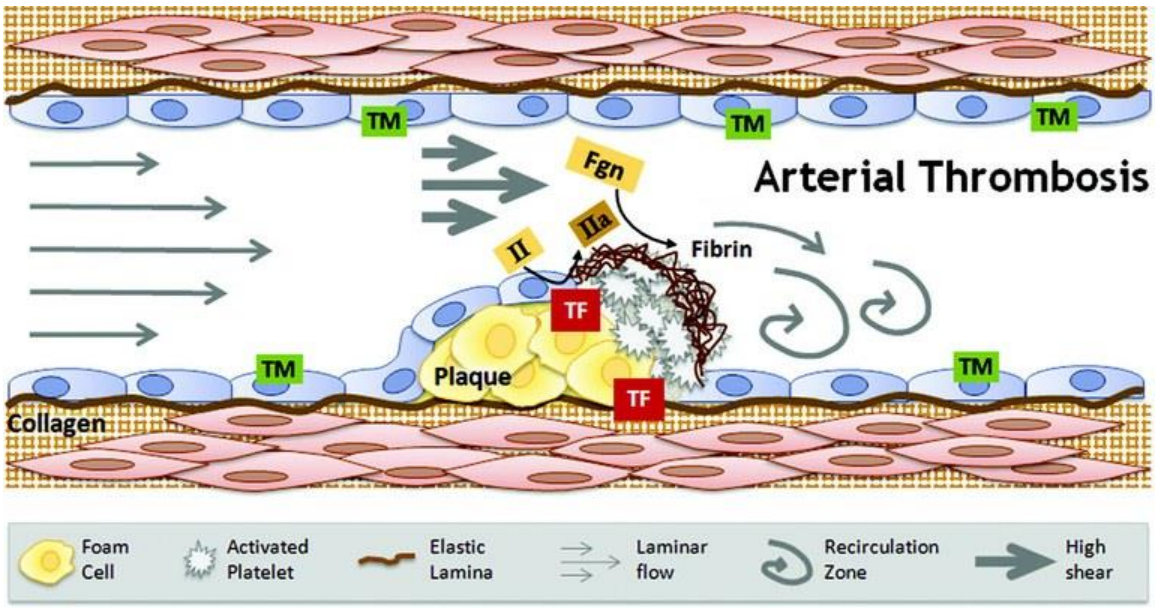
On the other hand there have been some studies, albeit in small groups and dating from periods when HD was more bio-incompatible, showing that renal transplantation and PD improved platelet function, while the HD procedure itself impaired platelet function (115).

Damage to blood vessel walls exposes tissue factor-containing cells from underlying cell layers to the bloodstream. Tissue factor (TF) is then able to bind in the presence of calcium to Factor VII, the calcium forming a bridge between TF and FVII. This sets off an extracellular cascade involving sequential serine protease activations leading to conversion of prothrombin (II) to thrombin (IIa), which converts fibrinogen (Fgn) to fibrin, leading to fibrin deposition and the activation of platelets to form thrombus (Figure 6.1).

Tissue factor pathway inhibitor (TFPI) is a potent anticoagulant protein. TFPI down-regulates the initiation of coagulation by inhibition of FVIIa/TF/FXa and blockage of TFPI enhances FXa and thrombin generation. Decreased TFPI activity contributes to the development of both arterial and venous thrombosis and has been implicated in the thrombotic events occurring in women using oral contraceptives and in patients with paroxysmal nocturnal haemoglobinuria.

Figure 6.1*: Arterial thrombosis involves the formation of platelet-rich clot that forms after rupture of atherosclerotic plaques and exposure of procoagulant material such as lipid-rich macrophages (foam cells), collagen, tissue factor, and/or endothelial breach, in a high shear environment. TM = thrombomodulin; II = prothrombin; IIa = thrombin; Fgn = fibrinogen; TF = tissue factor.

* Adapted from *Wolberg AS, Aleman MM, Thrombosis Research 2010 Apr; 125 Suppl 1:S35-7*



Recently in a single center cross sectional study, it was observed that compared with healthy control subjects, patients on both forms of dialysis showed pro-thrombotic coagulation protein profiles.

In this study the tissue-factor pathway was markedly elevated in both groups, but PD was associated with significantly greater concentrations of tissue factor (p= 0.0056) and tissue-factor pathway inhibitor (p= 0.0138). Similarly, compared with patients receiving HD, patients on

PD had greater concentrations of fibrinogen (p=0.0325), which corresponded with platelet hyper-function as measured by platelet contractile force and clot elastic modulus (p=0.003 and 0.017 respectively), compared with HD patients using thromboelastography. The high level of TFPI might thus counterbalance the increased activity of factor VII in uraemia, and may be seen as a defence mechanism against a hypercoagulable state.The authors, thus, concluded that compared with HD patients, patients on PD appear to have a more pro-thrombotic profile (116). Given PD patients are exposed to glucose-based dialysate; it is believed that they can experience metabolic derangements. Theoretically, this exposure should create a more prothrombotic environment than that occurs in HD patients.

Thus, currently there is lack of any consensus or large studies evaluating the platelet function in patients receiving RRT with PD compared to HD; in particular there are no studies which have assessed global thrombotic status, of patients in ESRD treated with different modalities of renal replacement.

6.6 Conclusion

The thrombotic status in the PD patients in this subgroup study did not differ significantly from patients on HD. An important limitation was the small sample size. This study nevertheless provides an insight into

the possible similarity of thrombotic status in ESRD patients irrespective of dialysis modality. The timing of blood sampling was different in the groups and also in the same group due to variable dialysis schedule and clinic appointments. As it has been shown in previous studies that spontaneous fibrinolytic activity in blood shows a sinusoidal variation within a period of 24 hour (117), it is possible that this might have affected the results. No co-morbidity data or drug history was collected in the PD group which certainly could have influenced on the results. The patients on PD were not followed up for clinical events and thus no conclusion can be drawn regarding the association between impaired endogenous thrombolytic ability in this sub group and cardiovascular or peripheral thrombotic events. Nevertheless based on this small pilot study it can be speculated that the modality of renal replacement does not affect the thrombotic status in ESRD. Further work though is necessary to confirm these findings.

Chapter 7:

Sub-study: Comparison of thrombotic status in patients post renal transplant and those on haemodialysis

7.1

Background

Renal transplant is one of the three modalities used for RRT in ESRD patients. Most dialysis patients would choose to receive a kidney transplant if possible with very few patients who are receiving intensive HD opting against transplantation and choosing to remain on dialysis. There are no randomized trials directly comparing transplantation to intensive HD. Database and prospective nonrandomized studies support a number of conclusions. Compared to conventional HD, survival appears to be better with either transplantation or intensive HD (118). Conventional maintenance HD patients typically receive three sessions per week, each lasting 2.5-5.5 hours. Recently, the uptake of more intensive HD (>5.5 hours, three to seven times per week) has increased. Survival appears to be similar between intensive HD and deceased donor kidney transplantation, but the best survival is reported with live donor transplantation. Secondly, people with a kidney transplant or receiving intensive HD report a higher quality of life than people on conventional HD (118). Finally, renal transplantation is more costly in the first year, but after about 2 years should be less costly than any form of HD (98).

Despite RRT, the increased risk of thrombotic events persists in patients with ESRD. A number of observational studies suggest that

CVD is more common in renal transplant patients than in the general population.

Cardiovascular events develop at annual rate of 3.5% to 5%, putting kidney recipients at a 50-fold excess (119) compared to the general population. Pre-transplant CVD is a well-established risk factor for post-transplant CVD and cardiovascular mortality. In addition, post-transplant dyslipidaemias, (120) hypertension, (121) allograft dysfunction, delayed or slow graft function and post-transplant erythrocytosis (122) are some of the more specific factors that increase cardiovascular risk in renal transplant recipients as compared to the general population**.** Apart from the traditional risk factors in pre-transplant state, the non- traditional risks factors like the renal allograft dysfunction, proteinuria, anemia, moderate hyperhomocysteinemia and elevated serum C-reactive protein concentrations, each dependently confer greater risk of CVD morbidity and mortality in the post-transplant period (123) .

However, there is insufficient evidence to show whether undergoing renal transplantation reduces the risk of future cardiovascular or thrombotic events in these patients compared to dialysis patients. Renal transplantation aims to normalise renal function and thus reduce the risks associated with ESRD. It is well known that the renal function normalisation is very variable post-transplant and pre-transplant

disease recurrence contributes to future cardiovascular risks in transplant recipients (124). It may also be the case that by the time transplantation is performed; risk factors have been so prevalent that chronic inflammation and atherosclerosis is already widespread and irreversible.

7.2 Aim

The aim of this sub-study was to investigate whether there are differences in the global thrombotic status of ESRD patients receiving HD compared to ESRD patients who have received renal transplantation (with functioning transplanted organ).

7.3 Methods

Renal transplant patients under supervision of the Lister Hospital were approached for participation in the study on the day of their out-patient appointment at the hospital. They were provided information in same manner as described in the chapter 3 for the HD patients. They were re-approached for consent and sampling on the day of their next clinic appointment.

Blood samples were collected for GTT analysis from a peripheral vein as described in chapter 2. Forty renal transplant recipients were enrolled in this sub-study. The samples were collected during the day

time while the patients attended their out-patient appointment. Due to the clinic schedule all patients were sampled between 14:00 – 17:00 pm. OT and LT values were obtained to assess the global thrombotic status of these patients using the global thrombosis test as described previously in chapter 3. These patients were then compared with the 216 HD patients who formed the part of the main study group. A comparison was also made to the patients receiving PD.

7.4 Results

The average age of the transplant recipients was younger compared to the patients on HD. The mean age in this group was 50 ± 14 years (compared to 64 +/- 25 years in the HD group). The OT of the renal transplant patients was 422 ± 130 seconds. This is very similar to the results in the main study population where the OT was 491± 177 seconds or the PD patients with OT of 419±134seconds. The median LT in the transplant recipients was 2071 seconds compared to 1820 seconds in the main study group (p=ns) and 2561 in the PD group (p=ns).

The global thrombotic status of the renal transplant recipients as assessed by GTT was similar to that of patients receiving HD or PD.

7.5 Discussion

Despite functioning transplants, renal transplant recipients are at an increased risk of thromboembolic events as a consequence of pro-thrombotic clotting and fibrinolytic abnormalities. This hypercoagulable state is, to an extent, associated with immunosuppressant drugs required by these patients as they tend to induce endothelial damage or augment platelet aggregation.

In a small study assessing the effect of immunosuppressive drugs on platelet function post renal transplantation, soluble P selectin levels were measured and platelet aggregation studies performed using a whole blood platelet lumi-aggregometer in 40 renal transplant patients. P-selectin is a constituent of the platelet granule membrane that is transported to the platelet surface after stimulation, and acts as a ligand to generate pro-inflammatory and pro-coagulatory platelet-leukocyte aggregates. Patients were divided in two groups based on the use of immunosuppressive agents, in group 1 ($n = 24$) were patients treated with cyclosporine, and group 2 ($n = 16$) had patients treated with tacrolimus. Effects were compared with those in control groups of hypertensive subjects and healthy subjects. P-selectin levels were appreciably higher in cyclosporine-treated patients, and statistically significant differences were observed compared with those of tacrolimus-treated patients ($p < 0.05$), hypertensive subjects

($p < 0.01$), and healthy subjects ($p < 0.05$). The authors concluded that cyclosporine-treated renal transplant patients show enhanced platelet activation (125). It has been shown in previous in-vitro studies that cyclosporine enhances platelet aggregation and ADP-induced platelet TxA_2 release (126). The immunosuppressive regimens *per se* can cause or worsen hyperlipidemia, hypertension, anemia and diabetes, each of which may in turn hasten the progression of CVD (125).

In a small study examining the blood coagulation, fibrinolytic, and inhibitory systems in post renal transplant patients, plasma antigen concentrations and activities of various proteins in the above pathways were measured. Significant elevations of factor IX activity, von Willebrand factor (vWF), D-dimer, protein C and tissue type plasminogen activator (t-PA) levels were found in these patients compared with normal healthy controls. In addition, the patients receiving cyclosporine showed a significant elevation of alpha 2-macroglobulin activity and patients on azathioprine showed a significant reduction in factor XII activity when compared with the normal controls. It was concluded that transplant recipients treated with long-term cyclosporine and prednisone exhibited significant elevation of plasma vWF, D-dimer and protein C concentrations. In addition, transplant recipients, irrespective of immunosuppressive

used showed increased plasma concentrations of D-dimer t-PA suggesting in vivo thrombin generation, fibrin formation and degradation (127).

Platelet function studies have shown that the numbers of activated glycoprotein (GP) IIb/IIIa receptors increased in renal transplant patients treated with cyclosporine (128).

There is a significant body of evidence suggesting that renal transplant is associated with impaired fibrinolysis. There are several factors affecting fibrinolysis in transplant patients. The predominant long-term change is hypo fibrinolysis secondary to excessive plasminogen activator inhibitor-I (PAI-I). Steroid and cyclosporine mediated immunosuppression plays a role in increase plasma PAI-1 levels in transplant patients most likely due to metabolic derangements like insulin resistance and dyslipoproteinemia and also genetic factors (129).

In a recent, single centre study it was seen that the use of an m TOR inhibitor (mTOR-i) everolimus was associated with significantly higher levels of vWF, prothrombin fragments, thrombin-activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 compared to

a non-mTOR inhibitor based immunosuppressive regimen in renal transplant recipients (130). The higher levels of vWF in the mTOR-i group are compatible with either increased endothelial cell activation or increased release from activated platelets. Increased levels of prothrombin fragment in the mTOR-i group indicate enhanced *in vivo* thrombin formation leading to fibrin generation and platelet activation. In addition, increased thrombin generation leads to augmented activation of TAFI, which impairs fibrinolysis. Both TAFI and PAI-1, inhibitors of fibrinolysis, were increased in the everolimus treated patients. This suggests that treatment with an mTOR-i leads to increased endothelial activation, thrombin formation and impaired fibrinolysis in renal transplant recipients suggesting an increased risk of thrombotic events in renal transplant recipients treated with mTOR inhibitors.

Reduction of fibrinolytic factors and activation of platelets may therefore shift the balance of the haemostatic system in the post-transplant patients toward a pro-thrombotic state. Acute graft dysfunction can be caused by ischaemic damage or immunological injury leading to serious consequences both in the short and long term. Early allograft loss, due to acute thrombotic complications, remains a constant and proportionally increasing complication of renal

transplantation. This is improving with advances in immuno-suppression therapy.

Thrombotic microangiopathy (TMA) is very rare but serious complication of renal transplantation, usually with poor outcome. It involves small vessels and is characterized by intravascular thrombi of aggregated platelets leading to thrombocytopenia and variable degrees of organ ischemia and anemia, which is due to erythrocyte fragmentation in microcirculation. Histologically it includes severe microvascular injury characterized by microvascular thrombi (in small arteries and/or glomeruli), fibrinoid necrosis of the wall of small arteries and, in later stages, severe mucoid to concentric thickening of the small arteries. It has been observed that this condition in renal allografts is associated with increase of pro-thrombotic factors and hypofibrinolysis (131).

In a recently conducted, single centre, nested case control study, it was shown that the risk of thrombo-embolic events is eight times higher in transplant recipients compared with the general population. This study showed that the risk is particularly high in the first year after transplantation, but remains elevated even after many years of follow-up, and is not fully explained by the greater probability of

transplant recipients to be hospitalized (132). This highlights the fact that thrombo-embolic events remain a high burden in these patients.

By 3 years after transplantation, nearly 40 % of patients have experienced an adverse cardiovascular event (133). Although there are robust data on the frequency of risk factors and their contributions to CVD in renal transplant recipients, few trials have demonstrated the benefit of modifying these risk factors to reduce cardiovascular events (134). This suggests there are still unknown mechanisms which could be responsible for the increased cardiovascular and thrombotic risks in this population despite correction of renal function and modifying the traditional risk factors. Although the present study does not have sufficient power to draw definitive conclusions, it suggests that perhaps the impaired thrombolytic profile in renal transplant recipients, similar to the HD population of the main study, might account in part for the increased thrombotic events observed in renal transplant patients.

7.6 Conclusions

The global thrombotic status of renal transplant recipients was not different to that of the HD or PD patients, suggesting that the modality of RRT does not alter the thrombotic status in ESRD. It has been

shown that renal transplantation is associated with hypofibrinolysis, and the findings of my study would support this. These suggest impaired endogenous thrombolysis in this group. This study did not investigate the role of immunosuppression, which is likely a contributory factor in the impaired thrombolysis observed and thus would limit the generalisation of the results to all transplant recipients. The number of patients, in this sub-study, was small and so definitive conclusions cannot be made. Further larger studies could be performed assessing thrombotic status, using GTT, comparing renal transplant patients to those receiving patients receiving other form of renal replacement. It would have been interesting to assess the thrombotic status in patients with end stage disease before and after renal transplantation. Unfortunately, it is difficult to know which patients will be transplanted, and thus, it was not possible for me to do this during my period of research.

It is important and clinically relevant to firstly establish whether transplantation alters thrombotic status, and secondly, to possibly monitor or initiate therapeutic measures in patients who remain pro-thrombotic after transplantation and have an increased risk of cardiovascular and/or vascular thrombotic events.

Chapter 8: Discussion

In this thesis, I have shown that, firstly, ESRD patients have markedly impaired endogenous thrombolysis, compared with healthy volunteers and secondly, that such impaired thrombolysis is associated with a high risk of cardiovascular and peripheral thrombotic events.

Furthermore, in the subgroup studies it was shown that the thrombotic status in ESRD is not influenced by the modality of RRT. Findings were similar in HD, PD and transplant patients.

Some aspects of my findings are not surprising. The fact that there is a high risk of thrombotic complications in ESRD patients is to be expected. However, what is remarkable is that with a novel, physiological, near patient test, we can identify patients at increased risk of such complications.

What mechanisms may explain these findings with thrombotic status in ESRD?

It has been observed in the past that CKD is often associated with both bleeding tendency and thrombotic events. It is suggested that early stages of CKD are typically associated with a pro-thrombotic tendency, whereas in its more advanced stage patients have higher risk of thrombotic complications but also suffer from a bleeding diathesis (135). Although most commonly manifested clinically as minor bleeding from skin and mucus membranes, it can frequently

occur as gastrointestinal, retro-peritoneal or cerebral haemorrhage, and bleeding at other systemic sites too.

Under normal conditions, after a vascular injury, the haemostatic mechanism initiates a cascade of coordinated events aimed at sealing the injury site (Figure 6.1) Once the endothelium is breached, the sub-endothelial elements of the vasculature such as collagen (particularly types I, III and VI), laminins, and microfibrils are exposed, which leads to a change in platelet morphology and provides support for platelet adhesion. Several glycoprotein (GP) receptors are present on the Platelets, including GP-VI that binds collagen and mediates both platelet adhesion and activation at the site of the injury and GPIb-V-IX that interacts with collagen bound von Willebrand factor (vWF) and is also required for platelet adhesion, (136). In addition to collagen-mediated platelet activation, tissue factor (TF) triggers another independent and distinct pathway for platelet activation where it forms a complex with the active factor VII and initiating a cascade by activating factor X, interacting with several enzymes within the haemostasis pathways, and ultimately generating thrombin. Thrombin in its turn binds to its receptor, protease-activated receptor-1 (PAR-1), on platelets and results in the release of adenosine diphosphate (ADP), serotonin, and thromboxane A2. Thromboxane A2 is synthesized in the

platelets functions as a platelet agonist and as a vasoconstrictor. These platelet agonists amplify the signal for thrombus formation by activating other platelets and recruiting them to the site of clot formation (137). Platelet activation also involves a conformational change in GPIIb/IIIa that increases its affinity for fibrinogen and vWF, and as such it enhances platelet-platelet affinity. Other substances released from platelets that play important roles are, fibronectin, which stabilizes platelet aggregates and platelet-derived growth factor, which mediates tissue repair physiologically. Termination of the process of clot formation involves multiple factors including anti-thrombin (AT), tissue factor pathway inhibitor (TFPI), and the protein C/protein S system. Clot organization and removal is conducted by the proteolytic enzyme plasmin.

In addition to the hemostatic mechanisms, there are several homeostatic mechanisms that maintain the balance between clot formation and bleeding. Tissue plasminogen activator, urokinase plasminogen activator, prostacyclin (PGI_2), nitric oxide (NO) and ectoapyrases are released by normal, functioning endothelial cells to maintain a local antithrombotic intravascular surface and degrade ADP (138).

Another homeostatic mechanism influencing hemostasis is laminar blood flow. This is partially influenced by hematocrit and local endogenous vasodilators, which modulate blood viscosity and vasomotor tone, respectively. A normal hematocrit facilitates the flow of red blood cells midstream, which displaces platelets such that they are closer to the endothelium; consequently, platelets can react quickly to damage to the vasculature (139, 140). The second aspect of laminar blood flow that influences hemostasis is vessel radius, which is regulated by a number of neurological and chemical mediators including, but not limited to, PGI_2 and NO. Each of the above areas commonly altered in patients with uremia.

As CKD advances, the pro-coagulant abnormalities persist, but in addition, patients start to exhibit platelet dysfunction that typically manifests with an increased risk of cutaneous, mucosal, or serosal bleeding. Several factors are thought to contribute to platelet dysfunction in patients with advanced CKD. In patients with platelet dysfunction secondary to uremia, there is thought to be a functional defect associated with vWF; either decreased binding affinity for GPIb/IX receptors or reduced expression of GPIb/IX receptors on platelets (141). Weakened interaction between vWF and GPIb/IX receptors alters cytosolic calcium concentrations, resulting in decreased production of TxA_2 and ADP. One study showed impaired

function of platelet aggregation at least partially due to an intrinsic GPIIb/IIIa dysfunction and the presence of a putative uremic toxin that inhibits fibrinogen binding to GPIIb/IIIa (142). Altered release of ADP and serotonin from platelet granules, (143) and faulty arachidonic acid and prostaglandin metabolism, (144) have also been reported , which all lead to impaired platelet adhesion and aggregation.

P-selectin is found in the α-granules of platelets. A deficiency in α-granules could lead to ineffective haemostasis. Two different explanations are possible for the deficient platelet α-granules release found in CKD. This could be due to depletion of α-granules itself, or due to a deficiency in the release of α-granules.

In a study by Bladel et al (145) platelet reactivity was measured using flow cytometric analysis. Platelets in whole blood were triggered with different concentrations of agonists (TRAP, ADP, CRP) and the platelet activation was quantified with staining for P-selectin, measuring the mean fluorescence intensity. In this study the authors found that the expression of P-selectin on the platelet surface measured as mean fluorescence intensity was significantly less in CKD patients compared to controls after maximal stimulation with the agonists suggesting impaired platelet reactivity.

In a similar study, Aggarwal and colleagues found a higher P-selectin expression in patients with ESRD receiving HD compared to healthy controls after stimulation with a single concentration of ADP suggesting an increased reactivity (146). Moal et. al performed a similar study in which ADP (200 μM) and TRAP (50 μM) in a single concentration were used to stimulate platelets in healthy controls and ESRD patients receiving HD. They found a lower P-selectin expression in patients compared to controls, indicating reduced platelet reactivity in patients with CKD (147).

Thus, there is conflicting evidence in literature with regards to the mechanism of haemostasis abnormalities in ESRD. This may be attributed to the heterogeneity of the study population, differences in methodology of handling platelets and other confounding factors such as use of antiplatelet or anticoagulant drugs and other comorbidities.

In addition to haemostatic changes caused by uraemia in the HD patient group, HD therapy itself leads to various haemostatic changes. These include coagulation cascade activation as a result of contact between the dialysis membrane and blood elements, the effect of anticoagulants used to prevent coagulation developing due to this cascade activation and a decrease in the negative effects on platelet

functions of uremic toxins, thought to be eliminated during HD. The HD procedure itself may also directly activate tPA, but it is unknown whether this activation contributes to an increased bleeding tendency in patients receiving it (148).

Anaemia may also play a pathogenic role in the increased risk of bleeding in patients with advanced CKD because correcting it results in improved platelet function in this population.

In a large meta-analysis examining the efficacy of antiplatelet agents in preserving dialysis access patency, antiplatelet agents appeared effective in reducing thrombosis in central venous catheters and AV shunts, but not in preventing AV graft thrombosis (149). The bleeding risk for patients on dialysis treated with antiplatelet agents appears to be related to the prescribed number and type of antiplatelet agents used.

In the studies described in this thesis it was observed that the OT was significantly prolonged in the ESRD patients on HD compared to the healthy volunteers. The prolonged OT in ESRD patients suggests either impaired primary haemostasis or reflects the fact that half the patients were on at least one antiplatelet agent, or a combination of these factors. It is difficult to ascertain the exact pathogenesis of this as the

study was not designed to establish the mechanism. The findings suggest that the platelet reactivity is reduced in this group of patients, as previously described by others. The effect of anaemia on the platelet reactivity could not be established as almost all the patients in this study were on erythropoietin and similarly the effect of HD cannot be established as all the patient were already on established HD. The small subgroups of PD patients and the post renal transplant patients did not show any difference in the OT when compared to the main group of HD patients. A shortcoming of the study was the lack of a control group of patients with earlier stages of CKD which may have allowed to distinction between the effects of impaired kidney function and the effects of RRT modality on the global thrombotic status.

Prolonged OT signifies delayed thrombus formation in this group and logically would imply an increased tendency towards bleeding. The study was not specifically designed to look for any bleeding complications and may have lacked adequate power to detect this. There were only three deaths in the study population secondary to bleeding complications. Two of these patients had gastrointestinal haemorrhage due to unspecified reasons and the third patient had bleeding secondary to chronic liver disease. All three of these patients had prolonged OT (661, 687 and 692 seconds) compared to the study

population but interestingly the two patients with GI bleeding had LT >3000 seconds. Prolonged OT would explain tendency towards bleeding but delayed LT would, in theory offer protection from bleeding by increasing thrombotic tendency. This may point towards separate mechanisms controlling platelet activation and spontaneous lysis of the formed platelet rich thrombus. However, the study was not designed to come to conclusions about the bleeding tendency in these patients and these may be just chance findings.

OT was prolonged in the group of patients taking clopidogrel, compared to those without clopidogrel. Interestingly Aspirin use (approximately just over half the patients were on aspirin {51%} compared to only 11% taking clopidogrel) did not have any correlation with the OT values in the study. This may suggest that platelet aggregation in HD patients is predominantly mediated by ADP- P2Y12 pathway which is blocked by clopidogrel and thus prolonging OT. This is similar to the effect of some uremic toxins. The uremic toxins guanidosuccinic acid and phenolic acid lead to platelet aggregation defect by inhibiting ADP-induced platelet aggregation (150).

Alternatively, patients with ESRD on HD may have "resistance" to the effects of aspirin. Aspirin use is standard in patients with coronary artery disease and has been shown to reduce the risk of myocardial

infarction, stroke, and vascular related deaths in patients with CVD. But a significant number of patients prescribed aspirin as antithrombotic therapy have major adverse vascular related events each year. It is unclear whether these patients simply receive too low an aspirin dose, are not compliant, have differing abilities to absorb aspirin, or have an underlying genetic disposition that renders aspirin ineffective. Such patients have been labelled aspirin "resistant". In a meta-analysis done in 2007 it has been shown that approximately 28% of patients taking aspirin can be classified as aspirin resistant. Aspirin resistance was higher in patients with previous renal impairment ($P<0.03$) (151).

OT in this study was found to be inversely related to sodium, urea and diuretic use. It is known that the fluid status plays a role in the residual renal function and thus the observation here in this study could reflect a relationship between the fluid status and OT, although the study did not have enough power to establish this.

OT in this study was not predictive of MACE. This was similar to the findings of a similar study involving patients with acute coronary syndrome on dual antiplatelet medication (76). As all the endpoints described for the purpose of this study were thrombotic complications, a tendency towards delayed thrombosis (prolonged OT) could not be used as an explanation for the number of events found in the study

and thus suggests that their possibly is another patho-mechanism in these patients causing the study endpoints.

Despite the relatively high prevalence of cardiovascular risk factors in ESRD, (152) the Framingham risk score projected cardiovascular risk in ESRD is similar or somewhat higher than reference populations from the Framingham cohort or from the National Health and Nutrition Examination Survey (NHANES) III (153). Even in studies which predict higher risk the incidence of CVD observed in dialysis patients or even transplant recipients may be underestimated (154). This emphasizes the contributory role of non-traditional risk factors to cardiovascular risk in ESRD, which traditional predictive models do not cover.

Although aggressive risk factor modification in CKD with statins, (155) angiotensin-converting enzyme inhibitors, (154) and normalization of haemoglobin with erythropoietin (156) reduce cardiovascular events, (157) thrombotic events continue to occur. Thus, the optimal management of cardiovascular risk in ESRD not only requires traditional risk factor modification, but also should aim to identify newer, previously unknown, risk factors in this complex group. These non-traditional risk factors may explain the excess cardiovascular events, which are higher than in any other disease state.

To define a non-traditional factor as a risk factor, all of the following conditions should be met, ideally: (1) biological plausibility as to why the factor may promote CVD risk; (2) demonstration that the risk factor level increases with severity of kidney disease; (3) demonstration of an association between the risk factor and CVD in CKD in observational studies; and (4) demonstration in placebo-controlled clinical trials that treatment of the risk factor decreases CVD outcomes. (158). Although, the first two conditions are met for the most part when one considers the non-traditional risk factors there remain many gaps in the CKD literature regarding condition 3, and particularly regarding condition 4. This is, therefore, an active area of interest and research.

In this study, it has been shown that impaired LT is strongly correlated with adverse thrombotic events in ESRD, independent of other risk factors. This observation that the endogenous ability to lyse any thrombus formed (prolonged LT) in these patients is impaired can provide a logical explanation as to why cardiovascular and other thrombotic events rate are higher in ESRD patients. But due to the non-randomized population and presence of multiple traditional risk factors in the patients it could also be a chance observation.

The observed cardiovascular events risk in the study is predominantly attributable to an increased risk of MI in those with LT≥3000 seconds, although it is very unlikely that the LT< 3000 seconds group is completely protected from MI. The MACE rate in those with LT< 3000 seconds was much lower than anticipated and may have contributed to the lower than expected significance of the results. Although the hazard ratio for MACE is high, the sample size is relatively small and the confidence intervals large, thus the results could be due to chance.

Although studies cannot be directly compared, these findings are supported by the prior study in ACS patients, where the same cut-off value (LT≥3000 s) was correlated with increased cardiovascular risk but imparted a much greater risk in ESRD than in ACS patients (HR 4.25 vs. 2.5) and LT was more frequently impaired and longer in ESRD than in ACS patients (96).

The distribution of events was not even in the subgroups based on LT (Figure 5.2 and table5.2 chapter 5 – results), with a break in events between 3000 and 5000 seconds, as there were no events in LT 4000–5000 seconds group. The reduction in hazard when LT> 4000 seconds is likely, at least in part to reflect the uneven distribution of events,

with small numbers of patients and even smaller numbers of events in these groups.

Components of the fibrinolysis system include t-PA, urokinase, u-PA receptor, plasminogen, and inhibitors of plasmin generation such as PAI-1 and TAFI (159). During normal haemostasis pro-enzyme plasminogen is converted to active plasmin by thrombin. Plasmin degrades the cross-linked fibrin into soluble degradation products by the tissue-type (TPA) and the urokinase type plasminogen activators. It is TPA that is mainly responsible for the dissolution of fibrin formed in the circulation. This fibrinolytic system can be inhibited either by plasmin through alpha 2-antiplasmin or by specific plasminogen activator inhibitors (PAI). The thrombin activatable fibrinolysis inhibitor (TAFI) is another important inhibitor of the fibrinolytic system and forms a link between blood coagulation and fibrinolysis. Thrombin forms fibrin to stabilize the platelet-rich thrombus and also produces TAFI to protect that fibrin network. The TAFI circulates as an inactive pro-enzyme and becomes activated by thrombin during blood clotting. The active form (TAFIa) inhibits fibrinolysis by cleaving off C-terminal lysine residues from partially degraded fibrin that stimulate the TPA-mediated conversion of plasminogen to plasmin. Consequently,

removal of these lysines leads to less plasmin formation and subsequently to protection of the fibrin clot from breakdown (160).

There have been studies showing increased fibrinogen, plasminogen activator inhibitor-1(PAI-1), and reduced tissue plasminogen activator in ESRD, (157-158, 161-164). In recent data on fibrin clot properties both ESRD (165,166) and thromboembolic coronary events (167, 168, 169) have been associated with the formation of dense fibrin clots, resistant to fibrinolysis. In a study by Undas et al. (170) it was shown, by using turbidometric plasma clot lysis, fibrin clot permeability and perfusion clot lysis assays, that ACS and CKD patients have higher PAI-1 and TPA levels and formed fibrin clots that were less porous and more resistant to fibrinolysis, compared to a control group of ACS patients with normal renal function. The fibrin clot in CKD patients exhibited smaller pore size, larger number of protofibrils per fibrin fibre, increased fibre size and clot mass. These studies support the findings of reported in this thesis, showing that there is delayed or impaired endogenous thrombolytic activity in ESRD as evident by the prolonged LT in this group. In this study population, high fibrinogen levels (5.7±2.2 g/l) were observed at baseline (table1 demographics-chapter 4). The observation of raised fibrinogen level in the study group and impaired endogenous thrombolysis is interesting. Based on

epidemiological data, fibrinogen has been associated with increased cardiovascular and arterial thrombotic risk, but whether this relationship is causal is not established. Recently high fibrinogen levels have been linked with resistance to thrombolytic therapy and adverse outcome in patients with acute ischaemic stroke (171). Patients with high fibrinogen levels displayed worse clinical response and had 2.7-fold risk to mortality when they received thrombolytic therapy.

In previous studies high fibrinogen levels are shown to lead to a less porous and therefore less permeable fibrin clot with thin fibers in particular genotypic individuals (172) A tendency to form of tight, rigid and space-filling fibrin network structures with small pores has been shown to be associated with premature coronary artery disease (173, 174).

Furthermore, in a murine model, artificial increase in fibrinogen level directly promoted thrombosis and thrombolysis resistance, via enhanced fibrin formation and stability (175). In this study the investigators raised plasma fibrinogen levels in mice via intravenous infusion and induced thrombosis by application of shear forces; hyperfibrinogenemia significantly shortened the time to occlusion in the both high and low shear exposed mice. Using

immunohistochemistry, turbidity, confocal microscopy, and elastometry it was shown that hyperfibrinogenemia increased thrombus fibrin content, promoted faster fibrin formation, and increased fibrin network density, strength, and stability. Hyperfibrinogenemia also increased thrombus resistance to tenecteplase-induced thrombolysis in vivo.

However, in a transgenic mouse model of hyperfibrinogenaemia, mice with high fibrinogen level did not demonstrate accelerated platelet thrombus formation in response to injury, compared with wild-type. (176). Surprisingly, transgenic mice demonstrated suppression of thrombin generation in plasma and activation of the fibrinolytic system. Furthermore, alterations in gene expression and coding function, splice variants such as the gamma' splice variation in fibrinogen gene transcription, and posttranslational modifications of protein products all influence fibrin structure/function and result in more highly cross-linked and stable fibrin clots, with reduced pore size, that are more resistant to lysis (177).

A study of gamma A/gamma' fibrinogen levels, in patients undergoing coronary angiography, has previously shown that gamma A/gamma' fibrinogen levels were higher on average in coronary artery disease patients than in patients without coronary artery disease, and that this association was independent of total fibrinogen levels (178).

In the present study to investigate further the relationship between fibrinogen level and impaired endogenous thrombolysis (as measured by LT), an in vitro experimental correction was performed to raise fibrinogen level and assess the effect on LT. In ten healthy volunteers, in parallel measurements, increase in plasma fibrinogen concentration in vitro by 1 g/L did not significantly alter LT compared with control samples [median LT 1081 (IQR 907–1300) secs and 1297 [1208–1660] secs respectively, P = 0.06]. Thus increasing fibrinogen concentration showed a trend towards enhanced rather than inhibited spontaneous thrombolytic activity. These findings in healthy blood are similar to previous studies claiming that hyperfibrinogenemia per se does not inhibit fibrinolysis (176). Thus, it is more likely that it is not just the elevated plasma fibrinogen concentration per se, but the quality of the fibrin clot architecture that determines risk.

It has been shown in previous studies that in ESRD, fibrinogen structure and function are altered, making clots more resistant to lysis and that the fibrin structure characteristics in the patients are associated primarily with the inflammatory plasma milieu rather than with level of azotemia (179). This may be causally related to increased thrombotic risk. In a small study it has been shown that in patients on chronic HD fibrin clot properties are markedly altered.

Fibrin clots from plasma of the patients display significantly reduced permeability, faster proto-fibril formation, increased fibre size and clot mass, along with decreased susceptibility to fibrinolysis, compared with healthy well-matched individuals. These findings indicated that clots from HD patients are much tighter than those from controls. None of the clot variables showed any association with the duration of HD treatment or the cause of ESRD. Although this was a small study, there was an association between mortality and reduced clot permeability and prolonged LT (180).

This may explain the functional significance of the impaired thrombolytic state observed in ESRD and can be possibly used as a marker of excess thrombotic event risk in this population. In this study I did not compare LT with plasma markers of fibrinolysis, since the value of fibrinolysis activity markers is very limited in aiding diagnosis and risk stratification in the individual patient. The overall outcome of studies looking at prognostic and diagnostic value of fibrinolytic markers is highly controversial. The number of negative studies unable to demonstrate an association or predictive power is practically equal to those reporting a positive association and prognostic value. Even some of the positive studies report relative risks that, although

significant, are so weak (HR: 1.1 to 1.7) that they indicate only a statistical trend but have little practical usefulness (139).

Prolonged LT in the current study was associated with prior CAD but it is difficult to say whether it is a causative phenomenon. In a similar study lysis time in ACS patients was not related to prior CAD (96).
Proton pump inhibitor use was related to prolonged LT. This study is too small to analyze whether this reflects a possible effect of proton pump inhibitors inhibiting the CYP2C19 iso-enzyme, thereby reducing the ability of clopidogrel to inhibit platelet aggregation.

Higher serum calcium concentration was associated with prolonged LT. This may be functionally important. Ionized calcium (Ca2+) holds together the fibrinogen binding receptor glycoprotein IIb/IIIa complex, it is essential for agonist-induced conversion of the glycoprotein IIb/IIIa complex into the functional fibrinogen receptor, and is required for the binding of fibrinogen to its receptor, as well as for the coagulation cascade (68).
C-reactive protein was related to LT suggesting a relationship between inflammation and thrombosis. Inflammation biomarkers are strong predictors of MI or thrombotic stroke (181) and platelets are known to initiate an inflammatory response at the endothelial level by secretion of various pro-inflammatory factors and forming a platelet–leucocyte

aggregate (PLA) and thus initiating processes pivotal for thrombus formation and atherogenesis (182). It is difficult to establish, based on this study, whether the relationship between CRP and LT is a cause or effect phenomenon in these patients.

Limitations of the study

The two major limitations of this study are its observational nature and the sample size. The patients enrolled in the study were known to have multiple risk factors for cardiovascular or thrombotic events. The observational nature of the findings in a high risk group with multiple risk factors for the primary end points makes one question the role of these known risk factors in influencing the result despite the statistical significant correlation between impaired endogenous thrombolysis and observed events in this study.

Since the sample size in the study was relatively small, particularly in the sub-studies, a further larger trial would be needed to confirm these findings.

Although we have tried to use appropriate controls in the main study and in the sub-studies, there were a few factors which proved difficult to control for. For example, age, co-morbidities and aetiology of ESRD. Moreover the control group did not have any renal impairment or risk factors associated with it. The main study group patients (HD group)

were sampled only once, pre-dialysis, and only a very small group was studied pre and post dialysis. The fact that mainly ESRD patients on HD were studied means that the results cannot be generalized to different modalities of renal replacement or to different stages of CKD. More than 90% patients in the HD group received erythropoietin, which is known to increase the number of circulating platelets, improves platelet function transiently bringing the bleeding time towards normal,(183) It has also been shown to have varying effects on platelet reactivity and fibrinolysis(184). It has also been proposed that in patients treated with erythropoietin, increased activity of C-reactive protein, nitric-oxide, and thrombin-activatable fibrinolysis inhibitor leads to a fibrinolytic deficit with resultant increase in thrombosis (185).

The effect of antiplatelet medication on LT is not fully established, but unlikely to be significant given the findings in a previous study of patients with ACS (76) and here in ESRD. It is well known that individual patients on antiplatelet therapy have varying response to the medications in terms of platelet inhibition and such variation is possible in this study but this study was not designed to study effects of antiplatelet medications on thrombotic status. Moreover, the effect could also vary depending on stage of CKD, modality of RRT and any

previous use of such medications in the study population at the time of sampling.

The test used (GTT) is a near patient test and requires meticulous sampling, and is subject to error due to effects of tourniquet application time or sample transit time, and results can vary depending on the site and state of the sample. The timing of blood sampling was different in the different groups and also in the same group due to variable dialysis schedules and clinic appointments. As it has been shown in previous studies that spontaneous fibrinolytic activity in blood shows a sinusoidal variation within a period of 24 hour (97), it is possible that this might have affected the results.

The GTT machine has been used and validated by the manufacturer previously and a small study on same patients assessed by more than one machine could help to establish the precision of the GTT analysis.

The small sub-groups studied, including the PD and the renal transplant recipients, were not followed up to investigate the relationship between global thrombotic status and any future thromboembolic or bleeding events. No data on co-morbidity or medication details were collected in the sub-groups which certainly could have influenced the findings as groups were not matched for comorbidities or medications that could have affected thrombotic status. Similarly smoking history, which is a well-known risk factor for

thrombotic events, was not collected in this study and it would have been interesting and probably relevant to establish a relationship between smoking and the GTT results in a high risk population.

Future work

A larger study assessing thrombotic status in patients undergoing different RRT modalities could help establish the effect of RRT modality on overall thrombotic status. Furthermore, it would be interesting to assess global thrombotic status in patients at different stages of CKD repeating the test as the condition progresses, to study the effect of progression on thrombotic status.

The role of antiplatelet medications in patients with renal disease and the effect of antiplatelet medication on LT are important clinical questions. Ideally a prospective study randomizing patients with CKD to antiplatelet regimens and assessing the effects of such intervention on global thrombotic status would be desirable. Otherwise, larger studies would be required where groups matched for CKD status, risk factors and medications, but varying with respect to antiplatelet medication, could be compared for overall thrombotic status assessment.

Other medications should also be studied to investigate their effects on global thrombotic status, in order to find a potential candidate drug that can favourably modulate thrombotic status and may thus reduce cardiovascular risk in high risk groups with impaired LT. Such medications include oral anticoagulation using vitamin K antagonists or novel anticoagulants.

Conclusion

In summary, this study has identified impaired thrombolysis as a novel risk factor in ESRD albeit with limitations, which may have important implications for screening and risk stratification. Thrombotic status does not appear to be affected by HD. Future studies are required to investigate medical therapies to which can improve endogenous thrombolysis, and to establish whether such agents may reduce the risk of cardiovascular events in these high-risk patients.

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Appendix

Appendix index

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REC Ref 09/H0311/25

PATIENT INFORMATION SHEET

You are being invited to take part in a research study. Before deciding whether to take part, you need to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP as you wish. Ask us if there is anything that is not clear or if you would like more information. Take your time deciding whether or not you wish to take part.

National Institute of health research has got an advisory group called INVOLVE (funded by Department of health) which provides information on public involvement in NHS, social care, and public health research and development (R&D).They Have produced Various publications including a Public Information Pack (PIP) titled: How to get actively involved in
NHS, public health and social care research (2007).This and all of their other publications can be downloaded free form there website http://www.invo.org.uk or a free copy can be ordered from – INVOLVE, Wessex House, Upper Market Street Eastleigh Hampshire SO50 9FD.

Thank you for reading this.

Study Title

Risk of thrombotic events in chronic renal failure (CRF) patients on renal replacement therapy (RRT)

(The meaning of the title is fully explained below)

What is the purpose of this study?

Kidney failure patients despite being on dialysis or having undergone a renal transplant remain at increased risk of heart problems. The reason for this is unclear, but likely to be related to either increased platelet reactivity, impaired thrombolysis or both. There is currently no way to identify which patients are likely to clot off their arteries, and thus whom to target with medication.

We propose to do blood tests to identify patients who remain prone to forming clots and/or have impaired ability to dissolve clots despite being on dialysis or having received a kidney transplant for kidney failure. We will do two blood tests, one before the start of dialysis or receiving a transplant and another after either being established on dialysis or becoming stable after the transplant depending on the treatment (dialysis or transplant in this case) patients receive. We will then follow patients up for a year and try and correlate whether the blood test results can predict who is at increased risk of future heart attacks and stroke and also to see if the blood test results were different before and after receiving renal replacement therapy (dialysis or transplant)
If these blood tests can identify which patients at are risks of future events, this group can be targeted in the future to individualize and optimize their medication, to reduce their risk of a future heart attack, stroke or death.

Why have I been chosen?

You have been chosen to participate since you are suffering with a kidney problem and are at higher risk of heart problems than the general population. We are studying why patients with renal failure develop heart problems earlier, and whether we can detect who is at risk of future problems.

Do I have to take part?

It is entirely up to you to decide whether or not you wish to take part in this research study, and you should not feel under any obligation to take part. Neither your decision to take part, nor your decision to not take part, will in any way affect your current or future medical care.

What will happen to me if I take part?

We will take 10 ml of blood from a vein in your arm, on two separate occasions, while you are attending hospital.
After you leave hospital, we would like to know how you are getting on. One of our research team will therefore contact you every few months (up to 1 year from now) either by phone or letter, to ask you a few simple questions about your health.

What do I have to do?

You do not need to do anything. If you decide to take part, we will take a 10 ml blood sample during your current hospital attendance and on one more occasion and then contact you, as above, every 3 months, until 1 year from now. No additional trips to hospital and no additional medication will be required.

What are the side effects of taking part?

The only risk is that of having a blood test. Like with any blood test, you may experience some minor discomfort, and there is a small risk of bruising or bleeding from the puncture site.

What are the possible disadvantages and risks of taking part?

The only risk is that of having a blood test. Like with any blood test, you may experience some minor discomfort, and there is a small risk of bruising or bleeding from the puncture site.

Are there any benefits to me in taking part?

There is no direct benefit to you for taking part. However, we hope the results of our research will further the medical profession's understanding of why some patients have an increased tendency to form blood clots and this may lead to a better way to treat these patients.

What happens when the study stops?

Once the research is completed and the results analysed, we will write you a letter explaining our findings in layman's terms. You will continue to have medical follow up with your GP or hospital doctor as is necessary.

What if something goes wrong?

If you have any cause to complain about any aspect of the way you have been approached or treated during the course of this study the normal National Health Service complaints mechanisms are available to you and you are not compromised in any way because you have taken part in a research study. If you are harmed due to someone's negligence, then indemnity and/or compensation for negligence on the part of NHS staff will be provided by the East and North Hertfordshire NHS Trust.

Formal complaints may be addressed to the Chief Executive, Lister Hospital, and Corey's Mill Lane, Stevenage, SG1 4AB.Tel. No. 01438 781541

Should you require independent advice about making a complaint or seeking compensation, you may wish to contact the Independent Complaints Advocacy service (ICAS) for Bedfordshire & Hertfordshire at Pohwer ICAS. Suite 19, The Pixmore Centre, Pixmore Avenue, Letchworth, Herts, SG6 1JG (Tel 0845 456 1082).

Will my taking part in this study be kept confidential?

The results of this study will be kept strictly confidential, although we will inform your general practitioner of your participation in this research.

What will happen to the results of the study?

We hope that the results of the research as a whole will be published in a peer-reviewed scientific journal. This will not contain any patient-specific data

Who is organising and funding the research?

The research is organised by and funded by East and North Hertfordshire NHS Trust.

Who has reviewed the study?

The study has been reviewed and given a favourable ethical opinion by the Hertfordshire Research Ethics Committee.

Contact for further information

If you or your relatives wish to discuss any part of this research further, please contact the Cardiology Research Team on bleep 0235 or 0031 via Hospital Switch Board 01438-314333 or else contact Dr Gorog's or Prof. Farrington's secretaries via hospital switch board.

East and North Hertfordshire NHS
NHS Trust

Lister Hospital
Corey's Mill Lane
Stevenage
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Queen Elizabeth II Hospoital
Howlands
Welwyn garden City
Herts AL7 4HQ

Tel: 01707 328111
www.enherts-tr.nhs.uk

Study Number: REC Ref 09/H0311/25

Centre Number:

Patient Identification Number:

CONSENT FORM

Title of Project:

Risk of thrombotic events in Chronic renal failure (CRF) patients on Renal replacement therapy (RRT)

Name of Researcher: Dr D A Gorog / Dr Ken Farrington

Please initial box

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to ☐
Withdraw my consent at any time, without giving any reason and without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked ☐
at by responsible individuals from the, sponsor (East & North Herts NHS Trust) or from regulatory authorities (East & North Herts NHS Trust) where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study. ☐

5. I agree for my GP to be informed of my participation in this study. ☐

Name of Patient	Date	Signature

Name of person taking consent (If different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

STUDY NO.		statin	
Hosp. No		BB	
Initials		Folic acid	
DOB		Diuretics	
Gender		Alfa-calcidol	
Age		Iron	
Ethnicity		B12	
Contact No.		Phosp. Binder	
Cause of ESRF		Tinzaparin	
Medical History		Other Meds	
Co-Morbidity 1		Hb	
Co-Morbidity 2		WCC	
Co-Morbidity 3		Platelet	
Co-Morbidity 4		Na	
Diabetic		K	
HTN		Bicarb.	
Prior CAD		Urea	
CVA/TIA		Creatinine	
PVD		Ca	
Lupus/APAb		Phosphate	
Dialysis start		CRP	
Access History (incld. date/ site)		ALT	
TransplantHistory (incld.date/type)		KRU	
Previous CV Intervention		Other bloods	
ASA		Dry Weight	
Clopidogrel		Pre HD Obs	
EPO / Dose		Post HD Obs.	
Insulin		**GTT OT**	
OHA		**GTT LT**	
ACE/ARB		Date/time Test	

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www.enherts-tr.nhs.uk

REC Ref 09/H0311/25

GP INFORMATION SHEET.

Patient Name:……
Patient DOB:…….
Your patient participated in the research below and gave _ ml venous/fistula blood to be tested as part of the research study below, on …….. (date)

Study Title: Risk of thrombotic events in chronic renal failure (CRF) patients

Patients with Chronic renal failure (CRF) despite being on optimal renal replacement therapy (RRT) remain at increased risk of acute thrombotic events. Patients with renal impairment have a high mortality from cardiovascular disease – such that a dialysis patient aged 30 has a relative risk of mortality over 100 times that of his peer without renal disease. The reason for this is unclear, but likely to be related to the increased platelet reactivity, impaired thrombolysis or both. There is currently no way to identify which patients are likely to have thrombotic event, and thus whom to target with more aggressive anti-platelet treatment. We propose to identify patients, through blood tests, who remain pro-thrombotic and/or have impaired fibrinolysis despite optimal treatment and observe whether this is related to an increase in acute thrombotic events. If these tests can identify which patients are at risk of further events, this group can be targeted to individualise and optimize the anti-platelet medication to achieve a more aggressive anti-platelet effect, in order to reduce the risk of further thrombosis.

The research involves taking a one-off blood sample, and telephone follow-up of patients. The research is been organised and funded by East and North Hertfordshire NHS Trust and the study has been approved by the Hertfordshire Research Ethics Committee.

If you wish to discuss any part of this research further, please contact Cardiology Research Team at the QE2 hospital on 01707 328 111 and ask for bleep 0031/0235, Dr Diana Gorog, Consultant Cardiologist via her secretary on 01707 365 036 or Prof. Ken Farrington, Consultant Nephrologist via hospital switchboard 01438 314333.

Version1.0 dated 01/02/2009

Hertfordshire Hospitals R&D Consortium NHS
Incorporating West Herts Hospitals NHS Trust and East & North Herts NHS Trust

R&D Office, Clinical Governance Support Centre (L57D)
Maternity Unit, York suite
Lister Hospital
East & North Herts NHS Trust
Coreys Mill Lane
Stevenage
Herts
SG1 4AB

Tel: Internal: Lister X4956
Direct Line: 01438 781640
Fax Internal: 4306

23/04/2009

Dr Sumeet Sharma
Cardiac Research Registrar
Queen Elizabeth II Hospital
Howlands
Welwyn Garden City
AL7 4HQ

Dear Dr Sumeet Sharma

Re: RD2009-21 **Risk of thrombotic events in chronic renal failure (CRF) patients on renal replacement therapy (RRT) - Risk of thrombotic events in chronic renal failure (CRF) patients on renal replacement therapy (RRT)**

Following review by the Research and Development Committee, I am pleased to confirm that the above project now has Trust approval to recruit patients at East and North Hertfordshire Hospitals NHS Trust.

May we remind you that the Principal Investigator is responsible for ensuring that research is conducted in accordance with the Department for Health Research Governance Framework. It must also comply with the law, all internal Trust policies and processes and any relevant good practice guidance. The research may be subject to internal or external monitoring.

Should you have any queries or require further information, please contact the Research & Development office on the above numbers.

Best wishes for a successful project.

Yours sincerely,

Dr S. Gowrie-Mohan
Associate Director of R&D

NHS
National Research Ethics Service
Hertfordshire REC
East of England REC Office No 3
9th Floor, Terminus House
The High
Harlow
Essex
CM20 1XA

Telephone: 01279 418 439
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02 March 2009

Dr Diana Gorog
Consultant Cardiologist
East & North Herts NHS Trust
QEII Hospital
Howlands
Welwyn Garden City
AL7 4HQ

Dear Dr Gorog

Full title of study: **Risk of thrombotic events in chronic renal failure (CRF) patients on renal replacement therapy (RRT)**

REC reference number: **09/H0311/25**

The Research Ethics Committee reviewed the above application at the meeting held on 25 February 2009. Thank you for arranging for Dr Sumeet Sharma to attend to discuss the study.

Ethical opinion

The Committee discussed with Dr Sharma the possibility of a seriously ill or deceased patient being phoned as part of the follow up. The Committee have suggested that when the current status of a patient is not known, a fax should be sent to their GP to establish their state of health.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The Committee agreed that all sites in this study should be exempt from site-specific assessment (SSA). There is no need to submit the Site-Specific Information Form to any Research Ethics Committee. The favourable opinion for the study applies to all sites involved in the research.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Other conditions specified by the REC

1. In the Participant Information Sheet it refers to CERES, which is no longer in existence, and it should be replaced by information about INVOLVE.
2. The Participant Information Sheet states that the study has been reviewed by the Ethics Committee of the East & North Hertfordshire NHS Trust this should be amended to the Hertfordshire Research Ethics Committee.
3. In the consent form point 3 it should state the name of the companies concerned.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Student CV	SS	09 February 2009
Protocol Co-Author CV	KF	
Participant Consent Form	1.0	01 February 2009
Participant Information Sheet	1.0	01 February 2009
GP/Consultant Information Sheets	1.0	01 February 2009
Covering Letter		05 February 2009
Protocol		01 February 2009
Investigator CV	DAG	09 February 2009
Application		09 February 2009

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed

guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0311/25 **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Dr Steve Eckersall
Chair

Email: jenny.austin@eoe.nhs.uk

Enclosures: List of names and professions of members who were present at the meeting and those who submitted written comments

"After ethical review – guidance for researchers" (SL-AR2)

Copy to: Fiona Smith, R&D Manager, HCC
The Clock Tower
Mount Vernon Hospital
Rickmansworth Road
Northwood, Middx
HA6 2RN

Hertfordshire REC

Attendance at Committee meeting on 25 February 2009

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Linda Bose		Yes	
Reverend Elizabeth Bradley	Hospital Chaplain	Yes	
Mr Michael Buck	Retired Solicitor & Chartered Arbitrator	Yes	
Mrs Heidi Ebrahim		Yes	
Dr Steve Eckersall	Consultant Anaesthetist	Yes	
Mrs Vivien Felgate	Senior Pharmacist	Yes	
Mr David Grayson		Yes	
Prof Russell Jones	GP	Yes	
Dr Wassim Matta	General Practitioner	Yes	
Dr Gordon Nichols	Business Manager	Yes	
Dr Mark Slater	Consultant Psychiatrist	Yes	
Dr Mark Tanner	Consultant Psychiatrist	Yes	
Dr Sunda Uthayakumar	Consultant GU Medicine	Yes	
Ms Melanie Whittick	Pharmacist	Yes	
Dr Patricia Wilson	Senior Nurse Lecturer	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Mrs Jenny Austin	Committee Co-ordinator
Eleanor Harrison	Network Co-ordinator

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European Heart Journal
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CLINICAL RESEARCH

Impaired thrombolysis: a novel cardiovascular risk factor in end-stage renal disease

Sumeet Sharma[1], Ken Farrington[2,3], Robert Kozarski[4], Christos Christopoulos[1], Maria Niespialowska-Steuden[1], Daniel Moffat[1], and Diana A. Gorog[1,3,5]*

[1]Cardiology and [2]Nephrology Department, East and North Hertfordshire NHS Trust, UK; [3]School of Postgraduate Medicine, University of Hertfordshire, UK; [4]Health Research and Development Support Unit, University of Hertfordshire, UK; and [5]National Heart and Lung Institute, Imperial College, UK

Received 31 March 2012; revised 3 August 2012; accepted 15 August 2012

Aims End-stage renal disease (ESRD) patients have an excess cardiovascular risk, above that predicted by traditional risk factor models. Prothrombotic status may contribute to this increased risk. Global thrombotic status assessment, including measurement of occlusion time (OT) and thrombolytic status, may identify vulnerable patients. Our aim was to assess overall thrombotic status in ESRD and relate this to cardiovascular risk.

Methods and results Thrombotic and thrombolytic status of ESRD patients ($n = 216$) on haemodialysis was assessed using the Global Thrombosis Test. This novel, near-patient test measures the time required to form (OT) and time required to lyse (lysis time, LT) an occlusive platelet thrombus. Patients were followed-up for 276 ± 166 days for major adverse cardiovascular events (MACE, composite of cardiovascular death, non-fatal MI, or stroke). Peripheral arterial or arterio-venous fistula thrombosis was a secondary endpoint. Occlusion time was reduced (491 ± 177 vs. 378 ± 96 s, $P < 0.001$) and endogenous thrombolysis was impaired (LT median 1820 vs.1053 s, $P < 0.001$) in ESRD compared with normal subjects. LT ≥ 3000 s occurred in 42% of ESRD patients, and none of the controls. Impaired endogenous thrombolysis (LT ≥ 3000 s) was strongly associated MACE (HR = 4.25, 95% CI = 1.58–11.46, $P = 0.004$), non-fatal MI and stroke (HR = 14.28, 95% CI = 1.86–109.90, $P = 0.01$), and peripheral thrombosis (HR = 9.08, 95% CI = 2.08–39.75, $P = 0.003$). No association was found between OT and MACE.

Conclusion Impaired endogenous thrombolysis is a novel risk factor in ESRD, strongly associated with cardiovascular events.

Keywords Platelets • Thrombosis • End-stage renal disease • Haemodialysis • Cardiovascular risk

Introduction

The risk of cardiac death in dialysis patients <45 years is 100-fold greater[1] and the risk of non-fatal cardiovascular disease is 10–30 times higher in patients with end-stage renal disease (ESRD) compared with general population.[2] Presence of angiographically significant coronary artery disease (CAD) ranges from 25% in young non-diabetic haemodialysis patients to 85% in older, diabetics with ESRD.[3] Many risk factors are more prevalent in ESRD and may explain some, but not all, the increased cardiovascular risk,[4] pointing towards the contributory role of non-traditional risk factors in ESRD, which traditional predictive models, such as the Framingham risk score, do not cover.

The identification of blood markers of inflammation, platelet hyper-reactivity, and hypercoagulability has received much attention to identify vulnerable patients.[5–7] Thrombosis and thrombolysis are dynamic processes occurring simultaneously. The thrombotic–thrombolytic equilibrium may determine the clinical manifestation of an acute thrombotic event.[8]

Bleeding tendency associated with chronic kidney disease (CKD) is attributed to a deficiency in primary haemostasis. Paradoxically, ESRD has been termed a hypercoagulable state, associated with thrombosis.[9] Enhanced platelet aggregation and elevation of thrombotic markers including thrombin–antithrombin III complex, D-dimer, and tumour necrosis factor has been demonstrated in dialysis patients[10–14] although dialysis may improve the thrombotic profile.[12] Chronic kidney disease patients also express markers of impaired fibrinolysis such as increased fibrinogen, plasminogen activator inhibitor-1, and reduced tissue plasminogen activator.[15–18]

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Despite several studies demonstrating a significant relationship between non-responsiveness to antiplatelet medication and subsequent thrombotic events, great differences exist between the predictive powers of different platelet function tests. Those currently in clinical use measure the response of platelets to a specific agonist,[19] despite the involvement of many other physiologically important factors such as high shear stress and thrombin generation. The first and predominant stimulus for platelet activation in a severely stenosed artery is the pathological high shear stress ($>10\,000\,s^{-1}$) creating rapid and strong bonds between platelets without prior activation.[20,21] Such shear-induced platelet activation brings about the release of soluble agonists (thromboxane, ADP) from circulating platelets and generation of thrombin.[22] Extracellular matrix proteins mediate adhesion of platelets and primary platelet aggregates to the vessel wall.[22] A major limitation of all point-of-care platelet function tests is the use of anticoagulated blood (citrate, heparin, hirudin, or other anticoagulant), which prevents the assessment of thrombin generation by activated platelets, the ultimate and major determinant of occlusive platelet-rich thrombus formation.[19] Further, there is no test in current use to measure endogenous thrombolytic activity (lysis of a platelet-rich arterial thrombus) as opposed to fibrinolysis of a venous (red cells rich) thrombus. Since the measurement of individual components of the fibrinolytic pathway fails to give a realistic assessment of overall thrombolytic status, its value is questionable.

The Global Thrombosis Test (GTT) is the first clinically available, comprehensive, near-patient test to simultaneously measure thrombotic occlusion time (OT), coagulation, and spontaneous endogenous thrombolytic activity. As the test employs non-anticoagulated blood, it is different, and free from many of the shortcomings of conventional point-of-care platelet function assays that employ anticoagulated blood.[23,24] We showed that in acute coronary syndrome (ACS) patients, impaired endogenous thrombolysis is highly predictive of cardiovascular events.[25] The endogenous thrombolytic status of stable patients has not been well characterized, and may be particularly important in those at highest risk.

It was our aim to characterize thrombotic status in ESRD and investigate whether this novel test of overall thrombotic status could identify those who, despite renal replacement therapy, remain at risk of future cardiovascular events. We also included arterio-venous (AV) fistula thrombosis since this bears many similarities to arterial thrombosis. It occurs in the presence of arterial blood, under systolic pressure and pulsatile flow. In 80% of cases, it associated with a significant stenosis, at or close to the AV anastomosis, predominantly due to intimal hyperplasia and associated with inflammation.[26,27] Abnormal haemodynamic shear stress is the most important upstream factor responsible for AV fistula failure. High shear rates upstream of a stenosed AV fistula predispose to platelet thrombus formation (rather than erythrocyte- and fibrin-rich thrombi formed in low shear, venous settings) and this is supported by histological findings.[27–31]

Methods

Study population

Patients with ESRD aged 18–90 years established on haemodialysis for at least 3 months ($n=216$) were tested after obtaining written informed consent. Subjects were excluded for the following reasons: known haematological disorder, bleeding diathesis, blood dyscrasia (platelets <70, Hb <8 g/dL, INR >1.4, APTT $>\times 2$ UNL, leucocyte count $<3.5\times10^9$/L, neutrophil count $<1\times10^9$/L), warfarin or other anticoagulant treatment, thrombolysis or glycoprotein IIb/IIIa inhibitor prior to sampling, intercurrent illness, such as pneumonia, sepsis, ACS or heart failure in last 3 months; malignancy or other disease shortening life-expectancy to <12 months; participation in another study; inability to give informed consent or when follow-up over 1 year period was unlikely. A group of 10 ESRD patients receiving peritoneal dialysis (not taking antiplatelet medication) were also tested immediately before dialysis. Healthy volunteers ($n=100$, age 38 ± 11 years) not taking medication, matched proportionally for sex and race, were also tested. Subjects were recruited, through advertisement, from among hospital staff and from relatives and carers of patients attending the outpatient department. The local research Ethics Committee approved the study.

Blood collection

From each subject, 9 mL venous blood was obtained from a peripheral (non-fistula) vein and tested immediately after withdrawal. In ESRD patients, blood was obtained immediately prior to dialysis, before the routine low-molecular weight heparin was administered according to local haemodialysis protocol. Low-molecular weight heparin is used in the majority of renal centres in the UK, as a single intravenous bolus provides predictable anticoagulation, while use of unfractionated heparin requires repeated boluses or continuous infusion. Blood was taken using an 18-gauge butterfly cannula using a two-syringe technique. The first 6 mL was used for routine tests (blood count, biochemistry, bicarbonate, and bone-profile) and the next 3 mL for global thrombotic status assessment. Blood is aspirated into a standard polypropylene syringe, which is directly, and immediately inserted into the fitting in the GTT instrument (within <15 s of withdrawal), that is positioned next to the subject to minimize sampling delay. The measurement is automated and starts as soon as blood is introduced. To ensure that the low-molecular weight heparin received just prior to the last dialysis session did not interfere or affect our results, we sampled 10 patients immediately before dialysis, exactly 48 h after the last dose of low-molecular weight heparin.

Assessment of thrombotic and thrombolytic status

Global thrombosis test

Thrombotic status and endogenous thrombolytic activity were assessed using the GTT (Montrose Diagnostics Ltd, UK). This is a novel, point-of-care assay that employs non-anticoagulated blood. The instrument measures the time taken to create shear-induced thrombi under pathophysiological conditions (discussed later) and in the second phase of the test, measures the time to achieve spontaneous lysis of thrombi created during the first phase. The principle of GTT has previously been described in detail[25,32] and is shown in *Figure 1*. Blood is introduced into, and flows through, a plastic tube in which two metal balls are inserted in the conical part of the tube. There are four narrow gaps between the inner plastic surface and the balls. When blood flows through these gaps adjacent to the upper ball, the resulting high initial shear stress (180 dynes/cm^2) causes activation of platelets. In the space between the balls, due to the turbulent flow and low shear, the activated platelets aggregate. Thrombin is generated, which accelerates the formation of these aggregates and stabilizes them through fibrin. When these stable thrombi reach the gaps around the lower ball, they gradually occlude

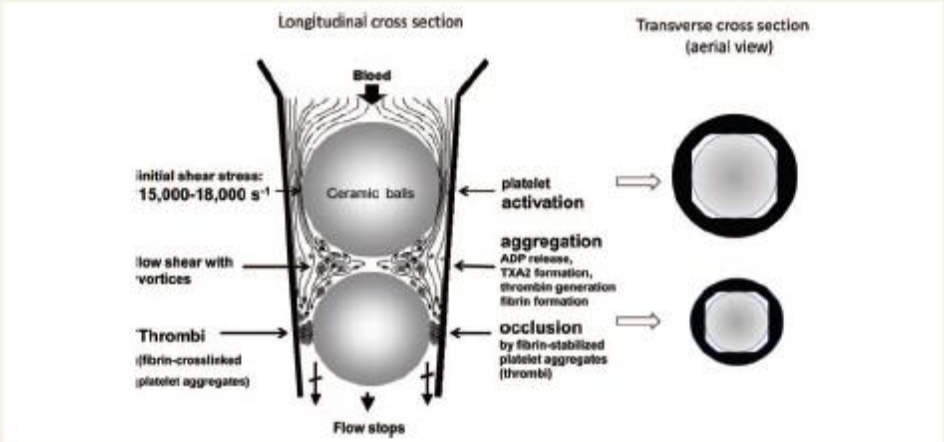

Figure 1 Schematic showing principle of the Global Thrombosis Test (see text).

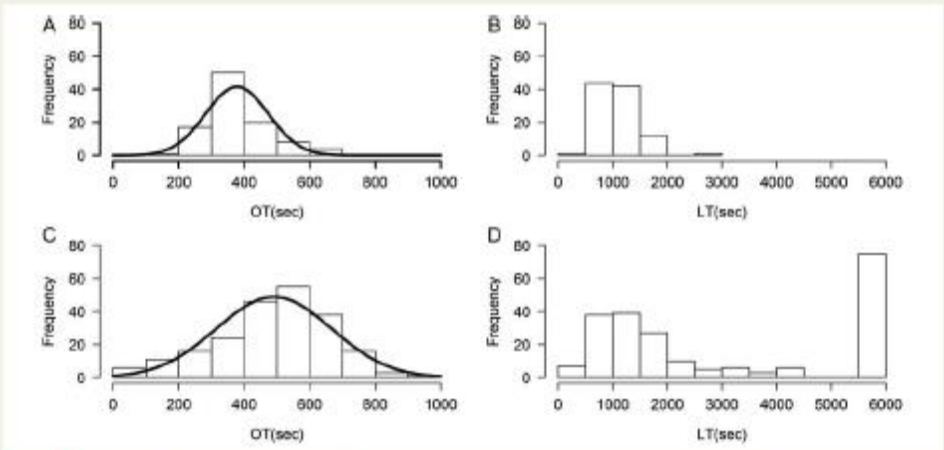

Figure 2 Distribution of (A) occlusion time and (B) lysis time of healthy volunteers and (C) occlusion time and (D) lysis time in end-stage renal disease. Y-axis shows number of subjects.

these gaps, reducing the flow rate and finally arresting flow. The instrument measures the time (d) between consecutive blood drops, which increases gradually as flow slows down and at an arbitrary point ($d \geq 15$ s) the endpoint of the measurement is displayed (OT; seconds). The principle of the technique is shown in *Figure 2*. The restart of blood flow following occlusion is due to spontaneous thrombolysis (lysis time, LT; seconds). If lysis does not occur until 6000 s following OT (LT cut-off time), 'no lysis' is recorded.

The GTT assesses thrombus formation under pathophysiological conditions, since (i) blood is non-anticoagulated, with physiological calcium-ion concentration, (ii) akin to pathological conditions, platelet-rich thrombus formation is initiated by high shear forces,

with release of soluble agonists playing only a secondary role, and (ii) thrombin generation from shear-activated platelets (procoagulant activity of platelets) plays a major role in the formation and stabilization of thrombi.

Coefficient of variation was assessed by testing 10 healthy volunteers twice, at 48 h intervals and also by testing 10 ESRD patients twice at 48 h intervals.

Data collection and follow-up

Baseline demographics, including dialysis vintage, were collected along with urea kinetic adequacy parameters relating to the previous month's routine testing. Follow-up was performed at 30 days and then at 3-monthly intervals, and documents of all adverse events obtained. To assess the effect of haemodialysis on thrombotic status, a subgroup of 20 patients was tested immediately pre- and 2 h after dialysis. We assessed the effect of antiplatelet therapy on thrombotic status, in 20 healthy volunteers. Volunteers were tested before and 12 h after a loading dose of 300 mg aspirin, and after a month washout, assessed again before and 12 h after a loading dose of 300 mg clopidogrel.

Study endpoints

The primary endpoint of the study was the occurrence of major adverse cardiovascular events (MACE) defined as the composite of cardiovascular death, non-fatal myocardial infarction (MI), or cerebrovascular event. The secondary endpoint was the occurrence of peripheral vascular thrombosis including acute ischaemic limb and arterio-venous (AV) fistula thrombosis. Collection and adjudication of events was performed blinded to GTT results.

Cardiovascular events

New cardiovascular events were diagnosed in the presence of (i) cardiovascular death, defined as death from MI based on the Universal Definition of Myocardial Infarction[33] (defined as sudden, unexpected cardiac death, involving cardiac arrest, often with symptoms suggestive of myocardial ischaemia, and accompanied by presumably new ST elevation, or new left bundle branch block, and/or evidence of fresh thrombus by coronary angiography and/or at autopsy, but death occurring before blood samples could be obtained, or at a time before the appearance of cardiac biomarkers in the blood), significant arrhythmia, or refractory congestive heart failure, or death attributed to cardiovascular cause at post-mortem; confirmed from death certificates as well as medical records and observers' accounts; sudden death was included as a cardiovascular event, or (ii) non-fatal MI, defined according to the Universal Definition of Myocardial Infarction[33] as a rise and/or fall of cardiac troponin with at least one value above the 99th percentile of the upper reference limit together with evidence of myocardial ischaemia with at least one of the following: symptoms of ischaemia; ECG changes indicative of new ischaemia (new ST-T changes or new left bundle branch block); development of pathological Q waves in the ECG; or imaging evidence of new loss of viable myocardium or new regional wall motion abnormality.

Cerebrovascular events

New-onset cerebrovascular event was suspected with recent onset of neurological symptoms or signs, e.g. aphasia, focal deficits, or unilateral paresis, thought to be vascular in origin and confirmed by computerized tomography or MRI. This included events with and without spontaneous clinical resolution, and thus included both stroke and transient ischaemic events.

Peripheral vascular events

Peripheral thrombotic events were defined as occurrence of acute ischaemic limb(s) or AV fistula thrombosis on clinical examination and confirmed by contrast angiography and/or duplex ultrasound.

Cause of death

Deaths were classified based on data obtained from post-mortem, from hospital notes, or from the general practitioner, regarding the patient's last illness, according to WHO criteria based on ICD 10 (2010).

Statistical analysis

A normal range for OT and LT was established from healthy volunteers. The required sample size was calculated based on the Cox PH prognostic model. On the basis of our earlier work,[25] the unadjusted HR for LT dichotomized by 3000 s was 2.25 (95% CI: 1.34–4.7). Based on the assumption that we would see around 14% MACE events per year in the ESRD cohort,[34,35] a sample size of 200 was predicted to yield 30 events/year and the final Cox PH model with four covariates would provide 5% significance and 80% power.[36,37]

Given 200 patients in the intervention group and arbitrary selected sample size of 100 healthy volunteers, the difference in LT (OT) between these groups is classified on the moderate (semi-large) effect size level. The Cox PH model was used to assess sample size and Mann–Whitney *U* test used to compare patients and healthy volunteers, with 5% significance and >90% power.

Unpaired *t*-test and Mann–Whitney *U* test were used for normally and non-normally distributed variables, respectively. Two-sided tests were used. Dichotomous variables were compared by χ^2 test or Fisher's exact test, as appropriate. The univariate linear regression model was used to assess the relationship between a continuous variable (dependent) given a dichotomized variable (independent). Where necessary, log transformations were applied. Correlations were analysed using Spearman's rank test. Ability of OT or LT to discriminate between patients with and without MACE was evaluated by receiver operating characteristic (ROC) curve analysis.[38] The optimal cut-off was determined by the value providing the greatest sum of sensitivity and specificity. Kaplan–Meier estimates with log rank tests were used to compare survival curves. Univariate Cox proportional hazard regression was performed on LT divided up into bands of 1000 s to investigate the relationship between LT and MACE, and to identify risk factors from which a multivariate Cox proportional hazard prognostic model was proposed. The hazard proportionality assumption was evaluated in the Cox model with scaled Schoenfeld residuals. The test was carried out for the univariate model including each of the candidate variables, and its multivariate versions: baseline (age, sex, haematocrit) and extended (age, sex, haematocrit, LT≥3000). In both setups, the hazard proportionality assumption was not rejected at 0.05 significance.

To assess the added predictive ability of LT≥3000 s for MACE, net reclassification improvement analysis[39] was performed. For chosen risk cut-offs, models do not recognize patients within the low-risk group (<5%), among patients with and without events. The effect of interventions (heparin, aspirin, clopidogrel, dialysis) on thrombotic status was evaluated with Wilcoxon signed rank test. All tests were two-sided and significance was defined as $P < 0.05$. Analyses were performed with R (R Foundation for Statistical Computing, Vienna, Austria).

Results

The characteristics of study population are shown in *Table 1*. Follow-up was available in all patients for 276 ± 166 days. The coefficient of variation for OT and LT was 8 and 10% in normal volunteers, and 6 and 5% in ESRD, similar to earlier studies using this technique.[32,40,41] There was no correlation between OT and LT (Spearman's, $P = 0.15$).

In ESRD, both OT (491 ± 177 vs.378 ± 96 s, $P < 0.001$) and LT were prolonged (median LT 1820 vs.1053 s, $P < 0.001$) compared with healthy volunteers (*Figure 2*). None of the controls, but 41.7% ESRD patients had LT≥3000 s, with 34% demonstrating markedly impaired thrombolytic status with LT≥6000 s. There were 12 non-cardiovascular deaths, attributable to sepsis (2), pneumonia (1), ESRD (4), haematemesis (1), gastrointestinal haemorrhage, unspecified (2), enterocolitis due to Clostridium difficile (1), and malignant neoplasm colon (1). In the remainder, 23 MACE and 17 peripheral thrombotic events occurred [15 AV-fistula thromboses (14 in natural fistulae, 1 in an AV graft) and two acute ischaemic limbs] (*Table 2* and *Figure 3A*).

Survival analysis demonstrated a strong relationship between LT and MACE (*vide infra*), but not between OT and MACE (HR = 1, 95% CI = 0.9976–1.002, $P = 0.969$). The optimal cut-point correlating with MACE was LT≥2940 s (rounded to 3000 s for clinical ease) (*Figure 4A* and *B*).

LT≥3000 s was associated with a significantly higher risk of MACE overall (HR = 4.25, 95% CI = 1.57–11.46, $P = 0.004$), non-fatal MI or cerebrovascular events (HR = 14.28, 95% CI = 1.86–109.9, $P = 0.01$), and peripheral thrombotic events (HR = 9.08, 95% CI = 2.08–39.75, $P = 0.003$) (*Table 2*). All non-fatal MIs and 15 of 17 peripheral thrombotic events occurred in those with LT≥3000 s. Non-cardiac death was not related to LT. As LT increased, HR increased up to LT≥3000 s (*Figure 2B*). There was no additional risk beyond 4000 s, possibly because a large number of subjects had severely impaired LT (LT≥6000 s, *Figure 1*). Impaired endogenous thrombolytic status was a very strongly associated with MACE (*Figure 5*).

All patient characteristics and variables were interrogated for effects on OT and LT, and shown in *Table 2*. Patients on clopidogrel exhibited longer (less thrombotic) OT than those not taking clopidogrel (564 ± 179 vs. 482 ± 175 s, $P = 0.021$). Occlusion time was also directly correlated with serum sodium and urea level. LT≥3000 s was significantly associated with raised fibrinogen levels, history of CAD, and proton pump inhibitor use.

A univariate model showed that only the following variables from *Table 2* were related to MACE: serum calcium ($P = 0.038$), (log) CRP ($P = 0.04$), haematocrit ($P = 0.013$), and a negative relation with diuretic treatment ($P = 0.044$).

After a back-step model selection procedure, the following three variables were then entered into the baseline multivariate Cox proportional hazard model: haematocrit (HR = 0.87, 95% CI = 0.78–0.97, $P = 0.015$), and two traditional risk factors, namely age (HR = 0.999, 95% CI = 0.97–1.03, $P = 0.944$) and male sex (HR = 1.24, 95% CI = 0.5–3.03, $P = 0.645$).

None of these basic covariates were correlated either with LT or its dichotomized version, which would have increased the standard error of the hazard ratio in the Cox proportional hazard model. Multivariate Cox proportional hazard analysis including the baseline covariates showed that LT≥3000 s remained strongly associated with MACE after adjustment for the baseline risk factors (HR = 4.37, 95% CI = 1.58–12.12, $P = 0.005$). The baseline model (age, sex, haematocrit) was then extended by including LT≥3000 s to give the final predictive model. Of the baseline model covariates, only haematocrit remained significant in the extended model adjusted for age, sex, and LT (HR = 0.89, 95% CI 0.79–0.99).

Receiver operating characteristic curve analysis (*Figure 3C*) indicates an improvement in the area under the curve with the extended model with borderline significance (DeLong test Likelihood ratio test $P = 0.058$) indicating the usefulness of net reclassification improvement analysis, as ROC may not be sensitive enough to detect the new marker improvement.[39] Reclassification analysis performed on the aforementioned baseline model with respect to the extended model (which included LT) showed that adding LT to the baseline risk factor model improves risk stratification of patients with ESRD in terms of cardiovascular risk. Inclusion of LT≥3000 s in the model containing three baseline predictors (haematocrit, age, sex) significantly added to the model effectiveness (net reclassification improvement = 0.61, $P < 0.001$) leading to improvement in reclassification mainly of non-event patients (see Supplementary material online, *Figure S1* and accompanying Table).

Thrombotic status was not affected by haemodialysis (pre- and post-dialysis OT: 570 ± 138 vs. 545 ± 126 s, $P = 0.368$ and LT 1725 vs. 1665 s, $P = 0.753$, respectively) nor by the low-molecular weight heparin administered 48 h earlier [anti-FXa level undetectable (0.0 iu/mL) in all samples]. In patients receiving peritoneal dialysis, thrombotic status did not differ significantly from patients on HD (OT 419 ± 134 vs. 491 ± 177, $P = 0.672$ and LT 2561 vs. 1820 s, $P = 0.574$). In volunteers, aspirin significantly prolonged OT (356 ± 54 vs.530 ± 99 s, $P < 0.0001$), but did not affect LT (1043 vs. 1049 s, $P = 0.741$). Clopidogrel also increased OT (365 ± 54 vs. 569 ± 84 s, $P < 0.001$) but did not affect LT (1043 vs.1067 s, $P = 0.731$). Occlusion time prolongation in response to clopidogrel was marginally more than in response to aspirin ($P = 0.02$).

Discussion

Our main findings are (i) that ESRD patients have markedly impaired endogenous thrombolysis, compared with healthy volunteers and (ii) that such impaired thrombolysis is associated with a high risk of cardiovascular and peripheral thrombotic events.

The prolonged OT in ESRD patients suggests either impaired primary haemostasis, as reported earlier, or reflects the fact that half the patients were on at least one antiplatelet agent, or a combination of these factors. In a large meta-analysis examining the efficacy of antiplatelet agents in preserving dialysis access patency, antiplatelet agents appeared effective in reducing thrombosis in central venous catheters and AV shunts, but not in preventing AV graft thrombosis.[42] OT was not predictive of MACE. This was similar to our findings in ACS patients on dual antiplatelet medication.[25]

Table 1 Baseline characteristics of end-stage renal disease patients and relationship to occlusion time and lysis time

	OT		Overall ($n = 216$)	LT < 3000 ($n = 126$)	LT ≥ 3000 ($n = 90$)	P-value
	Intercept (P-value)	Slope (P-value)				
Risk factors, *n* (%)						
Age (years)	555.4 (<0.001)	−1.0 (0.218)	64.4 ± 14.8	65.5	62.9	0.169
Male gender	459.8 (<0.001)	48.4 (0.053)	138 (63.9)	80 (63.5)	58 (64.4)	1.000
History of peripheral vascular disease	492.2 (<0.001)	−18.9 (0.672)	17 (7.9)	11 (8.7)	6 (6.7)	0.765
Hypertension	512.1 (<0.001)	−30.7 (0.242)	151 (69.9)	88 (69.8	63 (70.0)	1.000
Hyperlipidaemia	496.6 (<0.001)	−8.0 (0.793)	43 (19.9)	29 (23.0)	14 (15.5)	0.143
Diabetes	478.6 (<0.001)	42.2 (0.113)	62 (28.7)	35 (27.8)	27 (30.0)	0.839
Prior CAD	479.0 (<0.001)	40.8 (0.125)	62 (28.7)	29 (23.0)	33 (36.7)	0.042
Prior stroke	491.6 (<0.001)	−5.6 (0.862)	37 (17.1)	20 (15.9)	17 (18.9)	0.691
Prior MI	484.8 (<0.001)	50.8 (0.177)	25 (11.6)	10 (7.9)	15 (16.7)	0.078
LV dysfunction	487.3 (<0.001)	39.9 (0.255)	32/153 (21.1)	15/80 (18.8)	17/73 (23.6)	0.624
Body mass index	537.7 (<0.001)	−1.8 (0.381)	26 ± 6	26	26	0.736
Drugs, *n* (%)						
Aspirin	489.9 (<0.01)	1.6 (0.947)	111 (51.4)	58 (46.0)	53 (58.9)	0.084
Clopidogrel	481.5 (<0.001)	82.5 (0.0306)	24 (11.1)	12 (9.5)	12 (13.3)	0.510
ACE-inhibitor	488.0 (<0.001)	7.9 (0.758)	73 (33.8)	36 (28.6)	37 (41.1)	0.076
Statins	476.5 (<0.001)	29.1 (0.226)	105 (48.6)	60 (47.6)	45 (50.0)	0.836
β-blocker	493.5 (<0.001)	−8.0 (0.752)	76 (35.2)	45 (35.7)	31 (34.4)	0.962
Calcium channel blocker	493.0 (<0.001)	−7.7 (0.768)	65 (30.1)	38 (30.2)	27 (30.0)	1.000
Proton pump inhibitor	484.9 (<0.001)	10.7 (0.659)	118 (54.6)	56 (44.4)	62 (68.9)	0.001
Diuretic	485.1 (<0.001)	13.5 (0.582)	89 (41.2)	58 (46.0)	31 (34.4)	0.117
Insulin	484.1 (<0.001)	39.3 (0.224)	36 (16.7)	17 (13.5)	19 (21.1)	0.195
Erythropoietin	427.7 (<0.001)	67.0 (0.185)	203 (94.0)	120 (95.2)	83 (92.2)	0.530
Laboratory results						
Haemoglobin (g/dL)	422.5 (<0.001)	6.2 (0.542)	11.0 ± 1.2	11.0	10.9	0.710
Haematocrit	500.1 (<0.001)	−0.3 (0.934)	32.8 ± 3.5	33.0	32.4	0.178
Platelet count (× 109)	564.5 (<0.001)	−0.3 (0.0419)	227 ± 75.4	228.9	225.9	0.818
Urea (mmol/L)	377.1 (<0.001)	5.6 (0.005)	20.5 ± 6.8	20.6	20.4	0.894
Creatinine (μmol/L)	437.0 (<0.001)	0.1 (0.128)	732.1 ± 250	722.2	745.9	0.670
Sodium (mmol/L)	−804.3 (0.005)	9.5 (0.005)	136.5 ± 3.4	136.3	136.6	0.452
Potassium (mmol/L)	496.3 (<0.001)	−1.1 (0.944)	5.0 ± 0.8	5.0	5.1	0.074
Calcium (mmol/L)	820.8 (<0.001)	−136.5 (0.069)	2.4 ± 0.2	2.4	2.5	0.002
Phosphate (mmol/L)	489.8 (<0.001)	1.9 (0.961)	1.6 ± 0.5	1.6	1.7	0.211
C-reactive protein (mg/L)	502.0 (<0.001)	−19.0 (0.438)	18.0 ± 23.7	16.2	20.6	0.050
Albumin (g/L)	458.6 (<0.001)	0.9 (0.746)	36.0 ± 4.4	36.3	35.6	0.120
Bicarbonate (mmol/L)	541.9 (<0.001)	−2.2 (0.576)	23.6 ± 3.1	23.4	23.8	0.347
Cholesterol (mmol/L)	561.5 (<0.001)	−16.4 (0.121)	4.1 ± 1.2	4.3	3.8	0.010
LDL/HDL ratio	569.3 (<0.001)	−13.7 (0.302)	3.5 ± 1.1	3.6	3.3	0.164
Fibrinogen (g/L)	534.5 (<0.001)	−4.3 (0.527)	5.7 ± 2.2	5.3	6.2	0.013
Parathormone (pg/mL)	492.1 (<0.001)	−0.03 (0.899)	52.3 ± 51	57.1	49	0.559
Dialysis parameters						
Duration of HD (min)	581.0 (<0.001)	−0.5 (0.164)	187.9 ± 34.9	185.3	191.5	0.191
Kt/V	449.0 (<0.001)	30.3 (0.447)	1.3 ± 0.3	1.4	1.3	0.562
Dialysis vintage (months)	580.9 (<0.001)	−0.5 (0.164)	6.3 ± 1	6.2	6.3	0.854
KRU (mL/min/m^2)	494.3 (<0.001)	−2.3 (0.744)	1.4 ± 1.7	1.6	1.3	0.197
Fistula (natural)			142	89	53	0.099
Fistula (graft)			3	1	2	0.768

NS, non-significant; CAD, coronary artery disease; MI, myocardial infarction; LV, left ventricle; HD, haemodialysis; KRU, renal urea clearance; Dialysis vintage, time from start of dialysis to present (in months); Kt/V, number used to quantify dialysis treatment adequacy, where K is the dialyzer clearance of urea, t the dialysis time, and V the volume of distribution of urea, approximately equal to patient's total body water.

Table 2 Breakdown of major adverse cardiovascular events and hazard ratio based on lysis time

	Overall ($n = 216$)	LT < 3000 ($n = 126$)	LT ≥ 3000 ($n = 90$)	HR (CI)	P-value	HR (CI) Adjusted for sex, age, Hct	P-value
Cardiovascular death, non-fatal MI, cerebrovascular accident	23 (10.6)	5 (4.0)	18 (20)	4.25 (1.58–11.46)	0.004	4.37 (1.58–12.12)	0.005
Non-fatal MI and cerebrovascular accident	13 (6.0)	1 (0.8)	12 (13.3)	14.28 (1.86–109.90)	0.011	15.76 (2.00–124.06)	0.009
Cardiovascular death	10 (4.6)	4 (3.2)	6 (6.7)	1.75 (0.49–6.22)	0.385	1.64 (0.44–6.15)	0.465
Non-fatal MI	9 (4.2)	0 (0.0)	9 (10.0)	—	—		
Cerebrovascular accident	4 (1.9)	1 (0.8)	3 (3.3)	3.57 (0.37–34.42)	0.270	4.89 (0.46–51.4)	0.186
Peripheral thrombotic event	17 (7.9)	2 (1.6)	15 (16.7)	9.08 (2.08–39.75)	0.003	10.81 (2.45–47.68)	0.002

Values are given as *n* (%).
CI, confidence intervals; Hct, haematocrit; MI, non-fatal myocardial infarction; peripheral thrombotic events, composite of fistula thrombosis and acute ischaemic limb.

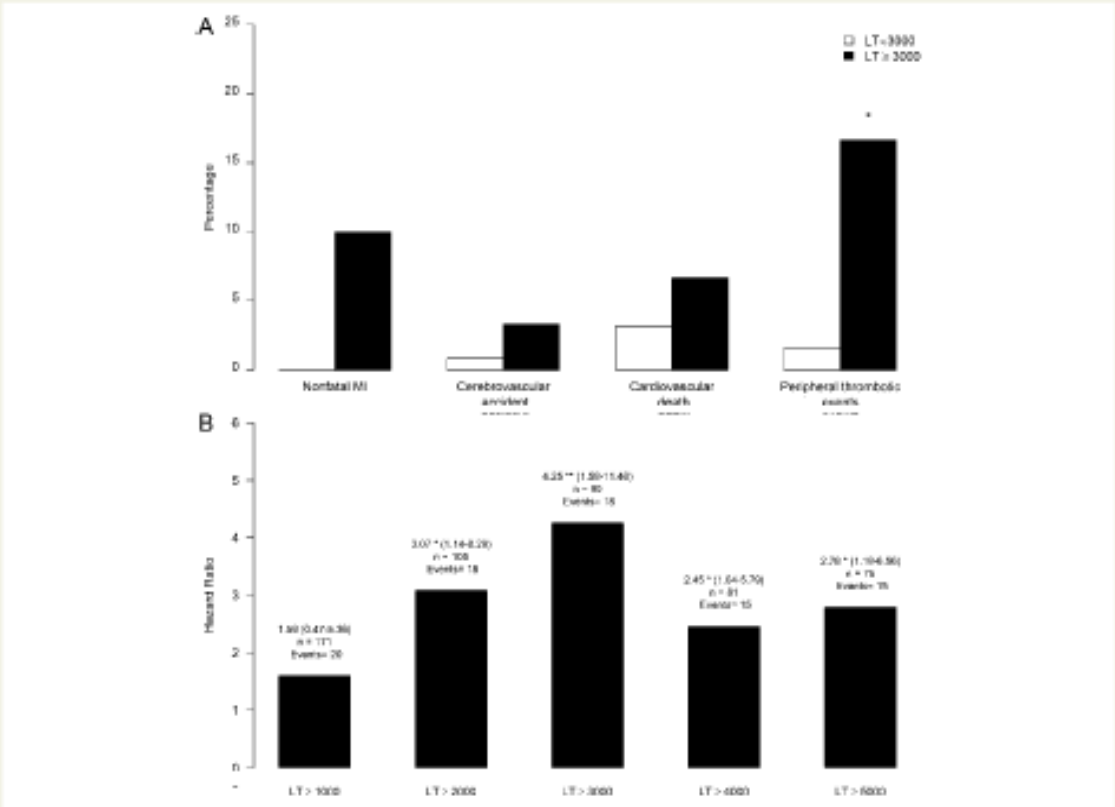

Figure 3 (*A*) Incidence of study endpoint events based on lysis time. MI, myocardial infarction, $^{*}P < 0.05$. (*B*) Hazard ratios (HR) for major adverse cardiovascular events by lysis time. LT ≥ 3000 s was the optimal cut-point to predict major adverse cardiovascular events. The 95% confidence interval is shown in brackets. $^{*}P < 0.001$; $^{**}P < 0.0001$.

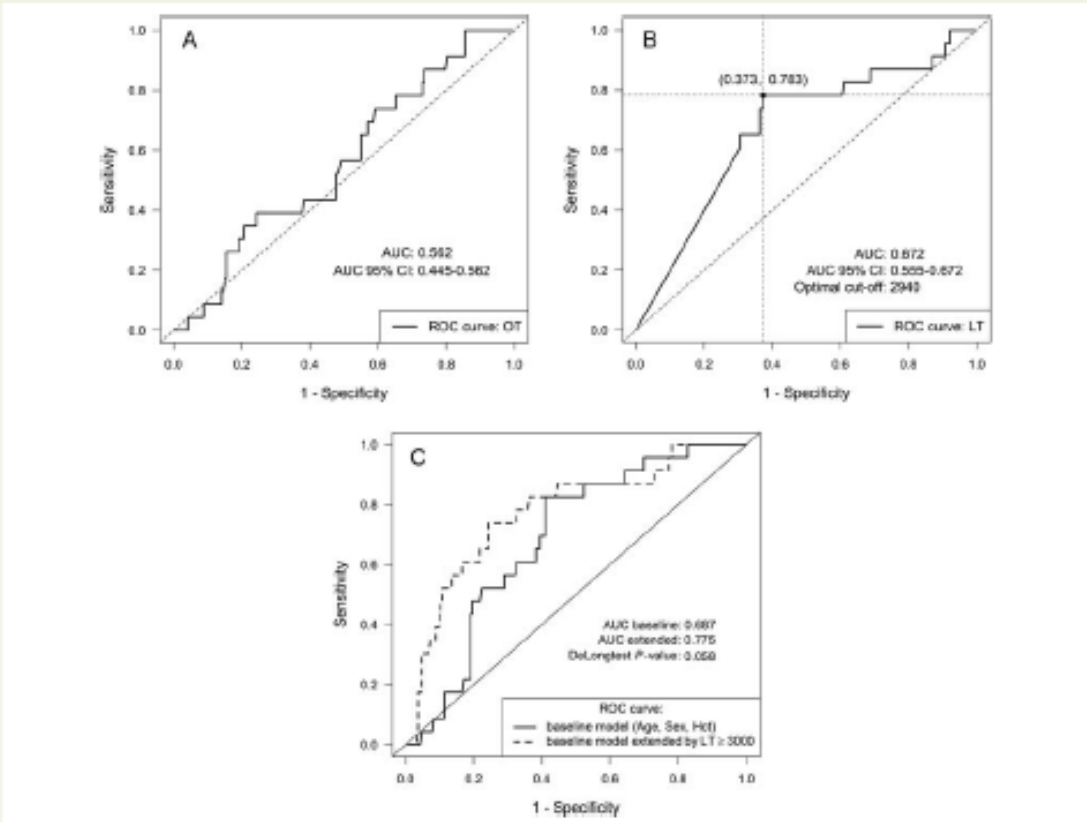

Figure 4 Receiver operating curves for (*A*) occlusion time and (*B*) lysis time and (*C*) receiver operating curve analysis showing improvement of the prognostic model by adding LT ≥ 3000 s. Lysis time significantly discriminated between patients with and without major adverse cardiovascular events. AUC, area under the curve.

Despite the relatively high prevalence of cardiovascular risk factors in ESRD,[4] the Framingham risk score projected cardiovascular risk in ESRD is similar or somewhat higher than reference populations from the Framingham cohort or from the National Health and Nutrition Examination Survey (NHANES) III.[43] Even studies predicting higher risk may underestimate the incidence of cardiovascular disease observed in dialysis patients or even transplant recipients.[44] These point towards the contributory role of non-traditional risk factors in promoting cardiovascular risk in ESRD, which traditional predictive models do not cover. Although aggressive risk factor modification in CKD with statins,[45] angiotensin-converting enzyme inhibitors,[44] and normalization of haemoglobin with erythropoietin[46] reduce cardiovascular events,[47] thrombotic events continue to occur. Thus, the optimal management of cardiovascular risk in ESRD not only requires traditional risk factor modification, but also identification of newer, previously unknown, risk factors in this complex group, that may explain the excess cardiovascular events, which are higher than in any other disease state.

In this study, we show that impaired LT is strongly correlated with adverse cardiovascular events in ESRD, independent of other risk factors. This is predominantly attributable to an increased risk of MI in those with LT≥3000 s, although it is very unlikely that the LT<3000 s group is completely protected from MI. The MACE rate in those with LT<3000 s was much lower than anticipated and may have contributed to the lower than expected significance of our results. Although the hazard ratio for MACE is high, the sample size is relatively small and the confidence intervals large, thus our results could be due to chance. Although studies cannot be directly compared, our findings are supported by the prior study in ACS patients, where the same cut-off value (LT≥3000 s) was correlated with increased cardiovascular risk[25] but imparted a much greater risk in ESRD than in ACS patients (HR = 4.25 vs. 2.5) and LT was more frequently impaired and longer in ESRD than in ACS patients.[25] The distribution of events was not even in our subgroups (*Figure 3B*), with a break in events between 3000 and 5000 s, as there were no events in LT 4000–5000 s group. The reduction in hazard when LT > 4000 s is likely, at least in part,

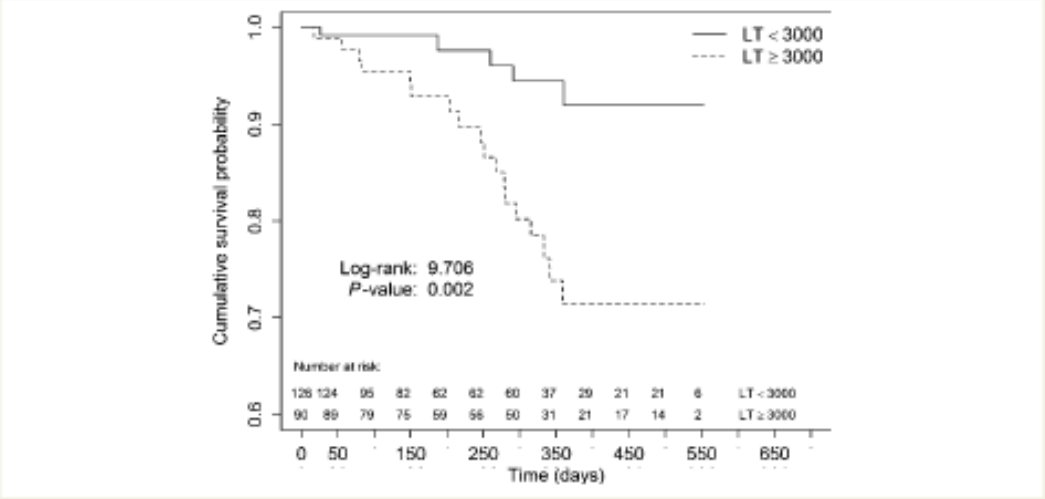

Figure 5 Kaplan–Meier curves showing probability of event-free survival in end-stage renal disease based on lysis time. Thrombolytic status was strongly predictive of major adverse cardiovascular events. LT ≥ 3000 s was associated with HR 4.25 compared with those with LT < 3000 s.

to reflect the uneven distribution of events, with small numbers of patients and even smaller numbers of events in these groups.

Our findings are supported not only by studies showing increased fibrinogen, plasminogen activator inhibitor-1, and reduced tissue plasminogen activator in ESRD,[46–51] but also recent data on fibrin clot properties. Both ESRD[52,53] and thromboembolic coronary events[54–56] have been associated with the formation of dense fibrin clots, resistant to fibrinolysis. Using turbidometric plasma clot lysis, fibrin clot permeability and perfusion clot lysis assays, Undas *et al.*[57] showed that ACS and CKD patients have higher plasminogen activator inhibitor-1 and tissue plasminogen activator levels and formed fibrin clots that were less porous and more resistant to fibrinolysis, than a control group of ACS patients with normal renal function. The fibrin clot in CKD patients exhibited smaller pore size, larger number of protofibrils per fibrin fibre, increased fibre size and clot mass. Our finding of an association between raised fibrinogen level and impaired endogenous thrombolysis is interesting. Based on epidemiological data, fibrinogen has been associated with increased cardiovascular and arterial thrombotic risk, but whether this relationship is causal is not established. Recently, high fibrinogen levels have been linked with resistance to thrombolytic therapy and adverse outcome in patients with ischaemic stroke.[58] Furthermore, in a murine model, artificial increase in fibrinogen level directly promoted thrombosis and thrombolysis-resistance, via enhanced fibrin formation and stability.[59] However, in a transgenic mouse model of hyperfibrinogenaemia, mice with high fibrinogen level did not demonstrate accelerated platelet thrombus formation in response to injury, compared with wild-type.[60] Surprisingly, transgenic mice demonstrated suppression of thrombin generation in plasma and activation of the fibrinolytic system. Furthermore, genetic variations such as the γ′ splice variation in fibrinogen gene transcription result in more highly cross-linked and stable fibrin clots, with reduced pore size, that are more resistant to lysis.[61] This variant is associated with an increased risk of thrombosis and MI, an effect that is independent of fibrinogen levels.[61,62]

To investigate further the relationship between fibrinogen level and impaired endogenous thrombolysis, we performed an *in vitro* experimental correction to raise fibrinogen level and assess the effect on LT. In healthy volunteers ($n = 10$), in parallel measurements, increase in plasma fibrinogen concentration *in vitro* by 1 g/L did not significantly alter LT compared with control samples [median LT 1081 (IQR 907–1300) s and 1297 [1208–1660] s respectively, $P = 0.06$]. Thus increasing fibrinogen concentration showed a trend towards enhanced rather than inhibited spontaneous thrombolytic activity.

Our *in vitro* findings in healthy blood support others claiming that hyperfibrinogenemia *per se* does not inhibit fibrinolysis.[60] Thus, it is more likely that it is not just the elevated plasma fibrinogen concentration *per se*, but the quality of the fibrin clot architecture that determines risk.

It is likely that in ESRD, fibrinogen structure and function are also altered, making clots more resistant to lysis[52] and this may be causally related to increased thrombotic risk.

Unfavourable clot properties were demonstrated in ESRD patients on haemodialysis, and although a small study, there was an association between mortality and reduced clot permeability and prolonged LT.[53] This may explain the functional significance of the impaired thrombolytic state observed in ESRD. We did not seek to compare LT with plasma markers of fibrinolysis, since the value of fibrinolysis activity markers is very limited in aiding diagnosis and risk stratification in the individual patient.[63]

Prolonged LT was associated with prior CAD but it is difficult to say whether it is a causative phenomenon. Lysis time in ACS patients was not related to prior CAD.[25] Proton pump inhibitor use was related to prolonged LT. Our study is too small to analyse whether this reflects a possible effect of proton pump inhibitors inhibiting the CYP2C19 isoenzyme, thereby reducing the ability of clopidogrel to inhibit platelet aggregation. That higher serum calcium concentration was associated with prolonged LT may be functionally important. Ionized calcium (Ca^{2+}) holds together the fibrinogen binding receptor glycoprotein IIb/IIIa complex, is essential for agonist-induced conversion of the glycoprotein IIb/IIIa complex into the functional fibrinogen receptor, and is required for the binding of fibrinogen to its receptor, as well as for the coagulation cascade.[19] C-reactive protein was related to LT suggests a relationship between inflammation and thrombosis. Inflammation biomarkers are strong predictors of MI or thrombotic stroke[64] and modification of platelet function has been reported to modulate inflammatory mediators.[65]

Limitations of our study include that normal volunteers were not age-matched, patients were sampled only once and predialysis, the fact that only CKD patients on haemodialysis were studied, and that most patients received erythropoietin, which is known to increase the number of circulating platelets, improves platelet function,[66] and has varying effects on platelet reactivity and fibrinolysis.[67] It has also been proposed that in patients treated with erythropoietin, increased activity of C-reactive protein, nitric oxide, and thrombin-activatable fibrinolysis inhibitor leads to a fibrinolytic deficit with resultant increase in thrombosis.[68] The effect of antiplatelet medication on LT is not fully established, but unlikely to be significant given the findings in patients with ACS[25] and here in ESRD. It will be interesting to see if LT is shortened by the emerging novel thrombin inhibitors once these are licensed for ACS, since a significant portion of patients with ACS have prolonged LT and this is a risk factor for recurrent cardiovascular events.[25]

Our identification of impaired thrombolysis as a novel risk factor in ESRD may have important implications for screening and risk stratification. Since thrombotic status does not appear to be affected by haemodialysis, future studies are required to investigate medical therapies to improve endogenous thrombolysis, to see whether this may reduce the risk of cardiovascular events in these high-risk patients.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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