HAEMOSTASIS IN PROGRESSIVE RENAL FAILURE

Thesis submitted for the degree of Doctor of Philosophy in the faculty of Medicine, University of London.

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ABSTRACT OF THESIS

This thesis describes a series of clinical and haematological studies of patients with chronic renal failure. My aim was to investigate the role of haemostatic abnormality in two aspects of renal disease:
(1) The bleeding tendency of uraemia.
(2) The progression of renal failure.

(1) In the first part of the thesis I show that anaemia appears to be the most important determinant of uraemic bleeding. The development of a prolonged bleeding time in the early stages of renal failure was documented, and experiments were performed to identify platelet functional abnormalities which might develop in parallel, and hence explain the haemorrhagic defect. The only significant relationship which emerged was a negative correlation between the haematocrit and the bleeding time. This was further investigated by studying platelet function during correction of anaemia with recombinant human erythropoietin. Bleeding time was shortened following erythropoietin, by a degree which correlated with the increase in the haematocrit, but not with any changes in platelet reactivity. A study of platelet aggregation in whole blood showed an aggregation-enhancing effect of uraemic red cells which was not evident with normal erythrocytes.

(2) In the second part of the thesis, increased blood viscosity and intravascular haemostatic activation were demonstrated in both diabetic and non-diabetic patients with progressive renal disease, abnormalities which might promote non-immune glomerular injury and hence the progression of renal failure. It was not clear, however, whether these changes represented cause or effect of disease, since in multiple regression analysis, proteinuria emerged as the major independent determinant of progression. A clinical study of anti-platelet therapy in progressive renal failure showed a slowing of progression in half the patients treated, and, in diabetic nephropathy, a significant reduction in proteinuria. These results suggest a partial role for haemostasis in the progression of renal injury.
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SECTION I INTRODUCTION
A bleeding tendency has long been recognised as a feature of renal failure. Richard Bright, in his early observations on patients with kidney disease, noted that many presented with purpura [1]. In 1907, Riesman highlighted the importance of haemorrhage as a manifestation of renal disease. To explain this, he postulated the action of a toxin, either on the blood vessels, or on the components of the blood [2]. The pattern of bleeding, involving mainly skin or mucous membrane sites, led to the early idea that the cause might be vascular damage. In 1957 Kuhlback reported increased capillary fragility in renal failure and, in the absence of thrombocytopenia, concluded that uraemic bleeding was the result of vascular damage, arising either as a direct effect of a toxin, or as part of a more general disease process that was also responsible for the destruction of the kidney [3]. The theory of vascular damage as the cause of uraemic bleeding was not, however, supported by other studies. In 1956, Lewis and colleagues had found a prolongation of the bleeding time in renal failure, but no increase in capillary fragility [4], and evidence of capillary fragility was also lacking in the report of Rath and coworkers published in 1957 [5]. Prolongation of the prothrombin time was sometimes reported [4-6], but this was sporadic and of only minor degree, and was probably due to liver insufficiency or vitamin K deficiency. Similarly, thrombocytopenia was neither sufficiently consistent nor severe to explain the haemorrhagic symptoms. Taken together, these findings pointed to a defect in platelet function as the major pathogenetic factor in uraemic bleeding.

The advent of dialysis therapy was accompanied by a reduction in the incidence of haemorrhagic complications, suggesting that platelet dysfunction was due to the action of retained toxins. Detailed study of the influence of the uraemic state on platelet biochemistry was, however, delayed until the introduction of aggregometry techniques in the early 1960's. Since that time there has been a rapid increase in our knowledge of haemostasis in renal failure, but many questions still remain.

It is paradoxical that, in the face of the haemorrhagic tendency of
uraemia, patients with chronic renal failure (CRF) on long-term haemodialysis run an increased risk of atherosclerotic complications [7]. Data relating to young adults aged 15 to 34 years, collected by the European Dialysis and Transplantation Association, illustrates this dramatically, by showing a risk of coronary death some 150 times higher, and a risk of cerebrovascular accident 250 times higher than healthy subjects of a similar age [8]. Uraemic patients thus display not only a bleeding tendency, but also an increased incidence of arterial thrombosis.

As well as atherosclerosis of the major blood vessels, vascular injury and thrombosis may occur within the kidney itself. Studies of experimental renal disease performed over the last 25 years have shown that glomerular deposition of fibrin and platelets may be a contributory pathogenetic factor in causing renal failure.

Vassalli and coworkers in the 1960's showed that induction of intravascular coagulation within the kidney led to glomerular platelet-fibrin thrombi and endothelial injury, and further to mesangial swelling and phagocytosis, cellular proliferation and eventually glomerular obliteration [9]. Experimental nephritis was also shown to be characterized by glomerular fibrin deposition, glomerular proliferation and crescent formation, which could be ameliorated by treatment with warfarin [10,11] or large doses of heparin [12,13]. Jorgensen induced intra-renal platelet activation in rabbits by infusing ADP into the aorta just proximal to the renal arteries. This led acutely to platelet thrombi, hypertension, segmental glomerular proliferation and some tubular necrosis. Mild segmental proliferation was still evident at 2 months [14].

Clinical investigation stimulated by these findings confirmed the involvement of the haemostatic mechanism in acute forms of renal disease [15]. The role of the platelet, in particular, as a mediator of inflammatory injury in glomerulonephritis is now well documented [16,17].

Observation of the natural history of renal disease indicates that once renal function is reduced to a critical level, progression of renal failure proceeds inexorably by mechanisms which may be independent of the initiating injury. During the last decade, a vast amount of research has attempted to elucidate the mechanisms by which
progression occurs. Progressive renal failure is characterized haemodynamically by high glomerular plasma flow and hydraulic pressure, clinically by hypertension and proteinuria, and histologically by focal and segmental glomerular sclerosis (FSGS). What is the role, if any, of the haemostatic mechanism in this process? Platelet involvement in primary FSGS was noted by Duffy in 1970 [18], and by George and co-workers in 1974 [19]. More recently, evidence from the sub-total nephrectomy model of progressive CRF has indicated a role for glomerular capillary thrombosis in the progressive loss of renal function (see below). It is not yet clear, however, whether this is also the case in human subjects with progressive CRF.

AIMS OF THESIS

There are two majors areas where abnormal haemostasis may be relevant in renal disease. These are first, the bleeding tendency of uraemia and second, the involvement of platelet and fibrin deposition in glomerular injury.

The aim of this thesis is to review haemostasis in patients with progressive renal failure, in order to investigate further these two areas.

(1) The Bleeding Tendency of Uraemia

The development of a bleeding tendency in the earlier phases of progressive renal failure will be documented, and experiments designed to investigate the mechanism of uraemic bleeding will be described. These studies focus mainly on the role of anaemia in prolonging the uraemic bleeding time.

(2) The Role of Blood Rheology and Haemostasis in Progressive Renal Failure.

The role of rheological abnormality and of haemostatic activation in chronic glomerular damage will be studied. There is little doubt that haemostasis is involved in acute forms of glomerular injury, but its role in chronic, progressive renal failure is uncertain. The studies reported in this section of the thesis will therefore be confined to this question.
CHAPTER 2 THE HAEMOSTATIC MECHANISM

Before discussing haemostatic abnormalities in renal failure, it is necessary first to give a brief description of normal haemostasis. Haemostasis is a complex response to vascular injury, which involves the balanced interaction of a variety of cellular and humoral components in order to prevent bleeding and restore vessel integrity. Loss of this balance results either in thrombosis, or in haemorrhage. In order to simplify description of the haemostatic system, the following components will be discussed separately:

(1) Platelets.
(2) Coagulation.
(3) Fibrinolysis.
(4) Endothelium.
(5) Blood rheology.

(1) Platelets.
Platelets are derived from bone marrow megakaryocytes, and circulate in the blood as small, disc-shaped, non-nucleated cells at a concentration of 150 to 400 x 10^9/l. They possess a typical bilamellar plasma membrane which extends by invagination into the cytoplasm, to form the surface connected canalicular system. Assymetric distribution of phospholipids within the membrane ensures that, in the unstimulated platelet, almost all of the negatively charged phosphatidylserine and phosphatidyinositol are confined to the inner leaflet of the bilayer. A variety of glycoproteins are located in the platelet membrane which confer antigenic specificity, contribute to surface negative charge and act as receptors for extracellular ligands, thus mediating platelet adhesion and aggregation [20]. The major glycoproteins of the platelet membrane, and their receptor functions, are shown in Table 2.1.
Platelet cytoplasm contains a number of storage granules which may release their contents following activation of the platelet. These granule contents possess biological properties which may promote the haemostatic process. Platelet granules are classified as (1) α granules; (2) dense granules and (3) lysosomes. The contents of these granules are shown in Table 2.2.
### Table 2.1 Platelet membrane glycoproteins.

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<tr>
<td>Ia</td>
<td>Collagen receptor (?)</td>
<td>[23]</td>
</tr>
<tr>
<td>Ia-IIa</td>
<td>Collagen receptor (?)</td>
<td>[24]</td>
</tr>
<tr>
<td>Ib</td>
<td>vWF receptor</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Thrombin receptor (?)</td>
<td>[26]</td>
</tr>
<tr>
<td>IIb/IIIa complex</td>
<td>Receptor for fibrinogen, fibronectin and vWF</td>
<td>[20]</td>
</tr>
<tr>
<td>IV</td>
<td>Thrombospondin receptor (?)</td>
<td>[27]</td>
</tr>
<tr>
<td>V</td>
<td>Thrombin receptor (?)</td>
<td>[27]</td>
</tr>
<tr>
<td>GMP-140</td>
<td>Displayed on platelet surface following platelet activation. Function uncertain</td>
<td>[28]</td>
</tr>
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(Question mark indicates that the function assigned to the glycoprotein is not yet fully established).

### Table 2.2 Platelet granules

<table>
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<td>α granules</td>
<td>β thromboglobulin; PF4; Low affinity PF4; PDGF; fibrinogen; fibronectin; vWFαg; thrombospondin; factor V; PAI - 1; albumin.</td>
</tr>
<tr>
<td>Dense granules</td>
<td>Ca²⁺; ATP; ADP; serotonin.</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>various lysosomal enzymes</td>
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The platelet possesses a cytoskeleton composed mainly of actin filaments, together with actin-binding protein, P235 and myosin. [21]. Two distinct pools of actin filaments are present: (1) the cytoplasmic network; (2) the membrane skeleton. In addition, there is a microtubule coil, comprised mainly of the protein tubulin, at the periphery of the platelet. In the unstimulated platelet, these cytoskeletal elements stabilize the plasma membrane, and maintain both platelet shape, and the distribution of glycoproteins and internal granules. During platelet activation, the membrane skeleton participates in the shape change response, and the cytoplasmic skeleton in centralization and secretion of granules, filopodia extension and clot retraction.

An early event in primary haemostasis is platelet adhesion to subendothelial connective tissue elements in the damaged vessel wall. The adhesive proteins vWF and fibronectin are important in mediating platelet adhesion, as is the platelet membrane receptor glycoprotein Ib [22]. Adhesion is promoted by the presence of red cells, and by increasing shear rate up to a value of about 2000 sec\(^{-1}\).

Platelet activation is initiated by the binding to a membrane receptor of a stimulatory agonist. In man, the most important physiological agonists are thrombin, collagen, ADP and thromboxane. Platelet activating factor (PAF) may also play a role, but is probably less important in man than in other species. Activation results in shape change, release of granule contents, platelet aggregation (brought about by the binding of fibrinogen to surface glycoprotein IIb/IIIa complexes), and the rearrangement of membrane phospholipids to expose sites for the binding and accelerated activity of coagulation enzyme complexes.

The biochemical events which link receptor binding to these platelet reactions are termed stimulus-response coupling [29]. This begins with the activation, via a guanine-nucleotide binding transducer protein, of the enzyme phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)), both of which act as second messengers within the cell. DAG activates membrane bound protein kinase C, by reducing the Ca\(^{2+}\) requirement for activation of this enzyme to resting physiological levels (around 100nmol/l). Activated protein kinase C then phosphorylates a 47 kilodalton protein, an event which, though not
fully understood, appears to be important in promoting platelet activation. IP₃ is water-soluble and passes across the cytoplasm to the dense tubular system, where it causes release of stored Ca²⁺ ions. IP₃ and also, possibly, its phosphorylated derivative 1,3,4,5-tetrakisphosphate (IP₄), are also believed to be involved in promoting the influx of extracellular Ca²⁺ into the platelet. Thus IP₃ generation leads to an elevation of cytosolic Ca²⁺, which, when complexed to calmodulin, activates myosin light chain kinase and thus promotes the contractile events which are important in platelet shape change, release and aggregation.

Another Ca²⁺-dependent enzyme which may be activated following platelet stimulation is phospholipase A₂. This enzyme liberates arachidonic acid, principally from membrane phosphatidylcholine and phosphatidylethanolamine, making it available for conversion by cyclooxygenase to prostaglandin endoperoxides. In the platelet, these are enzymatically converted mainly to thromboxane A₂ (TxA₂). TxA₂ is an ephemeral compound with extremely potent platelet-activating and vasoconstrictor properties [30].

Intraplatelet cyclic AMP (cAMP) is important in the regulation of platelet function. Platelet inhibitors, such as prostacyclin (PGI₂), prostaglandin E₁ and adenosine, stimulate adenyl cyclase to convert ATP to cAMP. This molecule acts as an inhibitory second messenger within the platelet, by reducing cytosolic Ca²⁺, and inhibiting the activity of phospholipase C and protein kinase C [29]. Cyclic GMP (cGMP) appears to be a further inhibitory second messenger in platelets, and agents such as endothelium derived relaxing factor (EDRF), which raise intraplatelet cGMP, interact in a synergistic fashion with PGI₂ to inhibit platelets [31].

2) Coagulation.

The formation and stabilization of a fibrin clot is the culmination of a series of interactions within the blood coagulation enzyme system. This complex system will be described here only in general terms. There are two major mechanisms by which activation of coagulation may be initiated: (1) surface contact, which depends upon the surface-binding properties of factor XII, and its susceptibility to proteolytic cleavage once bound [32]; (2) tissue factor (procoagulant;
thromboplastin), a lipoprotein released from damaged tissue, and also made available on the surface of activated macrophages and endothelial cells. Tissue factor dramatically increases the activity of factor VIIa [33].

These two activating mechanisms are the initial events in two enzyme "cascades" known respectively as the "intrinsic" and "extrinsic coagulation systems (Fig. 2.1). Although it is convenient to describe coagulation in these terms, it is now clear that connections exist between the two systems, and an absolute division between them is untenable.

At key stages in the coagulation mechanism, proenzymes are activated by complexes consisting of an enzyme, a cofactor, Ca\(^{2+}\) and phospholipid. In each case, the proenzyme is a vitamin K-dependent factor (factors VII, IX, X and II) containing a gamma-carboxy glutamic acid residue that can bind Ca\(^{2+}\), thus inducing a conformation change that permits binding of the protein to negatively charged phospholipid. These phospholipid binding sites are provided by the membranes of activated platelets [34].

The intrinsic and extrinsic pathways meet at factor X, which, when activated and complexed with factor V, Ca\(^{2+}\) and platelet membrane phospholipid, converts prothrombin to thrombin (the "common" coagulation pathway). The generation of thrombin has a variety of effects. It cleaves fibrinopeptides A and B from the Aα and Bβ chains, respectively, of fibrinogen, leading to the formation of fibrin monomer, which is then crosslinked and stabilized by the action of (thrombin-activated) factor XIIIa. Thrombin provides further positive feedback to the coagulation process by activating factors V and VIII, vastly increasing their cofactor activity.
Figure 2.1 The blood coagulation mechanism. Dashed lines indicate connections between the "intrinsic" and "extrinsic" systems. PL = phospholipid. "a" indicates the factor in its activated form.
3) Fibrinolysis.
The blood fibrinolytic system consists of three main components: (1) the proenzyme plasminogen, which can be converted to the active enzyme plasmin; (2) plasminogen activators, of which the most important physiologically is tissue-type plasmingen activator (tPA) (urokinase-type plasminogen activator (uPA) and a less well defined factor XII dependent activator also contribute); (3) fibrinolytic inhibitors, either plasminogen activator inhibitors (PAI) or inhibitors of plasmin (the most important being α2 antiplasmin). These proteins possess lysine binding sites which play an important role in the biochemical mechanism of fibrinolysis. The major physiological sites of synthesis appear to be the liver (plasminogen, α2 antiplasmin, PAI), the kidney (uPA) and the endothelium (tPA, PAI) [35]. Despite the fact that both plasminogen and plasminogen activators are present in circulating blood, no free plasmin is detectable under normal conditions. This is due to the biochemical coordination of fibrinolysis that ensures that it is localised to the site of a fibrin clot. Fluid phase fibrinolysis is prevented, firstly by the immediate ($t_{1/2} 100$ msec.) inactivation of free plasmin by α2 antiplasmin, secondly by the binding of circulating tPA by an excess of PAI, and thirdly by the low affinity of tPA for circulating plasminogen [36,37]. Fibrin, once formed, mediates its own destruction by initiating a series of molecular interactions culminating in the local generation of plasmin and subsequent fibrinolysis [38]. tPA and plasminogen bind to fibrin via their lysine binding sites, and the affinity of plasminogen for tPA is enhanced by the interaction of tPA with fibrin. A ternary complex between tPA, plasminogen and fibrin is formed, accelerating the formation of plasmin both by spatial proximity and by conformational changes which increase the efficiency of plasminogen activation by tPA. Nascent plasmin remains transiently complexed to fibrin by its lysine binding site and its active centre, and is partially protected from α2 antiplasmin, though inactivation will slowly occur by α2 antiplasmin which has been bound and crosslinked to fibrin. Effective fibrinolysis therefore requires further binding of plasminogen, and this is promoted by at least two mechanisms: (1) partial digestion of fibrin increases the amount of plasminogen which may be bound; (2) cleavage of plasminogen by newly formed plasmin converts native glu-plasminogen (with glutamic acid as N-terminal
amino acid) to lys-plasminogen (with N-terminal lysine). Lys-plasminogen binds more avidly to fibrin. Fibrinolysis is thus localised to the site of a fibrin clot by the binding of active components, and by the inactivation (by α2 antiplasmin and PAI respectively) of any plasmin or tPA diffusing from the fibrin surface. Degradation of cross-linked fibrin by plasmin yields a series of fragments ranging in molecular size from the smallest, D dimer (molecular weight 190 x 10^3), to the very large X oligomers with molecular weights of about 2 x 10^6 [39].

4) Endothelium.
Vascular endothelium regulates haemostasis by a number of powerful mechanisms. Under normal conditions a continuous biocompatible lining to the vasculature is maintained which opposes platelet activation and intravascular coagulation. In certain prothrombotic states, activation of the endothelium may occur following the release of endotoxin, interleukin-1 (IL-1) or tumour necrosis factor (TNF), and this may change the balance of endothelial activities so that it promotes thrombosis [40]. The regulatory properties of the endothelium may therefore be divided into those that are antithrombotic, and those that are prothrombotic.

a) Antithrombotic properties of the endothelium.
Platelets Endothelial cells oppose platelet adhesion and aggregation by electrostatic repulsion (caused by their surface negative charge) [41], and by the synthesis of ADP degrading enzymes [42], prostacyclin (PGI₂) [43], endothelium-derived relaxing factor (EDRF) [31] and the lipoxygenase-derived chemorepellent substance 13-hydroxyoctadecadienoic acid [44]. In addition, both PGI₂ and EDRF possess potent vasodilatory properties.

Coagulation Blood coagulation is inhibited by two powerful activities located on the endothelial surface. The first is the presence in the extracellular matrix of sulphated glycosaminoglycans, which promote the inhibition of thrombin by antithrombin III [45] and heparin cofactor II [46]. The second is synthesis of thrombomodulin and protein S, cofactors in the activation by thrombin of the natural anticoagulant, protein C [47]. Activated protein C goes on to degrade and inhibit factors VIII and V, thus suppressing the coagulation
enzyme system. Thrombin loses its activity upon binding to thrombomodulin, and this represents a further antithrombotic property. Fibrinolysis Endothelial cells promote fibrinolysis by secreting tPA [48] as well as by participating in the activation of protein C (see above), which degrades PAI [49].

b) Prothrombotic properties of the endothelium.

Platelets Endothelial cells synthesise proteins such as vWF, fibronectin, collagen type IV and thrombospondin, which may participate in platelet adhesion. When activated, they may also produce PAF [50] and thromboxane [51], as well as receptors for fibrinogen binding [52] and leukocyte adhesion [40].

Coagulation The endothelial surface, like that of the platelet, can support the binding and interaction of coagulation enzyme complexes, and may thus facilitate the production of thrombin [53]. Factor V in these complexes may be supplied by the endothelial cell itself. When activated, endothelium may also promote coagulation by expressing tissue factor [54].

Fibrinolysis The endothelium is the major site of synthesis of the type 1 plasminogen activator inhibitor (PAI-1) which inhibits both tPA and uPA [36].

5) Blood Rheology.

The resistance to flow of a fluid is a measure of its viscosity. Clinical studies of blood viscosity have demonstrated its importance in diseases in which impairment of blood flow leads to ischaemia and thrombosis [55,56].

Determinants of blood viscosity The most important single determinant of blood viscosity is the haematocrit [57], the influence of which is stronger at lower shear rates [58]. Plasma viscosity, determined by temperature and plasma proteins, is an important component in blood viscosity. The contribution of any individual plasma protein depends upon its concentration, molecular weight and molecular assymetry. Thus albumin has little effect on plasma viscosity, whereas fibrinogen, α-2 macroglobulin and IgM all contribute significantly.

Red cell deformability is a further determinant of whole blood viscosity, especially when measured at high shear rate. A number of
factors influence erythrocyte deformability, including cell shape and size, internal viscosity and cellular metabolic state [59]. Under conditions of high shear, red cell membranes deform and rotate about their contents in the direction of flow, and thus reduce the resistance of blood to flow [60].

Blood behaves rheologically as a "non-newtonian" fluid, in that its viscosity varies as a shearing force is applied to it. At low shear rates (below about 50s⁻¹) red cell aggregation causes viscosity to rise exponentially. This aggregation is brought about by plasma proteins (mainly fibrinogen) which adsorb to the cell surface, reduce mutual electrostatic repulsion and link the cells together [58].

Rheology and blood flow. The Poisuelle equation predicts that an increase in blood viscosity will reduce flow, unless compensated for by vasodilation or increased driving pressure. Organ perfusion studies have shown that blood hyperviscosity does indeed result in reduced flow, but that this reduction is less than predicted, suggesting the existence of compensatory mechanisms.

Resistance to blood flow is minimized in two main ways:

1) Shear rates in all vessels are high enough to ensure minimum viscosity.

2) In the microcirculation, the haematocrit, and hence also the viscosity, are lower than those in larger vessels (the Fahraeus - Lindqvist effect) [61,62]. This is due to "plasma skimming" at the capillary entrance, and also to axial migration of red cells so that they traverse the centre of the capillary surrounded and lubricated by a low viscosity zone of plasma. As vessel diameter approaches that of the red cell, the cells flow in single file. The major rheological determinants of blood flow in the microcirculation are therefore those of plasma viscosity and cellular (erythrocyte and leucocyte) deformability [56].
CHAPTER 3 THE BLEEDING TENDENCY OF URAEMIA

Ecchymoses, purpura, epistaxis and bleeding from venupuncture sites are all common manifestations of the uraemic haemorrhagic tendency. More serious and, in some cases, life threatening complications may also arise. These include gastrointestinal bleeding, haemopericardium, haemorrhagic pleural effusion, retroperitoneal bleeding, subdural haematoma and bleeding into the anterior chamber of the eye [63]. Although it is generally agreed that the haemorrhagic tendency of uraemia is due to a defect in the interaction of platelets with the blood vessel wall, the precise nature of this defect is still uncertain. Many different aspects of platelet function have been reported to be abnormal in CRF, and the only individual laboratory test which has proved to be of value in the prediction and monitoring of uraemic bleeding is the skin capillary bleeding time [64]. It must therefore be concluded that the bleeding tendency of uraemia is the result of a number of abnormalities acting together to reduce haemostatic function. Current knowledge of the pathogenesis of platelet dysfunction in uraemia will therefore be reviewed under the following headings:

1. Thrombocytopenia.
3. Platelet biochemical abnormalities.
4. Abnormalities of the interaction of platelets with the vessel wall.
5. The role of anaemia in uraemic bleeding.
6. The role of drugs in uraemic bleeding.

1. Thrombocytopenia.
Platelet counts in CRF are usually within the normal range [65,66], but mild thrombocytopenia is sometimes reported. A recent study found mean platelet counts of 175 x 10^9/l and 181 x 10^9/l in haemodialysis patients and non-dialysed CRF patients respectively, compared with 253 x 10^9/l in healthy controls. 31% of the haemodialysis patients had counts lower than 150 x 10^9/l [67]. Although the difference from controls was statistically significant, this level of thrombocytopenia is unlikely to compromise haemostasis to any important degree.
Abnormalities of platelet aggregation.

Platelet aggregation in uraemia is a vexed question. Reduced aggregation in response to thrombin and ADP has been reported [68,69], and Di Minno and colleagues showed threshold aggregating concentrations of collagen, ADP and adrenaline two to three times higher than normal in uraemic haemodialysis patients [70]. In a further report, the reduction in ADP- and collagen - induced aggregation in uraemia was attributed to an impairment in the exposure of fibrinogen receptors on the platelet surface after stimulation with these agonists. Correction of this defect by the simultaneous addition of arachidonic acid suggested to these authors that a failure to liberate endogenous arachidonate might explain uraemic platelet dysfunction [71].

Defective platelet aggregation was, in some cases, found to be corrected by dialysis therapy. Lindsay and coworkers published a series of papers during the 1970's, in which they demonstrated reduced platelet aggregation to ADP in patients with serum creatinine concentrations greater than 6 mg/dl (530 umol/l), but normal aggregation in patients with serum creatinine levels below this value [65]. An increase in the duration [65] or the frequency [72] of haemodialysis, or the use of peritoneal dialysis [73], was reported to correct ADP-induced aggregation. These findings suggested a relatively simple explanation of uraemic bleeding, in which the accumulation of dialysable toxins in CRF caused inhibition of platelet aggregation and hence of haemostasis. A number of dialysable substances retained in renal failure had been postulated as the cause of uraemic platelet dysfunction. These included urea [66], creatinine, methylguanidine, phenolic compounds [74] and guanidinosuccinic acid [75]. Horowitz concluded that only the latter two met the most rigid criteria for causative agents [76], but in the light of current knowledge, the experiments performed with these compounds are suspect, due to the use of imidazole buffered saline as a diluting buffer. Imidazole is now known to be an inhibitor of thromboxane synthetase, and the results obtained in these studies are therefore difficult to interpret. Other workers have looked at the possible role of the less easily dialysable "middle molecules" in impairing platelet aggregation in renal failure [77].

The picture of abnormal platelet aggregation in uraemia resulting from
the action of retained metabolites was further confused by later studies in which haemodialysis was found to correct in vitro platelet function only partially [78], or not at all [79]. Other workers failed to demonstrate defective platelet aggregation in haemodialysis patients [80], or else found platelet aggregation to be enhanced [81]. In a study of the effects of arachidonic acid on uraemic platelets, Remuzzi and coworkers found aggregation and malondialdehyde production to be enhanced in response to low agonist doses, but depressed in response to high doses [82]. This paradox was not explained by the authors, but the paper was important in that it highlighted the difficulties involved in interpreting in vitro platelet function results. Clearly, conclusions drawn from platelet responses to a restricted range of agonist types and doses might be subject to error.

What other factors, apart from the choice of aggregating agent, might explain the conflicting results of platelet aggregation studies in uraemia? In many of the papers involved there is no indication that technical considerations, such as the need to standardize the platelet count and the citrate anticoagulant concentration of the test samples, had been taken into account. Differences in the patient groups studied may also have confused the results obtained. For example, it is important to consider the influence on platelet studies of the underlying cause of renal failure, since platelet function may be influenced by active immunological disease [16], diabetes [83] and the nephrotic syndrome, in which platelet hyperaggregability arising from both thromboxane dependent [84] and thromboxane independent mechanisms [85] has been found. The effects of platelet contact with the dialysis membrane [86], of heparinization [87] and of lipid disturbance in uraemia [88] must also be considered. Some studies of haemostasis in uraemia have been performed on patients who were actually suffering from haemorrhagic episodes, whereas others have looked at patients without these symptoms.

(3) Platelet Biochemical Abnormalities.
a. Granule contents.
Dense granule content was measured by both Eknoyan [89] and Di Minno [70], and found to be reduced compared with that in platelets from healthy subjects. Eknoyan reported a reduction in platelet ADP
and serotonin, and an increase in the ratio of ATP to ADP, results characteristic of a storage pool defect. Di Minno and colleagues also showed a reduction in platelet adenine nucleotides, and in addition showed that the release of platelet ATP following stimulation with thrombin was subnormal. These authors suggested that, as well as a storage pool defect, uraemic platelets exhibit a defect in the mechanism involved in granule secretion. This has recently been supported by the findings of Kyrie et al, who found that the release of the platelet α-granule protein, beta thromboglobulin, was impaired in haemodialysis patients [90].

b. Carbohydrate metabolism.
Glucose utilization by platelets is inhibited by certain substances which are retained in renal failure. Of these, o-hydroxyphenolic acid, guanidinosuccinic acid, and an unidentified peptide of middle molecular weight (about 1000 daltons), caused inhibition at concentrations typical of those found in the plasma of CRF patients [91]. Interruption of platelet energy metabolism in uraemia may therefore contribute to haemostatic dysfunction.

c. Cyclic AMP and adenylate cyclase.
Intra-platelet cAMP is elevated in haemodialysis patients [92], and this may be related to an abnormality in the regulation of adenyl cyclase. Jacobsson and colleagues demonstrated that, in the uraemic platelet membrane, this enzyme had an increased capacity for activation [93]. The density and affinity of catecholamine receptors, which when stimulated cause depression of adenyl cyclase activity, were normal. The authors therefore concluded that a defect in an inhibitory nucleotide binding protein might explain their findings.

d. Parathyroid hormone and platelet calcium.
A failure by the kidney to hydroxylate vitamin D at the 1- position reduces calcium absorption from the gut in CRF, resulting in secondary hyperparathyroidism and excessive calcium deposition in various tissues [94]. Gura et al found that platelet calcium content was increased in uraemia, and that this abnormality was corrected by treating the patients with 1-α vitamin D [95]. The authors speculated that parathyroid hormone (PTH) might cause the elevation of intraplatelet calcium, and that the effect of 1-α vitamin D might be mediated by a suppression of PTH levels.
Bovine PTH inhibited in vitro platelet aggregation in response to various stimuli, whereas the terminal synthetic fragment 1 - 34b PTH was ineffective [96]. The mechanism of platelet inhibition was not elucidated, but did not appear to involve elevation of intra-platelet cAMP.

On the basis of these studies, a role for PTH in uraemic bleeding was postulated, but subsequent results have not supported this theory. Docci et al found no relationship between platelet aggregation and the degree of secondary hyperparathyroidism in uraemic subjects, nor any change in platelet responses following effective treatment with 1,25-dihydroxycholecalciferol [97]. Leithner and colleagues found inhibition of in vitro platelet aggregation with bovine parathyroid extracts, but not with synthetic human PTH, and concluded that the platelet inhibitory activity previously reported was due to contaminants in the bovine PTH preparation [98]. This group also found no change in ex vivo platelet aggregation following surgical correction of primary hyperparathyroidism.

Abnormalities have been demonstrated in cytoplasmic calcium responses following stimulation of uraemic platelets loaded with the calcium-sensitive probes indo-1 and aequorin [99]. In uraemic patients with greatly prolonged bleeding times, agonist-stimulated elevation of intra-platelet free calcium was smaller than that in platelets from uraemic subjects with less prolonged bleeding times or from normal controls. This appeared to be an intrinsic platelet defect, since it was not corrected by incubation in normal plasma, nor was it transferable to normal platelets by incubation in uraemic plasma.

e. Platelet arachidonic acid metabolism.

Abnormalities in the metabolism of arachidonic acid have been reported in uraemic platelets, but there are inconsistencies between the findings of different authors.

The first studies were performed by Remuzzi et al, who found subnormal generation of malondialdehyde, an arachidonic acid derivative whose production parallels that of thromboxane, after stimulation of uraemic platelets with maximal doses of arachidonic acid, collagen and thrombin [100]. They subsequently reported that malondialdehyde generation in arachidonic acid stimulated uraemic platelets could be either depressed, or enhanced in comparison with
healthy controls, depending upon the strength of the stimulus [82].

The authors discussed the possible role of the enrichment of plasma albumin with free fatty acids in influencing the metabolism of arachidonic acid by uraemic platelets, but failed to produce a conclusive explanation for these paradoxical findings. No correlation was found by Fernandez et al between reduced platelet malondialdehyde production and bleeding time prolongation [101].

In a comprehensive study published in 1983, Remuzzi and colleagues confirmed their previous findings by showing again a divergence in the comparative production of immunoreactive thromboxane (TxB2) by uraemic and normal platelets in response to different doses of arachidonic acid [102]. In addition, the synthesis and release of the cyclooxygenase products TxB2, PGE2 and 6-keto-PGF1α were all subnormal when measured in clotting blood. Platelet levels of the enzyme PGH synthetase were similar in uraemics and normal controls, as was the platelet TxA2 receptor, judging by normal responsiveness to the TxA2 mimetic U46619. The authors concluded that uraemic platelets were characterized by a functional cyclooxygenase defect. This study has been widely quoted in the literature, and although its conclusions are consistent with the findings of some authors, for example those of Smith and Dunn [103], they are inconsistent with those of others. Winter et al found reduced TxB2 generation in response to arachidonic acid in some conservatively treated uraemic patients, but haemodialysis patients produced increased amounts of TxB2 [104]. Di Minno and coworkers found TxB2 production to be reduced in response to thrombin and collagen, but normal in response to arachidonic acid, and concluded that a defect existed in the release of arachidonate from platelet membrane phospholipids, rather than at the level of cyclooxygenase [70]. Bloom et al also disagreed with the concept of a functional cyclooxygenase defect [105]. Their evidence pointed to a more specific abnormality in the response of uraemic platelets to thrombin.

f. Platelet membrane glycoproteins

Andrassy and coworkers examined platelet membrane glycoproteins by both silver staining and lectin binding to platelet lysates following gel electrophoresis and Western blotting, and showed that in platelets from CRF patients there was a reduction in glycoproteins Ib and
It should be noted, however, that these interesting findings have only been published in abstract form, and have not yet been confirmed by others.

(4) Abnormalities of the interaction of platelets with the vessel wall.

a. Platelet adhesion.
Platelet adhesion has been consistently found to be subnormal, whether measured by retention on glass beads [66,78,107] or by deposition of platelets onto subendothelium using Baumgartner's perfusion system [107]. This abnormality did not, however, correlate with prolongation of the bleeding time nor with levels of retained metabolites, nor was it fully corrected by haemodialysis therapy [78].

b. Vascular production of prostacyclin (PGI2).
Disturbance in platelet/vessel wall interactions in uraemia may be due, not only to platelet dysfunction, but also to abnormality of the vessel wall. Specimens of venous tissue from patients suffering from acute and chronic renal failure [108] and from rats with experimentally-induced renal failure [109] generated increased amounts of PGI2-like activity; this abnormality was corrected on restoration of normal renal function. Increased levels of PGI2 (measured as its stable metabolite 6-keto-PGF1α) were also demonstrated in uraemic blood emerging from standardized punctures made to the microvasculature of the skin, made to determine the bleeding time [90]. This enhancement of PGI2 production is thought to result from an increase in the plasma level of a factor which stimulates PGI2 release [110]. The stability of PGI2 in uraemic plasma is normal [109].

The relevance of these findings to uraemic bleeding has not been demonstrated conclusively, and a recent study using experimental rats made uraemic by renal ablation showed that treatment with conjugated oestrogens produced a significant shortening of the bleeding time, but without any change in the enhanced generation of PGI2 [111].

c. Factor VIII / von Willebrand complex (FVIII/vWF) in uraemia.
Clinical studies have shown that the bleeding tendency of uraemia may be corrected for some hours by the administration of cryoprecipitate [112], a plasma fraction containing high concentrations of fibrinogen, fibronectin and the FVIII/vWF complex, or by the administration of desamino-8-D-arginine vasopressin (DDAVP) [113], a synthetic analogue
of vasopressin which stimulates the release from vessel wall of a number of products, including vWF in the form of high molecular weight multimers [114]. Shortening of the uraemic bleeding time may be obtained for more prolonged periods by the administration of conjugated oestrogens [115].

One possible common factor underlying the success of these diverse treatments might be the alteration of the quantity or quality of vWF in the plasma of the recipient. The factor VIII/vWF complex has been extensively investigated in uraemia, although there are some inconsistencies between the results of different studies. Mean plasma concentrations of VIIIc (factor VIII coagulant activity) and of vWFAg have always been found to be higher in uraemic subjects than in healthy controls [116-121]. Elevation of VIIIc and vWFAg in CRF may result from vascular damage [122], from impaired clearance by the reticulo-endothelial system [123] or from an acute phase response [124]. The presence in CRF of abnormal forms of the VIIIc molecule, suggestive of proteolytic degradation, is interesting, but of uncertain pathological significance [125].

Plasma vWF activity, measured by its ability to support ristocetin-induced platelet agglutination, was reported by two groups to be reduced [116,119], but increased concentrations of this activity have been found in the majority of studies [117,118,120,121]. These results, together with the presence of normal amounts of vWFAg on the vascular intima of CRF patients [126], argued against a lack of plasma vWF being a cause of uraemic bleeding.

The multimeric structure of vWF, as well as its circulating concentration, is important in the functional activity of this protein [127], and this aspect of the vWF molecule has also been investigated in uraemic patients. A vWFAg component with increased mobility found on crossed immunoelectrophoresis of uraemic plasma was suggested to indicate an abnormal factor VIII molecule [121,128], perhaps due to an increased proportion of small multimers, or to the effects of proteolytic degradation of the molecule. Analysis of vWF by SDS agarose electrophoresis, however, found no abnormality in multimer pattern in CRF [113,115].

In a comprehensive investigation of platelet adhesion in blood from uraemic patients with bleeding symptoms, Castillo and colleagues found
evidence of both platelet and plasma abnormalities [107]. Ristocetin-induced agglutination of uraemic platelet rich plasma was abnormal, and was not corrected when the platelets were suspended in normal plasma, indicating that the platelets were unable to interact normally with vWF. Adhesion of uraemic platelets with subendothelium was also evaluated using Baumgartner's perfusion method. Not only was the interaction of uraemic platelets with subendothelium reduced in the presence of both uraemic and normal plasma, but the adhesion of normal platelets was also reduced in the presence of uraemic plasma. These experiments suggested that, in uraemia, both platelets and plasma fail fully to support the adhesion of platelets to subendothelium. The nature of the abnormality is still uncertain. Recent data indicate normal binding of vWF to GpIb on the uraemic platelet surface [129], but a defect in the interaction of vWF with GpIIb/IIIa [130]. Further, an abnormality of the vWF molecule might be induced that is not evident on SDS agarose electrophoresis, but which alters the ability of the molecule to bind to receptors on the subendothelium.

Is the factor VIII/vWF complex involved, then, in the correction of uraemic bleeding by cryoprecipitate, DDAVP and conjugated oestrogens? This is still unknown, but it is unlikely that these agents work simply by increasing the plasma concentration of this complex. In their report of the effects of DDAVP on uraemic haemostasis, Mannucci and colleagues commented upon the temporal association between the appearance of very large molecular weight vWF multimers and the shortening of the bleeding time. The effects of DDAVP may, therefore, result in part from qualitative changes in vWF protein. More recently it has been suggested that elevation of plasma catecholamine concentrations following DDAVP administration may also contribute to its haemostatic effects [131].

(5) The role of anaemia in uraemic bleeding.
Patients with severe renal failure are almost always anaemic. Studies by Hellem in the early part of the 1960's demonstrated a role for red cells in promoting platelet adhesiveness and haemostasis, and showed shortening of the bleeding time in anaemic patients following red cell transfusion [132]. More recent work has shown that erythrocytes may
enhance platelet function by chemical mechanisms, such as the release of ADP [133] and the binding and inactivation of PGI₂ [134], as well as by a physical mechanism involving the displacement of platelets towards the vessel wall by the action of flowing erythrocytes [135]. Livio and coworkers used this information to devise a treatment for the bleeding tendency of uraemia. They found that transfusion of washed red cells into anaemic haemodialysis patients brought about a shortening of the previously prolonged bleeding time [136]. This was accomplished without any significant change in platelet biochemical indices, suggesting that the correction of the bleeding time was solely due to the increase in the haematocrit. A significant negative correlation was demonstrated between bleeding time and haematocrit in the uraemic population, and the authors concluded that elevation of the haematocrit to 30% or above allowed successful management of the uraemic haemorrhagic tendency.

These results were confirmed in a later study by Fernandez et al [101], who measured a wide variety of haemostatic indices, but concluded that anaemia was the main determining factor of the prolonged bleeding time in uraemic patients.

More recently, the correction of the anaemia of renal failure has been achieved by the use of recombinant human erythropoietin. Moia and colleagues found a shortening of the bleeding time and an improvement of bleeding symptoms in patients treated in this way, and these changes were paralleled by an increase in platelet adhesion to subendothelium tested in an in vitro perfusion system [137]. A second study of the haemostatic effects of erythropoietin treatment were consistent with these results, although in this case an increase in ex vivo platelet aggregation responses was reported [138].

Thus anaemia appears to be an important, perhaps the most important, pathogenetic factor in uraemic bleeding. It should be noted however, that other mechanisms are operating, since bleeding times are longer in uraemic patients than in non-uraemic patients with comparable degrees of anaemia [136], and also since, in many patients, the bleeding time is not completely corrected even after successful treatment of anaemia.
The role of drugs in uraemic bleeding.

Haemostatic dysfunction may be an unwanted side effect associated with drug treatment, and there is evidence that patients suffering from CRF may be at increased risk of this type of complication. In a recent review, Andrassy highlighted the risk of bleeding associated with the accumulation of betalactam antibiotics in CRF. Drugs such as penicillins and cephalosporins may promote haemorrhage by inhibiting both platelet function and thrombin activity and by interfering with the synthesis of vitamin K dependent coagulation factors. [139]. Excessive prolongation of the bleeding time is also seen after the administration of aspirin to uraemic patients, who appear to be much more sensitive than healthy subjects to the antihaemostatic effects of this drug [140]. Gaspari et al found a temporal dissociation in CRF patients between bleeding time prolongation and inhibition of platelet TxB\textsubscript{2} generation following aspirin, and concluded that the effect was due neither to differences in the pharmacokinetics of aspirin, nor to increased sensitivity of cyclooxygenase enzyme in CRF, but that it depended upon the presence of aspirin in the blood [141].
CHAPTER 4 THE ROLE OF HAEMOSTASIS IN THE PROGRESSION OF CHRONIC RENAL FAILURE

(1) The progressive nature of CRF.
Glomerular injury is the most common cause of progressive renal failure, and may be initiated by a variety of mechanisms, either immunological or non-immunological. Once established, CRF shows a tendency to progress to end-stage renal failure. During the 1970's it was demonstrated that this progression occurred in an orderly fashion which could be mathematically defined. Thus Mitch et al [142] and Rutherford et al [143] found a linear relationship between duration of renal disease and either the reciprocal or the logarithm of the plasma creatinine concentration, and Jones and colleagues later showed that this relationship also held in patients with diabetic nephropathy [144]. These findings implied that functioning nephrons were being lost at a constant rate. The rate of progression of CRF differed greatly between patients, and could not be predicted by the nature of the initiating injury. This evidence, together with animal studies showing that renal failure could progress even after the original insult to the kidney had been removed, suggested that, once a certain critical degree of renal failure had been reached, deterioration continued independently of the original disease, by mechanisms arising from adaptations within the injured kidney. Although this hypothesis is now widely accepted, it may not constitute a complete explanation of progressive CRF. A recent British study of 108 patients with mild to moderate CRF showed differences in the mean rates of progression depending upon the original cause of renal failure, suggesting that continued activity of the underlying disease may contribute to progression [145].

(2) FSGS - the characteristic lesion of progressive renal disease.
In man and experimental animals, progressive CRF is characterized clinically by a decline in GFR, and by the development of proteinuria and hypertension. Histologically, the common end point is the development of focal and segmental glomerulosclerosis (FSGS)[146], the structural features of which have been recently reviewed by Olson and Heptinstall [147]. Adhesions develop between portions of the
glomerular tuft and Bowman's capsule and protein resorption droplets appear within the epithelial cells. Capillaries are dilated and may form microaneurysms, and become occluded by platelet and fibrin thrombi and by hyaline lesions containing lipid. There is an increase in mesangial matrix and cellularity. Arterioles and interlobular arteries may show fibrinoid necrosis and medial hypertrophy, lesions which probably arise as a consequence of hypertension. By electron microscopy, epithelial cell foot processes can be seen to become coarsened and to separate from the glomerular basement membrane (GBM). Separation from the GBM of endothelial cells also occurs; allowing an accumulation of fibrin and hyaline material. In addition, platelet aggregates are particularly seen in these areas where the endothelium has lifted away from the GBM.

Glomerulosclerosis may be focal (involving only a minority of glomeruli) and segmental (involving only segments of affected glomeruli), but may become diffuse, involving all glomeruli.

**FSGS in Man.** FSGS may occur as a specific lesion early in the course of the nephrotic syndrome [148], but more commonly develops in the later stages of a variety of human renal diseases, including both primary glomerular diseases and systemic diseases. These systemic disorders may be immunological (for example SLE), metabolic (diabetes mellitus), vascular (malignant hypertension) or hereditary (Fabry's disease) [146]. The slow development of FSGS appears also to be a normal feature of ageing [149].

**FSGS in experimental animals.** Research into the mechanisms of progressive renal failure has been greatly facilitated by the availability of a useful animal model. This model, which employs the reduction of functioning renal mass either by removal or infarction, was used as early as the 1930's to produce and study experimental renal failure [150]. More recently it has been shown that FSGS is the characteristic lesion arising either spontaneously in Wistar rats [151], or more acutely in Wistar rats subjected to extreme renal ablation, either by sub-total nephrectomy [152-154], or by unilateral nephrectomy together with partial infarction of the contralateral kidney [155]. Similar pathological changes arise in experimental models of hypertension [156], diabetes [157] and aminonucleoside administration [158].
(3) The pathogenesis of FSGS.

FSGS shows striking similarities to atherosclerosis [159], and a variety of factors have been proposed to explain the development of FSGS in remnant kidneys. These include both (i) non-haemostatic and (ii) haemostatic factors, and for the purpose of this discussion they will be described under these two headings. Non-haemostatic factors will be dealt with briefly before reviewing more fully the evidence for the involvement of haemostatic mechanisms in progressive glomerular injury.

(i) Non-haemostatic factors in the pathogenesis of FSGS.

a) Hyperfiltration.

The presence of superficial glomeruli in the Munich-Wistar rat, together with the development of micropuncture techniques, allowed the direct measurement of haemodynamic changes induced by renal ablation within individual nephrons. The results of these studies confirmed earlier reports of an increase in GFR in remnant kidneys [160], by showing an increase in single nephron GFR mediated by an elevation of both glomerular plasma flow and glomerular hydraulic pressure [161]. Similar haemodynamic changes were observed in diabetic rats [162], and intrarenal hypertension was postulated to be the underlying cause of the initiation and progression of glomerular injury in diabetic and non-diabetic glomerulopathies [163].

It is well recognised that systemic hypertension may accelerate the progression of CRF. Injury to small arteries and arterioles within the kidney is a common finding in all experimental models of hypertension, but the degree of glomerular injury appears to depend largely upon the degree to which systemic blood pressure is transmitted by the afferent arteriole [164]. Thus glomerular injury was not reduced by triple therapy with a combination of hydrochlorothiazide, reserpine and hydralazine (which also failed to reduce glomerular hydraulic pressure) [165], whereas treatment with enalapril reduced both glomerular capillary pressure and glomerular damage in rats with both reduced renal mass [166] and streptozotocin-induced diabetes [167]. Both regimens lowered systemic blood pressure equally.

Thus the case is strong for the involvement of glomerular hypertension and hyperfiltration in the pathogenesis of progressive glomerular injury. It should be noted, however, that this hypothesis
may require modification in the light of certain discrepant results [168].

b) Dietary factors.
The influence of diet on the course of experimental renal disease has long been recognised, and the usefulness of dietary manipulation in the clinical management of renal disease has excited interest in recent years [169]. Most attention has been given to the role of protein intake, since it is evident that the development of FSGS may be accelerated by an increase, and retarded by a decrease in protein intake [170]. Dietary protein may influence the course of renal disease by its effects on glomerular haemodynamics. Dietary intake of both phosphorus and of lipids may also be important.

c) Genetic factors.
Differences in the susceptibility of both experimental animals, and human patients, to develop progressive FSGS have suggested that genetic factors may influence this process. These may include genetic differences in structural, metabolic and haemostatic factors, as well as in glomerular haemodynamics [171].

d) Lipids.
Lipid abnormalities may arise in renal disease, particularly in response to proteinuria, and FSGS may be seen in the kidneys of both obese Zucker rats and of morbidly obese patients. Moorhead and colleagues proposed an hypothesis that lipid disturbance might mediate the progression of CRF by increasing the permeability of the GBM, accumulating in the mesangium and stimulating mesangial cells to proliferate [172]. In support of this hypothesis, treatment of hyperlipidaemia has been shown to reduce glomerular injury in obese Zucker rats [173] and in rats following renal ablation [174].

e) Hypertrophic factors.
Reduction in renal mass produces structural, as well as functional hypertrophy [175] and experimental models associated with severe glomerular injury also lead to an increase in glomerular volume [166]. Recent experimental evidence has shown a strong association between glomerular hypertrophy and the development of FSGS, leading to suggestions that such hypertrophy, rather than glomerular haemodynamic changes, is responsible for the development of FSGS [176,177]. It is not yet certain whether hypertrophy is due to the
action of some specific factor(s), or to an increase in the tissue sensitivity to normal circulating growth factors.

(ii) Haemostatic mechanisms in the pathogenesis of FSGS.

a) Evidence of platelet and fibrin deposition in FSGS.

The morphological features of FSGS, described above, give a clear basis for postulating the involvement of the haemostatic system in progressive glomerular sclerosis. Endothelial injury, perhaps arising as a result of increased intraglomerular blood pressure and flow, is associated with the accumulation of hyaline material, as well as frank platelet and fibrin thrombi within the affected capillary lumina. In diabetic patients with moderate diffuse glomerulosclerosis, 70% of glomeruli showed fibrinogen related antigen along the capillary basement membrane and in the mesangium [178].

b) Experimental evidence from the renal ablation model.

Having noted these appearances in experimental rats, Purkerson and co-workers went on to perform a series of studies in which they investigated the effects of anticoagulant or antiplatelet treatment on the renal ablation model of CRF. Administration of heparin to rats with 2/3 infarction of one kidney and contralateral nephrectomy, was effective in reducing blood urea concentrations, blood pressure and the proportion of abnormal glomeruli, compared with untreated rats [155]. These results were later confirmed by the demonstration that heparin, in a dose that caused a sustained prolongation of both clotting times (APTT and PT), and bleeding times, was able to prevent progressive renal failure, hypertension and glomerular injury in the renal ablation model [179]. In this study it was also shown that anticoagulation with coumadin, at doses that prolonged the prothrombin time and bleeding time, led to amelioration of progressive renal failure and hypertension, although the degree of protection was less than with heparin.

Similar protection against progressive renal failure, proteinuria, hypertension and glomerular injury was subsequently demonstrated with the use of the thromboxane synthetase inhibitor OKY 1581 [180]. This drug inhibited ex-vivo platelet aggregation in response to collagen and arachidonic acid for up to 7 hours (ADP-induced aggregation was unaffected). Of particular interest was the finding
that amelioration of progressive renal disease occurred in the face of increased calculated values for single nephron plasma flow and GFR in OKY 1581 treated rats, in other words increased hyperfiltration and hyperperfusion! The protection against glomerular injury was not due to a reduction in the degree of renal hypertrophy, nor was it explained by the fall in blood pressure, since the degree of protection was greater than that shown in previous similar trials using antihypertensive drugs. The authors concluded that the inhibition of platelet aggregation and intraglomerular thrombosis might represent the most important mechanism by which the benefit was achieved.

Protection against progressive renal injury was also demonstrated by Yamashita et al using the thromboxane synthesis inhibitor OKY-046 in the Dahl-S rat model [181].

More recently, Zoja and coworkers have confirmed the efficacy of anti-platelet therapy in rats with reduced renal mass [182]. They showed that ticlopidine, a drug which inhibits platelet function by a broad spectrum of pharmacological actions, prolonged the bleeding time, and inhibited ex-vivo aggregation in response to ADP and arachidonic acid in experimental rats whose functioning renal mass had been reduced by approximately 11/12. Associated with this anti-platelet activity, ticlopidine significantly ameliorated the hypertension, proteinuria, FSGS and renal functional impairment caused by the loss of renal tissue, a result which clearly strengthens the argument that intraglomerular thrombosis is a major factor in the development of FSGS. This study was extended to include a further group of rats who received GR 32191, a thromboxane receptor antagonist with much more restricted anti-platelet action. In these rats, treatment with GR 32191 failed to protect the remnant kidney, from which the authors concluded that thromboxane was not of direct pathogenetic importance in this model.

The renal ablation model was used by Olson to compare the protective effects of unfractionated heparin (UFH) with those of a low molecular weight heparin fraction (LMWH) characterized by increased anti-factor Xa activity, but reduced inhibitory activity against other serine proteases [183]. UFH reduced hypertension, proteinuria and renal damage, but LMWH failed to produce such benefit. Although best known as an anticoagulant agent, heparin has a number of other properties
which might participate in the prevention of renal damage. These include enhancement of negative charge on the vascular wall, lowering of blood pressure by the inhibition of renal kallikrein, and suppression of the proliferation of smooth muscle and of mesangial cells. These properties might all be potentially beneficial in the prevention of renal damage. The difference in the efficacy of UFH and LMWH shown by Olson might be due to discrepancies not only in their anticoagulant activity, but also in these other properties. This is supported by the finding that N-desulphated heparin, which has no anticoagulant activity, protected against glomerular injury in rats following renal ablation [184].

c) Evidence from clinical studies.
A small number of clinical trials have addressed the question of haemostatic involvement in progression of human CRF. In membranoproliferative glomerulonephritis (MPGN), treatment with either warfarin (at a dose which maintained the prothrombin time 1.5 -2 times the control value) and dipyridamole (300 - 400 mg/day) [185], or with aspirin (975 mg/day) and dipyridamole (225 mg/day) [186] was successful in stabilizing renal function. Children with MPGN treated with a combination of immunosuppression (corticosteroids and azathioprine) and anticoagulation (heparin followed by warfarin and dipyridamole) and followed for between 2 and 5 years, were well in 6 out of 10 cases, a result which was better than would be expected from the natural history of the disease [187]. In contrast, a controlled trial of antiplatelet therapy (aspirin 650 mg/day plus dipyridamole 75 - 225 mg/day) conferred no significant benefit over treatment with placebo (vitamin B) in IgA nephropathy [188]. In pediatric renal disease, dipyridamole (4 - 10 mg/kg daily) given alone reduced 24 hour protein excretion in 53% of patients [189]. This antiproteinuric effect of dipyridamole has been confirmed by others, but was associated with a fall in filtration fraction that correlated positively with the reduction in protein excretion [190]. This effect of dipyridamole might therefore result from changes in glomerular haemodynamics, rather than from inhibition of platelet function.

d) Mechanisms of haemostatic activation in FSGS.
How might activation of the haemostatic mechanism occur during chronic renal failure? Purkerson and colleagues have suggested that
increased glomerular hydraulic pressure in the remnant nephron causes endothelial damage, with subsequent activation of platelets and coagulation pathways [180]. This is supported by the fact that purified glomerular basement membrane is capable of activating platelets [191], and by ultrastructural evidence showing the association of platelet and fibrin thrombi with sites of endothelial injury (see above). In a recent review of the role of platelets in progressive FSGS, Clark and Naylor have proposed a number of additional mechanisms by which haemostatic activation may occur in the microcirculation of remnant nephrons [192]. These may be summarized as follows: (1) Kinetic activation due to high plasma flow rates; (2) close cell contact within the glomerulus following ultrafiltration; (3) concentration of macromolecules (especially aggregated IgG) which act as agonists for platelet release; (4) glomerular release of fatty acids that stimulate platelets to produce thromboxane; (5) induction of glomerular procoagulant and subsequent activation of both platelets and coagulation by generated thrombin.

A further possible pathway of haemostatic activation may be through the activity of leukocytes, in particular of macrophages, which in the remnant glomerulus may be either resident or infiltrating [146]. Macrophages may themselves release, or may stimulate other glomerular cell types (such as endothelial or mesangial cells) to release, factors which have a potent role in the modulation of the haemostatic process. These include cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF), which stimulate the endothelium to express adhesion molecules, and to produce procoagulant, TxA2 and plasminogen activator inhibitor; in addition they suppress endothelial production of prostacyclin, tissue plasminogen activator and thrombomodulin [40]. Thus the overall effect is to reduce the antithrombotic, and promote the prothrombotic potential of the endothelium. Macrophages may also stimulate platelet aggregation and release by producing platelet activating factor (PAF)[193].

e) Mechanisms by which haemostatic activation might promote FSGS.

Once haemostatic activation has occurred within the remnant nephron, there are a number of ways in which the development of FSGS may be promoted. First, and most simple, is the occlusion of glomerular capillaries by platelet/fibrin thrombi, leading to ischaemic damage. In
addition, activated platelets can release a number of mediators which may then participate in the alteration of glomerular structure and function. The involvement of these mediators has been described in detail in recent reviews [16,146,194]; important examples of their effects are (1) the neutralization of glomerular basement membrane negative charge, either by platelet-derived cationic proteins such as PF4 [195], or by the action of a heparinase enzyme; (2) stimulation of mesangial cell proliferation by platelet-derived growth factor (PDGF)[196]; (3) contraction of mesangial cells by TxA2 with consequent reduction in glomerular surface area [197]; (4) inflammation (chemotaxis, complement activation, increase in vascular permeability) due to the action of mediators such as platelet activating factor (PAF), histamine, 5-hydroxytryptamine, PF4 and PDGF [16,193].

f) Possible role of blood rheology in FSGS.

Blood viscosity is elevated in patients with nephrotic syndrome [198] and IgA nephropathy [199], as well as in experimental acute renal failure [200]. Hyperviscosity in these conditions may be related both to elevated levels of plasma proteins, such as fibrinogen and immunoglobulins, and also to red cell abnormalities such as red cell aggregation in the nephrotic syndrome [201], and reduced deformability in chronic renal failure (CRF)[202]. Whether red cell deformability is abnormal in CRF before the introduction of regular dialysis therapy is uncertain, since reports vary [202,203]. Suggested causes for loss of deformability in CRF include elevation of intra-cellular calcium [204], and loss of membrane fluidity [205].

Studies in experimental animals [206] and in patients with either polycythaemia [207] or anaemia [208] have indicated a positive relationship between haematocrit and filtration fraction. Since the driving force for filtration is glomerular hydrostatic pressure, this correlation suggests that elevation of haematocrit (and consequently of blood viscosity) results in increased intra-glomerular pressure. In dogs, isovolaemic elevation of either whole blood, or plasma viscosity was followed by a fall in renal vascular hindrance, and an increase in plasma renin activity, while arterial blood pressure and renal blood flow remained constant [209]. This was interpreted as evidence that the flow impedance caused by blood hyperviscosity led to a compensatory renal vasodilation to increase glomerular hydrostatic
pressure and maintain flow, and that plasma renin activity rose in response to this vasodilation.

Micropuncture experiments have shown an increase in efferent, relative to afferent, arteriolar pressure following haematocrit elevation, resulting in an increase in glomerular perfusion pressure and, hence, in filtration fraction [206]. An increment in efferent vascular resistance may be caused by blood hyperviscosity following the loss of filtrate during the passage of blood through the glomerulus. Simpson has proposed an hypothesis that rheological disturbance, whether due to polycythaemia, plasma hyperviscosity or loss of erythrocyte deformability, leads to an exaggeration in this post-glomerular rise in blood viscosity and in vascular resistance within the efferent arterioles and post-glomerular venules [210,211]. In order to overcome this resistance, a further rise in afferent blood pressure is required, leading to glomerular capillary hypertension and thus to proteinuria and glomerular sclerosis. If this sequence of events actually occurs in vivo, then it is possible to make the following predictions: 1) that gross rheological disturbance would result in renal functional abnormalities, and 2) that, in patients with renal failure from any cause, abnormal blood rheology would promote proteinuria and progression of their disease. Evidence exists in support of the first prediction. Renal functional abnormalities are seen in patients with polycythaemia [207], plasma hyperviscosity syndrome due to Waldenstrom's macroglobulinaemia [212], and in conditions involving a loss of red cell deformability such as Sickle cell disease [213].

Two recent studies have looked at the effect of haematocrit elevation on the progression of renal failure. Garcia et al investigated the effect of increasing haematocrit with recombinant human erythropoietin (rHuEPO), or inducing anaemia by iron restriction, on systemic blood pressure, renal haemodynamics and glomerular injury in rats with five-sixths nephrectomy [214]. rHuEPO worsened systemic and glomerular hypertension, and promoted proteinuria and glomerular sclerosis. Anaemia ameliorated each of these effects. The authors concluded that anaemia, by reducing blood viscosity, is a haemodynamically favorable adaptation to chronic renal disease, and that over-vigorous correction of anaemia in CRF results in
acceleration of the progression of renal failure.

Eschbach and colleagues studied the effects of rHUEPO administration in human subjects with progressive renal failure [215]. In spite of the fact that hypertension was worsened in more than half the patients, there was no increase in the rate of progression of renal failure, as measured by the slope of reciprocal creatinine. Similar results were reported by Frenken et al [216].

The discrepancy between these studies may be due to differences in the experimental setting (experimental animals and human patients), in the methods used to determine the influence of treatment on progression of renal failure and in the degree of haematocrit elevation by rHuEPO. The influence of blood viscosity on progressive renal failure is, therefore, not yet certain.
SECTION II LABORATORY METHODS
CHAPTER 5  LABORATORY METHODS - BLOOD RHEOLOGY

1. Venesection
Blood for rheological investigations was taken between 9 and 11 AM after overnight fasting. A 19 gauge needle and plastic syringe were used, and blood was anticoagulated with dipotassium EDTA (4 mmol/l final concentration). Samples were kept at room temperature and measurements performed within 5 hours of venesection.

Measurements of red cell count, white cell count, haematocrit (Hct), mean red cell volume (MCV) and mean red cell haemoglobin concentration (MCHC) were kindly performed by the Department of Haematology, St. Paul's Hospital, using an automatic electronic counter (Coulter electronics, Luton, UK).

Whole blood viscosity was measured on blood samples which had been thoroughly mixed immediately before use to ensure even distribution of red and white cells. Plasma viscosity was measured on plasma obtained after centrifugation of blood for 10 minutes at 1500g and room temperature. In each case, sample volume was 1.5 ml.
The Wells-Brookfield Viscometer. Viscosity was measured using a microviscometer (Wells-Brookfield, Stoughton, Massachusetts, USA) which consists of a rotating cone of angle 1.565°, and a convex plate, which remains stationary. Blood or plasma samples placed between these two surfaces are subjected to shear during rotation of the cone. The gap between the cone and plate is set precisely at 0.0005 inches before use (see below), and the resulting geometry ensures that the entire blood sample is subjected to a uniform rate of shear during testing. Temperature is maintained at 37 ± 0.5° C by means of a water jacket connected to a circulating water bath.
The instrument measures the torque produced by the viscosity of the sample during rotation of the cone at constant speed. The apparent viscosity is then calculated as:
Apparent viscosity = shear stress / shear rate
= K x  torque / rotational speed

49
where $K$ is a conversion factor dependent on the geometry of the viscometer system. Viscosity is expressed in units of millipascal seconds (mPas).

The Wells-Brookfield viscometer can measure blood viscosity at shear rates as low as $23\text{s}^{-1}$, but is unreliable at shear rates lower than this [55]. Shear rates of $23\text{s}^{-1}$ (low shear) and $230\text{s}^{-1}$ (high shear) were used in this study.

Prior to testing, the circulating water bath was turned on and left for 5 minutes to allow the temperature of the apparatus to equilibrate. With rotational speed at 12 rpm, the gap between the cone and plate was then set by closing the instrument's micrometer dial until the cone and plate were just touching (indicated by a slight deflection from zero of the instrument pointer with each revolution of the cone). The micrometer dial was then turned one division clockwise, at which point the gap was exactly 0.0005 inches.

The instrument settings were checked before use by measuring the viscosity of a mineral oil standard (Wells-Brookfield) at $230\text{s}^{-1}$. If this standard measurement was correct, sample testing could then proceed.

**Viscometer equilibration time.** Preliminary experiments were carried out on samples of whole blood and plasma to investigate the time required to allow the torque signal response to reach a steady value. 1.5 ml samples were introduced to the viscometer and shear applied for periods of 5 - 120 seconds, after which a viscosity reading was taken. Mean results from 3 experiments measuring blood viscosity at 23 and $230\text{s}^{-1}$ and plasma viscosity at $230\text{s}^{-1}$ are shown in Fig. 5.1. Readings fluctuated between 5 and 40 seconds, but stabilized by 60 seconds, with no further significant alteration up to 120 seconds. For all subsequent studies, viscosity was therefore measured on 1.5 ml samples of whole blood or plasma after 1 minute of shearing in the viscometer.

**Effects of delay before testing.** To investigate the effect of time delay between venesection and the measurement of viscosity, experiments were performed in which blood was taken and stored at room temperature for periods between 1 and 6 hours, prior to the measurement of whole blood and plasma viscosity. Mean results from 3 experiments are shown in Fig. 5.2. No significant change was seen in viscosity values during periods of storage up to 6 hours. In all subsequent studies, blood and plasma viscosity measurements were
performed within 5 hours of venesection.

Correction of results for the effects of haematocrit. A regression line relating blood viscosity to haematocrit was constructed in order to adjust measured viscosity values to a standard haematocrit of 0.45. Blood was taken from 20 normal subjects and haematocrit was modified in these samples by altering the proportions of plasma to packed red cells (obtained by centrifugation at 1500g for 15 minutes). Viscosity was then measured at haematocrit values of 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60. Analysis of mean results showed that the logarithm of blood viscosity bore a direct linear relationship to haematocrit, as has been previously reported [57]. Inglis and colleagues showed that the regression lines for measurements from healthy controls and vascular disease patients are approximately parallel at shear rates of 23s\(^{-1}\) and above [217]. In order to check this in our patient groups, regression lines were obtained from measurements of blood viscosity in 44 non-diabetic patients with chronic renal failure and 36 patients with diabetic nephropathy. These regression lines were then plotted against the correction curve used to calculate blood viscosity at a standard haematocrit of 0.45. The results showed that the slopes of these lines were similar at shear rates of both 23 and 230s\(^{-1}\) (Figs. 5.3 and 5.4), indicating the validity of the haematocrit correction curve.

Reproducibility of viscosity measurements. In order to assess the reproducibility of the methods used for the measurement of plasma and whole blood viscosity, 10 replicate plasma samples were measured at 230s\(^{-1}\) and 10 replicate whole blood samples were measured at 23 and 230 s\(^{-1}\). Coefficients of variation (CV) were calculated for each set of results by expressing the standard deviation as a percentage of the mean value. Results were as follows:

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma viscosity</td>
<td>10</td>
<td>1.53</td>
<td>0.03</td>
<td>2.0</td>
</tr>
<tr>
<td>Whole blood viscosity (23s(^{-1}))</td>
<td>10</td>
<td>7.62</td>
<td>0.25</td>
<td>3.3</td>
</tr>
<tr>
<td>Whole blood viscosity (230s(^{-1}))</td>
<td>10</td>
<td>4.60</td>
<td>0.04</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 5.1 Plasma viscosity (open circles) and whole blood viscosity measured at shear rates of 23s$^{-1}$ (closed circles) and 230s$^{-1}$ (open triangles) following equilibration of the viscometer for periods of 5 to 120 seconds. Values are the mean of three experiments.

Figure 5.2 Plasma viscosity (open circles) and whole blood viscosity measured at shear rates of 23s$^{-1}$ (closed circles) and 230s$^{-1}$ (open triangles) following increasing periods of delay after venesection. Values are the mean of three experiments.
Figure 5.3 Relationship between haematocrit and whole blood viscosity measured at a shear rate of 23 s\(^{-1}\) in healthy controls (open circles), patients with non-diabetic CRF (closed circles) and patients with diabetic nephropathy (open triangles). Solid line = correction curve obtained from healthy controls: dashed line = regression line for non-diabetic patients: dotted line = regression line for diabetic patients.

Figure 5.4 Relationship between haematocrit and whole blood viscosity measured at a shear rate of 230 s\(^{-1}\) in healthy controls (open circles), patients with non-diabetic CRF (closed circles) and patients with diabetic nephropathy (open triangles). Solid line = correction curve obtained from healthy controls: dashed line = regression line for non-diabetic patients: dotted line = regression line for diabetic patients.
In addition, the reproducibility of repeated plasma and whole blood viscosity measurements made in a single patient was assessed. Plasma and whole blood viscosity were measured on 4 to 6 occasions in 7 separate patients. There was no change in drug therapy during the period in which these measurements were made. CVs were calculated for these repeated measurements in each patient. The mean CV values obtained were as follows:

Plasma viscosity: 5.2% (range 2.7 - 8.2)
Whole blood viscosity 23s⁻¹: 10.9% (range 6.3 - 16.0)
Whole blood viscosity 230s⁻¹: 7.0% (range 4.9 - 8.1)

Measurement of red cell deformability.
Red cell deformability was measured by filtration of washed, leucocyte-depleted red cell suspensions through polycarbonate filters of 5 and 3 micron pore size (Nucleopore, Pleasanton, California, USA). Filters were taken from the same batch and discarded after a single use. Filtration measurements were performed using a St. George's filtrometer (Carrimed Ltd. Dorking, UK)

Preparation of red cell suspensions. Red cells were separated from plasma by centrifugation at room temperature for 10 minutes at 1500g. The plasma and buffy layer were removed using a transfer pipette, and replaced with an equal volume of phosphate buffered saline (PBS), made up as follows:

\[
\begin{align*}
\text{NaCl} & \quad 4.50g \\
\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O} & \quad 2.11g \\
\text{Na}_2\text{HPO}_4 & \quad 8.73g \\
\text{Distilled water} & \quad \text{to} \quad 11
\end{align*}
\]

pH was adjusted to 7.4 ± 0.05 and osmolarity adjusted to 300 ± 2 mOsm/Kg. PBS was filtered before use through a 0.45 micron pore size filter (Minisart P, Sartorius Instruments, Sutton, UK).
The tube was then gently inverted several times to allow thorough mixing of the contents, and re-centrifuged as before. This washing procedure was repeated twice, and white cells were removed by discarding the buffy layer after each wash. 1.5 ml of packed red cells were then removed from the centre of the red cell column, and diluted with roughly 2 ml of PBS. Red cell counts and white cell counts were performed on this sample using an electronic counter (Coulter...
electronics, Luton, UK). For filtration through 5 micron filters, the red cell count was adjusted to $1.2 \times 10^{12}/l$ by dilution with PBS. For filtration through 3 micron filters, red cell count was adjusted to $0.12 \times 10^{12}/l$. Care was taken to ensure that white cells in the final suspension had been depleted to less than $0.25 \times 10^9/l$ (for 5 micron filters) and $0.025 \times 10^9/l$ (for 3 micron filters). If white cell contamination was higher than these values, suspensions were re-centrifuged and the meniscus layer removed. This was usually sufficient to deplete white cells to an acceptable level.

**The St. George's Filtrometer.** The essential elements of this apparatus are shown in Fig. 5.5. After inserting a new filter, samples were introduced via the filling channel using a plastic syringe. Filtration of the sample was then initiated by opening the tap in the outflow channel. The effective filtration pressure can be set by varying the
Figure 5.5 The St. George's Filtrometer.
height of the receiving reservoir. In all experiments reported here, 4 cm of water pressure was used.

Filtration rate was measured by 4 pairs of fibre-optic light sources and detectors aligned along the flow-meter tube at approximately 1 cm intervals, which detected the liquid meniscus as it moved down the flow-meter tube during filtration. Filtration rate was calculated by computer from measurements of the time taken for passage of the meniscus between detectors.

To perform a measurement, the filtration rate of the suspending medium (filtered PBS) was first measured 3 times, and if results were consistent to within 1%, mean values were stored by the computer. The system was then flushed with red cell suspension to remove residual PBS, and the test sample of red cell suspension introduced. Care was taken to ensure that the flow-meter tube was filled with test suspension and that air bubbles were excluded. The tap in the outflow tube was opened, and filtration of the test sample initiated. Flow rates were measured, and results analysed by the computer and expressed in relation to the flow rate of PBS.

Red cell filtration was expressed in terms of the transit time across the filter of a red cell compared with that of suspending medium, at each of the 3 filtered volumes. If the filter clogging rate is assumed to be constant, and the filtered volumes known, then the initial relative filtration rate of the suspension (Fi) can be calculated.

When setting up the method, the volumes of the 3 sections of the flow-meter tube between the detectors had first to be measured accurately, and the values stored in the computer. This was performed by first measuring the total volume of the flow-meter tube. The tube was weighed accurately before and after being filled with distilled water, and the internal volume calculated from the difference in weight. The length of the tube was accurately measured, and internal volume/cm calculated. The total length across the detectors is 3 cm (manufacturer's information). Using the filtration times obtained between each of the detectors during buffer standardization, the exact distance between each pair of detectors could be calculated. Since the volume/cm of the flow-meter tube was known, the exact volume between each detector pair could be calculated, and these figures
stored in the computer for use in subsequent measurements.
The red cell transit time (RCTT), which is a measure of the initial
filtration rate prior to clogging of filter pores by rigid cells, could
then be expressed using the formula:

\[
\text{RCTT} = \left( \frac{1}{F_i} \right) - 1 + \frac{1}{\text{Hct}}
\]

where \( F_i \) was the initial relative filtration rate of the cell suspension,
and \( \text{Hct} \) was the haematocrit value for the cell suspension.
The clogging rate (CR) could be calculated using the \( F_i \) and relative
filtration rate (\( F_v \)) when a known volume (\( v \)) had been filtered, using
the formula:

\[
\text{CR} = 1 - \left( \frac{F_v}{F_i} \right)
\]

\[
\frac{v}{v}
\]

The parameters calculated by the St. George's filtrometer give
information on different aspects of the test sample. RCTT reflects the
average filterability of the sample as a whole, whereas CR is sensitive
to the presence of sub-populations of rigid particles [218].

Effects of delay before testing. Filterability of pure erythrocyte
suspensions has been reported to show no alteration for up to 6 hours
after venesection if blood is anticoagulated with either EDTA or
heparin and stored at room temperature [219]. In order to verify this,
blood was taken and anticoagulated with dipotassium EDTA (4 mmol/l
final concentration), and stored at room temperature for periods of
between 1 and 6 hours before the preparation and filtration of red
cell suspensions. Mean results from 3 experiments are shown in Fig.
5.6. RCTT using 5 micron filters showed a slight increase, and RCTT
using 3 micron filters showed a slight decrease between one and two
hours of storage, but no consistent change was noted thereafter. For
all subsequent studies, red cell filtration was performed between one
and 5 hours after venesection.

Reproducibility of red cell deformability measurements. In order to
assess the reproducibility of red cell deformability measurements, 10
replicate samples were tested using both 3 and 5 micron filters. The
coefficient of variation (CV) for each measurement was calculated by
expressing the standard deviation as a percentage of the mean value.
Mean CV results from 2 sets of experiments are given below:
The reproducibility of repeated measurements made in a single patient was also assessed. Red cell deformability was measured on 4 to 6 occasions in 7 separate patients. There was no change in drug therapy during the period in which the measurements were made. CVs were calculated for results in each patient. Mean CV values are given below:

5 micron filters  
RCTT 8.0% (range 5.0 - 10.3)  
CR 39.2% (range 11.4 - 84.5)  
3 micron filters  
RCTT 7.9% (range 2.2 - 16.1)  
CR 23.5% (range 14.4 - 37.1)  

Effects of contaminating white cells in the final red cell suspension. RCTT using 5 micron filters has been reported to be unaffected by levels of contaminating leucocytes below 0.4 x 10^9 /l. Clogging rate, however, was sensitive to a white cell count above 0.1 x 10^9 /l [218]. In our hands it was possible consistently to reduce white cells to < 0.25 x 10^9 /l in red cell suspensions for use with 5 micron filters, and < 0.025 x 10^9 /l in those for use with 3 micron filters. This represents a ratio of white cells to red cells of < 1:4800. In order to investigate whether these residual leucocytes influenced deformability measurements, linear regression analysis was performed on data obtained from 50 subjects (25 healthy controls and 25 CRF patients), correlating RCTT and CR values with levels of residual white cells (WBC). Results were as follows:

5 micron filters  
RCTT v WBC : r = -0.30, p<0.05.  
CR v WBC : r = 0.35, p<0.02.  
3 micron filters  
RCTT v WBC : r = 0.003, p NS.  
CR v WBC : r = 0.22, p NS.  

Thus filtration through 3 micron filters was unaffected by white cell contamination in the cell suspensions. Results obtained with 5 micron filters, however, showed a weak but significant correlation between filtration parameters and residual white cells suggesting that, at the levels achieved in our experiments, they may have exerted some degree of influence on the results. The importance of this is difficult to
assess because (1) the correlation coefficients obtained were low, and (2) the correlations were not consistent with one another (one was a negative correlation and the other was positive).
Figure 5.6 Red cell deformability measured using filters of 5 micron (open circles) and 3 micron pore size (closed circles) following increasing periods of delay after venesection. Values are the mean of three experiments.
1. The skin capillary bleeding time.

Principle

The skin capillary bleeding time may be measured by making a skin incision of standard size and depth, and measuring the time taken for bleeding to stop. Bleeding occurs from the capillaries, pre-capillary arterioles and post-capillary venules of the dermis, and ceases upon the formation of a platelet plug. The role of fibrin formation in determining the bleeding time appears to be secondary [220], and the test is therefore almost entirely dependent upon platelet number and function, although congenital abnormalities of connective tissue may also result in prolonged bleeding.

Lowering of the platelet count below $100 \times 10^9/1$ is associated with a progressive increase in the bleeding time [221] but prolongation of the bleeding time in the presence of a normal platelet count is due to platelet dysfunction. This may be caused by an abnormality in the platelets themselves, or by a non-platelet abnormality such as von Willebrand's disease, afibrinogenaemia, factor V deficiency, dysproteinaemia or severe anaemia.

In order to obtain reproducible results it is crucial to standardize the conditions of the test as far as possible. Important variables include the site, size, depth and direction of the incision, venous pressure at the site of the test, and skin temperature. There is marked variation in the normal range between laboratories [222]. Operator differences may be minimized by assigning one person to perform the tests.

The original method of Duke [223] involved measurement of the bleeding time following puncture of the ear lobe. The technique was later refined and improved by Ivy [224] who used three puncture wounds on the forearm, and induced venous stasis in the arm by the use of a sphygmomanometer inflated to 40 mm of mercury proximal to the puncture site. Further improvements included the use of incisions made with a template system and the development of a disposable template device which minimises error due to differences in operator technique [225].

Method

In the present studies, the following technique was used. Patients were
tested lying supine with the forearm exposed and firmly supported. A sphygmomanometer cuff was inflated to 40 mm of mercury on the upper arm proximal to the elbow. A section of the forearm free from detectable blood vessels was selected, cleaned with alcohol and allowed to dry. Duplicate incisions 5 mm long by 1 mm deep were then made using a disposable spring-loaded template device (Simplate II, General Diagnostics (Morris Plains, USA). The device was applied firmly to the skin, but without undue pressure, before making the incisions. In all cases, the incision was made in the vertical direction, parallel to the long axis of the arm. After allowing bleeding to proceed undisturbed for 30 seconds, blood was blotted with filter paper, and blotting was repeated every 30 seconds until bleeding had stopped. Care was taken to avoid touching the wound itself since this might have disturbed the formation of the platelet plug.

Reproducibility of bleeding time measurements.

Bleeding time was tested in 16 patients and the measurements then repeated after an interval of 3 months. During this time there had been no major change in renal function, and drug therapy had remained constant. The duplicate results are shown in Table 6.1. Although the values from each individual did vary, sometimes by a large margin, there was no significant difference between the mean results from the 16 patients at the two time points.
Table 6.1 Duplicate Bleeding time results (in minutes) measured at an interval of 3 months.

<table>
<thead>
<tr>
<th>Subject</th>
<th>1st measurement</th>
<th>2nd measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.45</td>
<td>5.42</td>
</tr>
<tr>
<td>2</td>
<td>4.13</td>
<td>4.50</td>
</tr>
<tr>
<td>3</td>
<td>6.63</td>
<td>6.66</td>
</tr>
<tr>
<td>4</td>
<td>4.36</td>
<td>5.50</td>
</tr>
<tr>
<td>5</td>
<td>7.08</td>
<td>5.25</td>
</tr>
<tr>
<td>6</td>
<td>8.33</td>
<td>7.58</td>
</tr>
<tr>
<td>7</td>
<td>4.50</td>
<td>6.66</td>
</tr>
<tr>
<td>8</td>
<td>4.42</td>
<td>4.66</td>
</tr>
<tr>
<td>9</td>
<td>2.75</td>
<td>4.00</td>
</tr>
<tr>
<td>10</td>
<td>5.42</td>
<td>5.67</td>
</tr>
<tr>
<td>11</td>
<td>3.65</td>
<td>4.05</td>
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<tr>
<td>12</td>
<td>3.05</td>
<td>3.48</td>
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<tr>
<td>13</td>
<td>2.83</td>
<td>4.78</td>
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<tr>
<td>14</td>
<td>10.35</td>
<td>9.75</td>
</tr>
<tr>
<td>15</td>
<td>3.20</td>
<td>4.33</td>
</tr>
<tr>
<td>16</td>
<td>4.75</td>
<td>7.12</td>
</tr>
</tbody>
</table>

Mean 5.12 5.59
SEM 0.54 0.41
2. Turbidometric Measurement of Platelet Aggregation.

Venesection. Blood for platelet aggregation studies was taken after overnight fasting into a plastic syringe using a 19 gauge needle and minimal stasis, and anticoagulated with 3.13% w/v trisodium citrate.

Correction of citrate anticoagulant concentration. The amount of citrate anticoagulant used was adjusted to take into account the effects of variation in the haematocrit. This was performed by the method of Kelton et al [226] where 9 mls blood were taken into 0.9 mls trisodium citrate, and a further volume of citrate then added depending on the value of the haematocrit. The extra volume of citrate anticoagulant added (v) was calculated from the haematocrit value using the following formula:

\[ v = (2 \times (1-HCT)) - 0.9 \text{ mls.} \]

Haematocrit values were measured using a micro-haematocrit centrifuge (Gelman Hawkesley, Lancing, UK).

Experiments to investigate the importance of correcting citrate anticoagulant concentration when measuring ex-vivo platelet aggregation in uraemic subjects.

Trisodium citrate is commonly used as an anticoagulant in platelet aggregation studies. Since citrate does not cross the red cell membrane, failure to take into account the concentration of red cells in the test blood sample results in a fixed volume of citrate being distributed in a volume of plasma that varies according to the patient's haematocrit. In anaemic subjects, this would lead to reduction of effective citrate concentration and an increase in ionised calcium concentration in the patient's PRP. Since many uraemic patients suffer from anaemia, this might represent a source of error in the measurement of ex-vivo platelet aggregation in this patient group.

In order to investigate this, ionized calcium concentration in platelet rich plasma (PRP) with or without correction of citrate concentration was measured in patients with renal failure and in a group of healthy, non-anaemic subjects.

Methods

Blood was obtained from 7 patients with chronic renal failure (CRF) and from 7 healthy (non-anaemic) subjects. Three samples of blood were obtained from each subject, and anticoagulated as follows:
Sample 1. (Citrate concentration uncorrected) 9 ml blood added to 1 ml 3.13% (w/v) trisodium citrate.
Sample 2. (Citrate concentration corrected)
9 ml blood added to 0.9 ml 3.13% (w/v) trisodium citrate, and a further volume of citrate added according to the method of Kelton et al [226]. This results in the standardization of citrate concentration to that which would be present at a haematocrit value of 0.55.
Sample 3.
5 ml blood were anticoagulated with EDTA (4 mmol/l final concentration).

Haematocrit was measured in EDTA - anticoagulated blood. PRP was prepared by centrifugation of blood for 10 minutes at 150g and room temperature. Ionized calcium was measured in PRP by means of an ion-selective electrode (Nova Biomedical, Newton, Massachusetts, USA), and results of free calcium from samples with corrected and uncorrected citrate anticoagulant concentration were compared by Student's paired t test.

In a further study, blood was obtained in a similar fashion from 10 anaemic haemodialysis (HD) patients and 10 healthy controls, for measurement of platelet aggregation. Platelet aggregation was measured using a turbidometric aggregometer, after stimulation with ADP (0.8, 2, and 5 umol/l) and collagen (0.5, 1 and 2 ug/ml). Comparisons were made between aggregation results within each group with and without citrate correction to see whether the change in citrate concentration was great enough to influence aggregation responses.

**Results**

1. **Ionized calcium.**
Mean haematocrit in the CRF group was 0.336 (range 0.230 - 0.400), and in healthy controls was 0.434 (range 0.410 - 0.470).
Ionized calcium concentrations in PRP with corrected and uncorrected citrate concentration are shown in Table 6.2.

2. **Aggregation**
Mean haematocrit in HD patients was 0.240 (range 0.14 - 0.33), and in healthy subjects was 0.418 (range 0.37 - 0.48).
There was no significant difference between platelet counts in the corrected and uncorrected PRP samples from HD patients (226 (19.9)
Platelet aggregation results in normal and HD PRP samples with corrected and uncorrected citrate concentration are shown in tables 6.3 and 6.4.

**Conclusions**

If citrate concentration is left uncorrected, mean free calcium is significantly higher in HD patients than in healthy subjects. Following correction of citrate concentration for differences in haematocrit, free calcium levels are not significantly different in HD patients and controls.

In healthy subjects, correction of citrate anticoagulant concentration to that which would be present at a haematocrit value of 0.55 did not alter platelet aggregation. This is probably explained by the fact that the difference in haematocrit between the patient values and the correction value was so small that its influence on ionized calcium, and hence platelet aggregation, was insignificant. In contrast, PRP from HD patients showed a significant difference in platelet aggregation between corrected and uncorrected samples, showing that citrate correction does have a significant effect on aggregation results in this patient group. Failure to standardize citrate concentration when making comparisons of platelet aggregation between normals and anaemic patient groups (such as uraemic patients) would therefore result in error due to the influence on platelet aggregation of differences in ionized calcium. Because of the variability in haematocrit values in patients with renal failure, this standardization was essential when studying platelet aggregation in these patients.
Table 6.2 Mean (SEM) ionized calcium (umol/l) in PRP samples from CRF patients and healthy controls with and without correction of citrate anticoagulant concentration.

<table>
<thead>
<tr>
<th></th>
<th>CRF</th>
<th>Controls</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Uncorrected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>67.7</td>
<td>61.0</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>SEM</td>
<td>1.52</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>Corrected</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>48.7</td>
<td>53.6</td>
<td>NS</td>
</tr>
<tr>
<td>SEM</td>
<td>1.64</td>
<td>3.67</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3 Mean (SEM) rate of aggregation in PRP from healthy subjects with or without correction of citrate concentration.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Corrected</th>
<th>Uncorrected</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ADP (0.8 umol/l)</td>
<td>60 (3.4)</td>
<td>63 (4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>ADP (2.0 umol/l)</td>
<td>85 (3.7)</td>
<td>88 (4.5)</td>
<td>NS</td>
</tr>
<tr>
<td>ADP (5.0 umol/l)</td>
<td>85 (3.0)</td>
<td>91 (4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen (0.5 ug/ml)</td>
<td>51 (10.0)</td>
<td>48 (9.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen (1.0 ug/ml)</td>
<td>65 (7.7)</td>
<td>70 (8.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen (2.0 ug/ml)</td>
<td>81 (7.7)</td>
<td>81 (9.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 6.4 Mean (SEM) rate of aggregation in PRP from HD patients, with or without correction of citrate concentration.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Corrected</th>
<th>Uncorrected</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>ADP (0.8 umol/l)</td>
<td>52 (5.0)</td>
<td>61 (7.8)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>ADP (2.0 umol/l)</td>
<td>69 (4.9)</td>
<td>82 (7.5)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>ADP (5.0 umol/l)</td>
<td>74 (6.5)</td>
<td>84 (7.4)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Collagen (0.5 ug/ml)</td>
<td>46 (8.5)</td>
<td>53 (9.3)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Collagen (1.0 ug/ml)</td>
<td>60 (8.8)</td>
<td>73 (9.7)</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Collagen (2.0 ug/ml)</td>
<td>74 (6.6)</td>
<td>86 (9.3)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
The turbidometric platelet aggregometer.

Principle

Turbidometric aggregometry was measured by the method of Born [227], in which light transmission through a cuvette containing a suspension of platelets in either plasma (PRP), or buffer, is measured (Fig. 6.1). The platelet suspension is placed in a heating block to maintain a constant temperature of 37°C, and mixed by means of a magnetic stirrer. A light beam is passed through the suspension and light transmission monitored by an optical detector. Light scattering by the platelets reduces transmission, but transmission is increased when the platelets are stimulated to aggregate. The change in optical transmission during an aggregation response may be monitored continuously, and displayed using a chart recorder. Calibration of the response may be achieved by assigning a value of 100% transmission to the platelet-free suspending medium, and 0% transmission to the initial platelet suspension. Subsequent platelet aggregation will result in an increase in light transmission which can be quantified by reference to these calibration values. Measurement of an aggregation response may be made in a number of ways. These include the initial rate of aggregation (expressed as either the angle of the aggregation slope or the rate of change in light transmission in %/minute), the maximum extent of aggregation (taken either after a standard time, or once the aggregation curve has flattened), the delay, or "lag phase", required after addition of agonist before aggregation begins, and the threshold concentration of an agonist, that is the lowest concentration of that agonist required to bring about a specified response, for example a primary, or a secondary wave of aggregation.

Method

(1) Preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP). Following correction of citrate anticoagulant concentration (see above), blood was centrifuged for 10 minutes at 150g and room temperature, and the PRP transferred into a separate plastic tube. Care was taken to avoid red cell contamination. The blood was then centrifuged for a further 10 minutes at 1500g and room temperature, and the resulting PPP separated. The platelet count in the PRP was adjusted to 200 x 10^9/l by the addition of an appropriate volume of PPP, prior to the measurement of aggregation responses.
(2) Induction of platelet aggregation.

To measure an aggregation response, 270 µl of PRP was placed in a glass cuvette containing a small magnetic stir bar which was rotating at a rate of 1000 revs/minute, and allowed to warm for 1 minute in a Payton dual channel aggregometer (Centronic Sales, New Addington, UK). 30 µl of aggregating agent was then added and the change in light transmission caused by the aggregation response continuously monitored using a Rikadenki chart recorder (Centronic Sales, New Addington, UK). Agonists used to induce platelet aggregation in PRP were as follows:

1. ADP (Sigma Chemicals, Poole, UK) (Derived from equine muscle). Dissolved in 9 g/l sodium chloride and stored in aliquots at -20°C at a concentration of 10mmol/l. Aliquots were thawed at 37°C prior to use and further diluted as required in 9 g/l sodium chloride.

2. Collagen (Hormon Chemie, Munich, West Germany) (Derived from equine tendon). Obtained as a suspension of fibrils in manufacturer's glucose buffer (pH 4.5). Stored at 4°C at a concentration of 1 mg/ml and diluted prior to use as required using manufacturer's buffer.

3. Ristocetin (Lundbeck Ltd, Luton, UK) Dissolved in 9 g/l sodium chloride and stored in aliquots at -20°C at a concentration of 30 mg/ml. Aliquots were thawed before use and diluted as required using 9 g/l sodium chloride.

Typical dose/response curves obtained as mean values from 20 healthy individuals are shown in Figs 6.2 - 6.4. The following indices were used to quantitate platelet aggregation responses, and to compare responses between groups:

1. ADP threshold (ADPt): minimum concentration of ADP required to give a secondary wave of aggregation.

2. Collagen ED50 (coll ED50): concentration of collagen giving 50% maximum rate of aggregation (maximum defined as that obtained using 20 µg/ml collagen).

3. Ristocetin induced platelet aggregation (RIPA): initial rate of aggregation obtained after stimulation of PRP with 1.25 mg/ml ristocetin.

4. Spontaneous aggregation (spont. aggn.): maximum extent of aggregation obtained after stirring PRP at 37°C for 15 minutes in the absence of any added agonist.
Figure 6.1 The turbidometric platelet aggregometer.
Figure 6.2 Platelet aggregation in response to increasing concentrations of ADP (mean and SEM from 20 healthy subjects).

Figure 6.3 Platelet aggregation in response to increasing concentrations of collagen (mean and SEM from 20 healthy subjects).
Figure 6.4 Platelet aggregation in response to increasing concentrations of ristocetin (mean and SEM from 20 healthy subjects).
Effect of time delay after venesection on aggregation responses in PRP.

To assess the effect of delay after venesection on platelet aggregation responses in the turbidometric aggregometer, PRP from 3 healthy volunteers was prepared from blood taken into trisodium citrate anticoagulant and stored in a sealed container at room temperature. Platelet aggregation was induced in each sample of PRP at a time after venesection of 30, 60 and 120 minutes. Platelet reactivity was assessed by initial rates of aggregation in response to ADP (1 and 10 umol/l), collagen (0.5 and 2 ug/ml) and ristocetin (1.25 mg/ml), and by ADP threshold and collagen ED50 values.

Results are shown in Figs. 6.5 and 6.6. Mean aggregation rates fell between 30 and 60 minutes, and then returned towards initial values over the next hour. Mean values of ADP threshold and collagen ED50 fell progressively over the period of study, suggesting an increase in the sensitivity of the platelets to these agents with time. Although the changes which occurred were not great, a protocol for the performance of platelet aggregation studies was devised in order to minimize, as far as possible, inaccuracies due to delay after venesection. PRP and PPP were prepared as soon as possible after venesection, and a uniform sequence for the addition of the various doses of different aggregation agents was rigidly adhered to.

Reproducibility of aggregation responses.

To assess the reproducibility of measurement of aggregation responses, aggregation of was induced 10 times with ADP (10 umol/l) using PRP samples from a single patient. Rate of aggregation was measured as described above, and the coefficient of variation (CV) of this parameter was calculated. Results were as follows:

\[
\begin{array}{ll}
\text{n} & 10 \\
\text{Mean} & 114 \\
\text{SD} & 9.4 \\
\text{CV} & 8.2\% \\
\end{array}
\]

3. Measurement of platelet aggregation using the impedance technique.

The impedance aggregometer

Principle

This instrument, developed by Cardinal and Flower [228], may be used for the measurement of aggregation responses in whole blood as well
as in PRP (Fig. 6.7). An electrode assembly, consisting of two parallel wires 0.25 mm in diameter and separated by a gap of 1 mm, is placed into the sample, and left to equilibrate for 1 to 2 minutes. This equilibration period is required to allow the electrodes achieve a temperature of 37°C and to become coated with a monolayer of platelets [228]. The aggregating agent is then added and the aggregation response monitored by the change in electrical impedance between the two immersed electrodes, which results from the adhesion and aggregation of platelets and other blood cells onto the electrodes. The magnitude of the change is proportional to the aggregate mass [229]. Calibration of the instrument is achieved by means of a 5 ohm resistor connected across the electrodes. The deflection on the chart recorder caused by platelet aggregation may be expressed in terms of ohms by reference to this calibration value. Calibration is performed prior to each separate aggregation response, after the electrodes have been allowed to equilibrate in the test sample for 1 to 2 minutes. The aggregation responses measured with the impedance aggregometer may be measured in terms of the rate of aggregation (in ohms/minute), or the maximum degree of aggregation (in ohms).

Method

Venesection and anticoagulation

Blood was taken and anticoagulated as described for turbidometric aggregation.

Induction of platelet aggregation.

1 ml samples of blood or PRP were placed in the heating block maintained at 37°C and stirred by means of a magnetic stirbar spinning at a rate of 600 rpm. The electrode assembly was introduced into the sample, allowed to equilibrate for 1 - 2 minutes, and then calibrated as described above. The aggregating agent was then introduced into the sample in a volume of 10 ul by means of a microsyringe (Hamilton, Reno, Nevada, USA). Impedance changes were monitored on a Rikadenki chart recorder (Centronic Sales, New Addington, UK) moving at a rate of 1 cm/minute. Rate of aggregation was measured in ohms/minute by means of a tangential line drawn at the steepest part of the aggregation curve. Maximum aggregation (in ohms) was measured by the difference in impedance between the baseline value and the plateau of the aggregation curve.
Figure 6.5 Rate of platelet aggregation in response to different agonists measured by turbidometric aggregometry following different periods of delay after venesection. Values are the mean of three experiments.

Figure 6.6 ADP threshold and collagen ED50 values measured by turbidometric aggregometry following different periods of delay after venesection. Values are the mean of three experiments.
Figure 6.7 The impedance platelet aggregometer.
The aggregating agents used in the impedance aggregometer were ADP (dissolved in 9 g/l sodium chloride as described above) at concentrations between 2 and 10 umol/l, and collagen (suspended in manufacturer's buffer as described above) at concentrations between 0.5 and 1.5 ug/ml.

Effect of time delay after venesection on aggregation measured by the impedance method.
In order to assess the effects of delay after venesection on platelet aggregation responses in the impedance aggregometer, blood was taken from 3 healthy volunteers and kept in a sealed container at room temperature. Aggregation was induced in each of the blood samples at times of 5, 30, 60 and 120 minutes after venesection. Agonists used were ADP (10 umol/l) and collagen (2 ug/ml), and the initial rate of aggregation was measured.

Results are shown in Fig. 6.8, and show that, although there was fluctuation in the aggregation responses over time, no consistent trends were seen.

Reproducibility of impedance aggregation responses.

a. Reproducibility of aggregation measurement on a single sample.

To assess the reproducibility of aggregation measurement aggregation was induced 10 times using ADP (10 umol/l) on samples of whole blood from a single patient, and the coefficient of variation (CV) of the rate of aggregation calculated. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>5.24</td>
</tr>
<tr>
<td>SD</td>
<td>0.57</td>
</tr>
<tr>
<td>CV</td>
<td>10.9 %</td>
</tr>
</tbody>
</table>
Figure 6.8 Rate of platelet aggregation in response to 10 umol/l ADP and 2 ug/ml collagen measured by impedance aggregometry following different periods of delay after venesection. Values are the mean of three experiments.

Principle
The assay is based on the competitive binding of unlabelled and $^3$H labelled TxB2 to specific anti-TxB2 antibody. Using fixed amounts of antibody and radiolabelled ligand, the amount of radioactivity bound is inversely proportional to the amount of unlabelled TxB2 derived from the test sample. Separation of the antibody-bound TxB2 from the unbound fraction is then achieved by absorption of free TxB2 on to dextran-coated charcoal, and the amount of bound ligand in the supernatant quantitated by radioactive counting. The concentration of TxB2 present in the test sample may be determined from a standard curve set up using samples with known TxB2 concentration.

Measurement of thromboxane production in clotting blood.

3 ml blood taken using a 19 gauge needle and plastic syringe (as described above for turbidometric aggregation) was put in a glass tube without any anticoagulant, placed in a 37°C water bath for 60 minutes and allowed to clot. After this time the clot was gently detached from the sides of the tube using a wooden applicator stick and the tube centrifuged for 15 minutes at 1500g and 4°C. 200 ul of serum was then transferred into a plastic tube and stored at -20°C. Prior to assay, samples were extracted by the addition of an equal volume (200 ul) of ice cold ethanol, vortex mixed and allowed to stand in an ice bath for 10 minutes before being centrifuged for 10 minutes at 1500g and 4°C to remove precipitated proteins.

Immunoreactive thromboxane (TxB2) was then measured in the extracted samples using a commercially available radioimmunoassay kit (Amersham plc, High Wycombe, UK). The time course of this reaction is shown in Fig. 6.9.

Measurement of thromboxane production in collagen-stimulated PRP.

PRP was prepared from blood anticoagulated with trisodium citrate, as described above for turbidometric aggregation. 270 ul of PRP were stimulated with 20 ug/ml (final concentration) collagen (added in a volume of 30 ul) and stirred at 37°C in an aggregometer cuvette. The relationship of collagen dose to TxB2 generation, and the time course of the reaction are shown in Figs 6.10 and 6.11. After 4 minutes the reaction was inhibited with EDTA (final concentration 13.4 mmol/l), the PRP transferred to a plastic tube and centrifuged at 1500g and 4°C for
10 minutes to remove platelets. 200 ul of plasma was then placed in a plastic tube and stored at -20\(^\circ\) until assayed. Prior to assay the plasma samples were extracted with ethanol as described above, and TxB2 measured using a commercial radioimmunoassay kit (Amersham plc, High Wycombe, UK).

**Materials**

Radiolabelled TxB2 (activity - 1uCi), TxB2 standards (0.05 - 3.00 ng/ml), anti - TxB2 antiserum and dextran coated charcoal (diluted in phosphate buffered saline pH 7.4) were all obtained from Amersham plc (High Wycombe, UK), as components of a commercially available radioimmunoassay kit (code TRK 780). Reagents were stored at -20\(^\circ\)C until used.

Phosphate buffered saline (0.15 mol/l, pH 7.4 ) (assay buffer) was made up freshly prior to each assay run in the following way:

\[
\begin{align*}
\text{NaH}_2\text{PO}_4\cdot2\text{H}_2\text{O} & \quad 0.42 \text{ g} \\
\text{Na}_2\text{HPO}_4 & \quad 1.75 \text{ g} \\
\text{NaCl} & \quad 0.90 \text{ g} \\
\text{Distilled water} & \quad \text{to 200 ml}
\end{align*}
\]

Xylene-based scintillation fluid (instagel) was obtained from Packard (Pangbourne, UK). Radioactivity was measured using a beta scintillation counter (LKB, Croydon, UK).

**Method**

Plasma and serum samples for measurement of TxB2 were obtained and extracted as described above. Assay reagents were allowed to reach room temperature prior to use. The following were measured in every run of assays:

1. Total counts (TC): a measure of the total amount of radioactivity added to each sample tube.
2. Non-specific binding (NSB): a measure of the binding of radioactivity to components other than specific antibody (for example, the walls of the sample tubes used in the assay). NSB should not exceed 5% of total counts; higher values indicate an inadequacy in the assay components, for example the assay buffer or the sample tubes.
3. Zero standard (Bo): represents the maximum binding of antibody to radiolabelled ligand, and should be around 40 - 50% of total counts.
4. Standards: assayed simultaneously with the test samples in order to obtain the standard curve.
5. Test samples: diluted 1/100 in assay buffer immediately prior to testing. Samples giving results which did not fall on the linear portion of the standard curve could be re-tested at a different dilution to allow accurate measurement.

All measurements were performed in duplicate. The protocol for the assay was as follows (all volumes are in µl):

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$^3$HTxB2</th>
<th>Standards</th>
<th>Samples</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NSB</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bo</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Standards</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Samples, standards and reagents were pipetted into 12 x 75 mm polypropylene tubes according to the protocol outlined above and vortex mixed for 5 seconds. The tubes were then placed into a refrigerated centrifuge and spun briefly at high speed by accelerating the centrifuge rapidly and turning off the motor as soon as it had reached a speed of 3000 rpm (1500 g).

The contents were vortex mixed again, incubated at room temperature for 60 minutes, and then incubated for a further 16 - 20 hours (overnight) at 4°C.

Separation of the free TxB2 fraction was then performed by the addition to each tube of 1 ml of ice-cold dextran-coated charcoal. This was performed for each sample except for the total count samples, from which 0.5ml was immediately pipetted into 5 ml of scintillation fluid, and set aside for counting.

The charcoal was kept in suspension by means of a magnetic stirrer, and added while the sample tube was being vortexed, in order to ensure a homogenous suspension. The mixture was then incubated in melting ice for 8 - 10 minutes before being centrifuged at 1000 g and 4°C for 10 minutes. 0.5 ml of each supernatent was then pipetted into 5 ml of scintillation fluid, and the radioactivity in each tube counted for 5 minutes.
Figure 6.9 Time course of thromboxane generation in clotting blood. Values are the mean of three experiments.

Figure 6.10 Thromboxane generation in platelet-rich plasma: response to increasing concentrations of collagen. Values are the mean of three experiments.
Figure 6.11 Time course of collagen-stimulated thromboxane generation in platelet-rich plasma. Values are the mean of three experiments.

Figure 6.12 Radioimmunoassay for thromboxane: standard curve.
Calculation of results Average counts per minute (cpm) were calculated for each standard and sample. To check the validity of the assay values of non-specific binding (NSB) and zero binding (Bo) were calculated for each assay run. Percent binding (%B/Bo) of standards and samples were then calculated using the following formula:

\[
\frac{(\text{Standard or sample cpm} - \text{NSB cpm})}{(\text{Bo cpm} - \text{NSB cpm})} \times 100
\]

A standard curve was then generated by plotting the logarithm of TxB2 concentration against %B/Bo. In order to obtain a linear relationship a logit transformation of the %B/Bo data was performed. This was conveniently accomplished by plotting the standard curve on log-logit graph paper. A representative standard curve is shown in Fig. 6.12. Results obtained were corrected for the dilution factor, the platelet count and (in the case of serum TxB2) the haematocrit, and expressed in terms of ng/10^9 platelets.

**Reproducibility of the assay**

a) **Intra-assay reproducibility**

TxB2 was measured in 10 replicate samples from a single patient, and gave the following values:

- \( n = 10 \)
- \( \text{Mean} = 163.3 \)
- \( \text{SD} = 13.5 \)
- \( \text{CV} = 8.3\% \)

b) **Intra-patient reproducibility**

Serial measurements of both serum thromboxane and collagen-induced thromboxane production were made, as described above, on four occasions in three patients, during which time there was no change in drug therapy, and no observable change in renal function.

For serum thromboxane, the results were as follows:

- **Patient 1** Mean 118, SD 29.7, CV 25.1\%
- **Patient 2** Mean 132, SD 40.4, CV 30.6\%
- **Patient 3** Mean 182, SD 51.3, CV 28.2\%
- **Mean CV = 28.0\%**
For collagen-induced thromboxane production, the results were as follows:

Patient 1  Mean 987, SD 50.2, CV 5.1%
Patient 2  Mean 999, SD 281.4, CV 28.2%
Patient 3  Mean 995, SD 111.4, CV 11.2%
Mean CV = 14.8%

**Specificity of the anti-TxB2 antiserum**

Manufacturer's data indicates that the antiserum used in the assay is highly specific, and that the only substance with cross-reactivity greater than 1% is prostaglandin D$_2$ (cross-reactivity 1.2%).

---

5. Measurement of platelet nucleotide content.

**Principle**

ATP is measured by bioluminescence using luciferase, after lysis of platelets with triton x-100, and extraction with ethanol. Firefly luciferase catalyses the activation of D-luciferin by ATP and its subsequent oxidation to electronically excited oxyluciferin. The transition of oxyluciferin to its ground state results in light emission [230].

Luciferase

\[
\text{ATP} + \text{luciferin} \rightarrow \text{AMP} + \text{PP} + \text{CO}_2 + \text{oxyluciferin} + \text{light}
\]

Light emmission is measured using a Luminometer (LKB, Croydon, UK). ADP is measured after its phosphorylation to ATP by puruvate kinase (PK) in the presence of phosphoenolpyruvate (PEP).

\[
\text{PK} \quad \text{Mg}^{2+}
\]

\[
\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate} \quad \text{K}^+
\]

**Materials**

1. Tris - EDTA assay buffer. 12.1 g Tris (BDH)
   0.744 g Disodium EDTA (BDH)
   1000 ml distilled water
   pH to 7.75 with acetic acid (BDH)
   Prior to use 1mg/ml bovine serum albumin (Sigma) is added.
2. 100 mMol/l EDTA
   3.72 g Disodium EDTA (BDH)
   100 ml distilled water

3. 20% v/v Triton x-100
   2 ml triton x-100 (BDH)
   8 ml distilled water

4. Absolute ethanol (BDH)

5. 2 mol/l potassium acetate
   19.63 g potassium acetate
   100 ml distilled water

6. 0.8 mmol/l PEP
   0.234 g PEP (Sigma)
   10 ml distilled water (gives 160 mmol/l solution). Prior to use dilute 1/10 (to 16 mmol/l) with assay buffer containing albumin.

7. PK
   20 ul PK (Sigma)
   180 ul assay buffer (with albumin).

8. Luciferase
   1 vial luciferase (LKB), dissolved in 10 ml distilled water.

9. ATP
   0.11g ATP (Sigma)
   20 ml distilled water (gives 10 mmol/l solution). Prior to use dilute to 10 umol/l with 0.9 g/l NaCl.

Method

1. Sample preparation.
PRP was prepared as described above, and a platelet count performed. To 200 ul of PRP were added 50 ul EDTA, 50 ul triton x-100 and 100 ul ethanol. The tube was vortex mixed for 5 seconds, incubated in melting ice for 10 minutes, and then centrifuged at 1500g and 4°C for 10 minutes. 10 ul of the supernatent was added to 490 ul of assay buffer,
and stored at -40°C until assayed.


The assay was carried out essentially as described by Summerfield et al [231], and readings made using a luminometer (LKB, Croydon, UK). Into a clean cuvette were placed 340 µl of assay buffer, 10 µl of potassium acetate and 100 µl of luciferase. These reagents alone should give a blank reading of <2 arbitrary units of light output.

50 µl of sample dilution were then added, and a reading taken, which represented the ATP content of the sample. ADP was then converted to ATP by the addition of 20 µl of a mixture of equal volumes of PEP and PK, and incubation for 2 minutes. A further reading was taken, and the increment in light emission was a measure of the ADP content of the sample. 5 µl of ATP standard were then added, and the further increment in light emission caused by the addition of this known concentration of ATP (95 nmol/l) was used to calibrate light emission in terms of ATP concentration and allow calculation of the ADP and ATP content of the sample.

Previous studies [231] have shown a linear relationship between light emission and ATP concentration up to 1 umol/l. In the assay system described here, the final concentration of ATP in the sample is of the same order as that of the added ATP standard, so that total ATP concentration in the assay cuvette rarely exceeded 200 nmol/l, and readings lie on the linear part of the calibration curve.

3. Reproducibility of the assay.

a) Intra-assay reproducibility. In order to assess intra-assay reproducibility, an extracted platelet sample was tested 5 times, and a coefficient of variation was calculated for each measured parameter. Results were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ATP Mean</th>
<th>ADP Mean</th>
<th>Ratio</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8.64</td>
<td>4.62</td>
<td>1.88</td>
<td>13.3</td>
</tr>
<tr>
<td>SD</td>
<td>0.24</td>
<td>0.27</td>
<td>0.12</td>
<td>0.40</td>
</tr>
<tr>
<td>CV%</td>
<td>2.8</td>
<td>6.0</td>
<td>6.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

b) Intra-patient reproducibility. In order to assess the variability of results from a single patient, platelet nucleotides were measured on PRP taken from a single patient on 5 occasions over a period of 12 months. A coefficient of variation was calculated for each of the
measured parameters. Results were as follows:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ATP</th>
<th>ADP</th>
<th>Ratio</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>18.5</td>
<td>9.4</td>
<td>2.06</td>
<td>27.9</td>
</tr>
<tr>
<td>SD</td>
<td>5.64</td>
<td>3.5</td>
<td>0.49</td>
<td>8.81</td>
</tr>
<tr>
<td>CV%</td>
<td>30.5</td>
<td>37.4</td>
<td>23.9</td>
<td>31.6</td>
</tr>
</tbody>
</table>

The poor intra-patient reproducibility may have been due to changes in the patient's state of health over the period during which blood samples were taken, or to differences in storage times before nucleotides were measured. Loss of ATP and ADP on storage has been shown in other studies [231], and it has been suggested that the most stable parameter is the ATP/ADP ratio. My findings are consistent with this.
CHAPTER 7  LABORATORY METHODS - HAEMOSTATIC FACTORS AND OTHER PLASMA PROTEINS.

1. Plasma fibrinogen.

**Principle** The assay is based on the method of von Clauss [232], in which the clotting time following the addition of thrombin to dilutions of patients plasma is measured. Fibrinogen concentration is calculated by means of a standard curve constructed from a reference preparation with known fibrinogen concentration.

**Sample preparation.** Blood was taken after overnight fasting into a 1/10 volume of HEPES buffered citrate (3.13% w/v trisodium citrate containing 5g/dl N - 2 - hydroxyethylpiperazine - N' -2 - ethanesulphonic acid). Platelet poor plasma (PPP) was then obtained by centrifugation for 25 minutes at 1500g and 4°C, and samples stored at -40°C until tested.

**Materials.** Imidazole buffer pH 7.3, 0.05mol/l was made up as follows:
3.4 g imidazole (BDH)
5.85 g sodium chloride (BDH)
900 ml distilled water
pH adjusted to 7.3 with concentrated HCl
Volume adjusted to 1000ml with distilled water.

Bovine thrombin was obtained from Leo laboratories (Aylesbury, UK) and diluted to 100 U/ml in 9 g/l NaCl.

Freeze-dried standard plasma (fibrinogen concentration 2.49 g/l) was obtained from Immuno (Sevenoaks, UK) and resuspended in distilled water immediately prior to use. Serial dilutions in imidazole buffer were used to produce a standard curve. A new curve was set up for each batch of assays.

Clotting times were measured using a Schnichter and Gross coagulometer (Burkard Scientific Sales, Uxbridge, UK).

**Assay procedure.** Dilutions of standard plasma (1/5 - 1/40) and test samples (1/10 and 1/20) were made in imidazole buffer, and kept on ice until tested. Duplicate 200 ul samples were then pipetted into a plastic test tube and warmed to 37°C in the heating block of the coagulometer. 100 ul of thrombin were added and the clotting time measured. Times obtained using dilutions of standard plasma were plotted against fibrinogen concentration, use a double logarithmic plot, and test
values read off this standard curve. A typical standard curve is shown in Fig. 7.1. Results were expressed in g/l.

Reproducibility of the assay.

a) Intra-assay reproducibility This was assessed by calculating the coefficient of variation on 10 replicate measurements performed on one sample. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.43</td>
<td>0.14</td>
<td>5.72</td>
</tr>
</tbody>
</table>

b) Inter-assay reproducibility This was assessed by calculating the coefficient of variation of measurements carried out during consecutive assay runs on aliquots of frozen plasma from a single plasma pool. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>4.10</td>
<td>0.24</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Figure 7.1 Clotting assay for fibrinogen: standard curve.

Figure 7.2 Clotting assay for factor VIIc: standard curve.
c) Intra-patient reproducibility. Repeated measurements were made in two patients over a period of 18 months, during which time there was no change in drug therapy. Reproducibility of these measurements was as follows:

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>3.1</td>
<td>5.0</td>
</tr>
<tr>
<td>SD</td>
<td>0.98</td>
<td>0.80</td>
</tr>
<tr>
<td>CV%</td>
<td>31.2</td>
<td>15.9</td>
</tr>
</tbody>
</table>

2. Factor VIIc assay.

Principle: A standard one stage clotting assay was used, using substrate plasma artificially depleted of FVII, as described by Brozovic et al [233]. Test values are derived from a standard curve set up using standard plasma of known FVIIc concentration.

Sample preparation: PPP was prepared as described above, and stored at -40°C until tested. Once samples had been thawed for testing they were kept at room temperature in order to avoid cold activation of FVII.

Materials: Imidazole buffer (pH 7.3, 0.05 mol/l) was made up as described above.

Thromboplastin was obtained from Diagen Ltd (Thame Oxon.) and diluted in imidazole buffer in order to give a buffer time in the assay of roughly 60 seconds. Dilutions of thromboplastin ranged from 1/4 to 1/32 in different assay runs.

Calcium chloride (BDH) was dissolved in distilled water to give a final concentration of 0.025 mol/l.

Freeze dried standard plasma was obtained from Immuno Ltd (Sevenoaks, UK) and resuspended in distilled water before use to give a FVIIc concentration of 1U/ml. Serial dilutions in imidazole buffer were used to generate a standard curve, and a new curve was set up for each batch of assays.

FVII depleted plasma was obtained from the Blood Products Laboratory (Elstree, UK). This was prepared as follows: factors II, VII, IX and X were removed from citrated normal human plasma by twice adsorbing with 10% v/v of 25% w/v aluminium hydroxide gel. A 2% volume of prothrombin complex concentrate (with high levels of factors II, IX and
X and a low level of FVII) was then added to raise levels of factors II and X to 0.3 - 0.4 u/ml.

Although FVII depletion was not complete, buffer times of 60 seconds were readily achieved by adjustment of thromboplastin concentration, and a linear dose response curves were obtainable over the range 0.02 - 0.2 u/ml. A typical standard curve is shown in Fig. 7.2.

Clotting times were measured using a Schnichter and Gross coagulometer (Burkard Scientific Sales, Uxbridge, UK).

**Assay procedure.** Dilutions of standard plasma (1/2 - 1/64) and test plasmas (1/8 and 1/16) were made in imidazole buffer and kept at room temperature. 100 ul each of plasma dilution, thromboplastin and FVII depleted plasma were pipetted into a plastic test tube and placed in the heating block of the coagulometer for 1 minute. 100ul of CaCl2 were then added, and the clotting time measured. Results from measurements of standard plasma dilutions were used to plot log. FVII concentration against log. clotting time, and FVII values for test samples read off this standard curve. Results were expressed in U/ml.

**Reproducibility of the assay.**

a) **Intra-assay reproducibility.** This was assessed by the coefficient of variation of 15 replicate measurements on one plasma sample. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.97</td>
</tr>
<tr>
<td>SD</td>
<td>0.05</td>
</tr>
<tr>
<td>CV%</td>
<td>5.3</td>
</tr>
</tbody>
</table>

b) **Inter-assay reproducibility** This was assessed by the coefficient of variation of repeated measurements made on aliquots of a single plasma pool during consecutive assay runs. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.88</td>
</tr>
<tr>
<td>SD</td>
<td>0.12</td>
</tr>
<tr>
<td>CV%</td>
<td>13.4</td>
</tr>
</tbody>
</table>

3. **von Willebrand factor antigen.**

**Principle** A standard double antibody sandwich type enzyme immunoassay (EIA) was used to detect von Willebrand factor antigen (vWF Ag) in plasma [234]. Test samples are incubated in the wells of
polystyrene 96-well microtitre plates which have been coated with rabbit IgG anti-human vWFAg. Binding of vWFAg to the wells occurs in proportion to the concentration of vWFAg in the sample. After washing off unbound antigen, rabbit IgG anti-vWFAg conjugated with horseradish peroxidase is added, and binding of this conjugated antibody occurs in proportion to the concentration of bound vWFAg. An enzymatic colour reaction is then performed by addition of a chromogenic substrate for peroxidase, and measured photometrically after termination of the reaction with acid. The concentration of vWFAg in the test sample is thus directly related to the amount of colour detected in the microtitre wells.

**Sample preparation.** Assays were carried out on PPP samples, obtained and stored as described above.

**Materials.** Buffers were prepared as follows:

a) 0.05 mol/l carbonate buffer pH 9.6 (coating buffer):
   - 1.59 g Na₂CO₃
   - 2.93 g NaHCO₃
   - 0.2 g NaN₃
   - Water to 1 l.

b) 0.5 mol/l phosphate buffered saline with tween pH 7.4 (wash buffer):
   - 8.0 g NaCl
   - 0.2 g KH₂PO₄
   - 1.15 g Na₂HPO₄
   - 0.2 g KCl
   - 0.5 ml Tween 20
   - Water to 1 l.

c) 0.1 mol/l phosphate citrate buffer pH 5.0:
   - 7.30 g citric acid (monohydrate)
   - 23.87 g Na₂HPO₄. 12H₂O
   - Water to 1 l.

Other reagents were as follows:

Rabbit IgG anti-vWFAg (HRP-conjugated and unconjugated): obtained from Dako Ltd (High Wycombe, UK) and diluted in coating buffer (unconjugated) or wash buffer (HRP-conjugated) as required.

Substrate: Orthophenylenediamine dihydrochloride (OPD) 80 mg
   - Phosphate citrate buffer 20 ml.
   - 20 vol. H₂O₂ 10 ul.
Made up immediately before use.
1 mol/l H₂SO₄: 55.5 ml concentrated sulphuric acid
Water to 1 l.
Standard: British standard for coagulation factors was obtained from
The National Institute for Biological Standards and Controls (NIBSC)
(Potters Bar, UK). The freeze dried material was dissolved in 1 ml
distilled water immediately before use, and serial dilutions in wash
buffer were used to produce a standard curve. A standard curve was
generated on every microtitre plate used.
Microtitre plates: Code M29A, obtained from Sterilin Ltd (Hounslow,
UK).
Microtitre plate reader: Minireader II, obtained from Dynatech Ltd
(Billingshurst, UK).
Assay procedure. Anti-vWFAg was diluted 1/10000 in coating buffer
(dilution determined by chequerboard experiment), and 100 ul of this
dilution incubated in the wells of a microtitre plate at 4°C overnight.
Wells were then emptied by inversion over a sink and tapping on a pad
of paper tissues, and washed by incubation with 200 ul of washing
buffer for 2 minutes. This standard washing procedure was then
repeated twice. 100 ul test plasma samples (diluted in wash buffer so
as to fall on the linear part of the standard curve - dilution was
usually between 1/50 - 1/200 ) were then added in duplicate to the
wells of the plate and incubated in a wet box at room temperature for
1 hour. The plate was then washed as described above and 100 ul of
HRP-conjugated anti-vWFAg (diluted 1/500 in wash buffer, dilution
determined by chequeboard experiment) was added to each well for 1
hour at room temperature. The plate was again washed, and 100 ul of
fresh OPD substrate added to each well, after which the plate was
incubated in the dark at room temperature for 15 minutes, or until
adequate colour had developed. The reaction was then stopped by the
addition of 50 ul of 1 mol/l H₂SO₄, and the amount of colour in each
well measured at 490 nM using the microtitre plate reader.
A typical standard curve is shown in Fig. 7.3. Results were expressed
in U/ml.
Chequer board experiment to determine antibody dilutions. An EIA
experiment, as described above, was carried out using a high
concentration of antigen (1/10 dilution of pooled uraemic plasma) to
ensure optimum binding and colour generation. A range of concentrations of coating antibody and of conjugated antibody were tested, in order to determine the optimum concentrations for use in the assay. Dilutions of coating antibody of $1/250 - 1/25000$ were used together with dilutions of conjugated antibody of $1/200 - 1/10000$. The aim of the experiment was to determine the minimum concentration of each reagent that was required to give adequate, but not excessive, colour generation (roughly 1 optical density unit at high antigen concentration). Results of a typical chequerboard experiment are shown in Fig. 7.4. A dilution of $1/500$ of conjugated antibody was the maximum dilution that gave a good colour reaction. Colour values dependent upon concentration of conjugated antibody were given with dilutions of coating antibody up to $1/10000$. This coating antibody dilution was therefore used.

Reproducibility of the assay.

a) Intra-assay reproducibility This was assessed by the coefficient of variation of 10 replicate measurements made on one plasma sample. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2.01</td>
<td>0.24</td>
<td>11.9%</td>
</tr>
</tbody>
</table>

b) Inter-assay reproducibility This was assessed by the coefficient of variation of measurements made during consecutive runs on aliquots from a single plasma pool. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.81</td>
<td>0.19</td>
<td>10.7%</td>
</tr>
</tbody>
</table>
Figure 7.3 Enzyme immunoassay for vWFAg: standard curve.

Coating antibody dilution:
- open circle 1/250
- filled circle 1/1000
- open triangle 1/2500
- filled triangle 1/5000
- open square 1/10000
- filled square 1/25000

Figure 7.4 Enzyme immunoassay for vWFAg: experiment to determine antibody dilutions.
c) Intra-patient reproducibility. Repeated measurements were made in 2 patients over a period of 18 months, during which time drug therapy remained constant. Reproducibility of these measurements was as follows:

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>1.32</td>
<td>1.02</td>
</tr>
<tr>
<td>SD</td>
<td>0.40</td>
<td>0.21</td>
</tr>
<tr>
<td>CV%</td>
<td>30.0</td>
<td>20.5</td>
</tr>
</tbody>
</table>

4. C Reactive Protein.

**Principle** A standard double-antibody sandwich type enzyme immunoassay was used for the measurement of C reactive protein (CRP). The principle of this assay is exactly the same as that described above for the measurement of vWFAg, except that the coating and conjugated antibodies employed were specific for CRP.

**Sample Preparation.** Blood was taken into plain glass tubes containing no anticoagulant, and allowed to clot at 37°C for 60 minutes. The tubes were then centrifuged for 15 minutes at 4°C and 1500g, and aliquots of serum stored at -40°C until tested.

**Materials.** Materials used in the assay were as described above for the assay of vWFAg, with the following exceptions:

- Rabbit IgG anti-CRP (unconjugated and HRP-conjugated): obtained from Dako Ltd (High Wycombe, UK) and diluted in coating buffer (unconjugated antibody) or wash buffer (HRP-conjugated antibody) as required.
- Standard: A standard serum with known concentration of CRP was obtained from Hoechst (Hounslow, UK).

**Assay Procedure.** The assay was carried out exactly as described above for the measurement of vWFAg. Coating antibody was used at a dilution of 1/2000, and HRP-conjugated antibody at a concentration of 1/500. These dilutions were determined by a chequerboard experiment as described above. Samples were diluted 1/50 and 1/100 in wash buffer prior to being assayed. A typical standard curve is shown in Fig. 7.5. Results were expressed in mg/l.
Figure 7.5 Enzyme immunoassay for C reactive protein: standard curve.

Figure 7.6 Enzyme immunoassay for D dimer: standard curve.
Reproducibility of the assay.

a) Intra-assay reproducibility This was assessed by the coefficient of variation of 8 measurements made on one serum sample. Results were as follows:

\[
\begin{align*}
\text{n} & : 8 \\
\text{Mean} & : 0.46 \\
\text{SD} & : 0.03 \\
\text{CV} & : 5.5\%
\end{align*}
\]

b) Inter-assay reproducibility This was assessed by the coefficient of variation of measurements made on aliquots from a single serum pool on 5 consecutive assay runs. Results were as follows:

\[
\begin{align*}
\text{n} & : 5 \\
\text{Mean} & : 0.34 \\
\text{SD} & : 0.04 \\
\text{CV} & : 12.5\%
\end{align*}
\]

c) Intra-patient reproducibility. Repeated measurements were made in 2 patients over a period of 18 months, during which time drug therapy remained constant. Reproducibility of these measurements was as follows:

\[
\begin{align*}
\text{Patient 1.} & & \text{Patient 2.} \\
\text{n} & : 6 & : 6 \\
\text{Mean} & : 2.10 & : 2.01 \\
\text{SD} & : 2.96 & : 1.12 \\
\text{CV}\% & : 140.8 & : 55.5
\end{align*}
\]

5. Cross-linked Fibrin Derivatives (D dimer).

Principle A double antibody sandwich EIA was used for the measurement of D dimer, as described by Whitaker et al [235], using reagents and microtitre plates pre-coated with monoclonal antibody to D dimer supplied by MAbCo (Springwood, Australia). The coating antibody used (DD-3B6/22) reacts with all derivatives of cross-linked fibrin, but has greatest affinity for D dimer [235]. The conjugated antibody is panspecific for fibrinogen and its derivatives.

Sample preparation. D dimer can be measured in either serum or plasma. A small study was therefore performed to compare D dimer
levels in matched samples of (1) plasma anticoagulated with EDTA, (2) plasma anticoagulated with trisodium citrate and (3) serum. Results are shown below:

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Citrated plasma</th>
<th>EDTA plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Mean (ng/ml)</td>
<td>88</td>
<td>186</td>
<td>192</td>
</tr>
<tr>
<td>SD</td>
<td>67.0</td>
<td>152.8</td>
<td>150.7</td>
</tr>
</tbody>
</table>

There was no significant difference between values measured in citrated and EDTA plasma, but serum values were significantly lower (p<0.02 by paired t test) than values from both types of plasma samples.

For all further studies, assays were performed on PPP samples anticoagulated with EDTA (4 mmol/l final concentration). Venesection, centrifugation and storage were exactly as described above for citrated PPP.

Materials. All materials for the assay were supplied in a kit, and were used in accordance with the manufacturer's instructions.

a) Wash buffer was prepared by adding the contents of a vial of buffer salts, together with 8mls of a 25% (v/v) solution of tween 20, to 1 litre of distilled water. This gives a phosphate buffer of the following composition:

0.5 mol/l sodium chloride
0.003 mol/l potassium chloride
0.008 mol/l disodium hydrogen phosphate
0.0015 mol/l potassium dihydrogen phosphate
2% (v/v) tween 20
pH 7.3

b) Microtitre plates were supplied pre-coated with monoclonal antibody to D dimer (DD 3B6/22).

c) HRP-conjugated antibody (DD 4D2) was diluted 1/10000 in wash buffer prior to use.

d) Substrate: One vial of azinodi-(3-ethylbenzthiazoline sulphonic acid) (ABTS) was activated with 10ul of 3% hydrogen peroxide immediately before use.

e) 30 mmol/l sodium fluoride was used to stop the enzyme reaction.

f) D dimer standard was diluted in wash buffer to give a range of concentrations between 0 and 5000 ng/ml.
Assay procedure.
Coated microtitre plates were washed 3 times, using 200 ul volumes of wash buffer, as described above. D dimer standards, and test plasma samples were diluted 1/5 in wash buffer, and 50 ul of each were incubated in duplicate in the coated wells of the plate for 60 minutes at room temperature in a moist chamber. The plates were again washed 3 times, and 50 ul of conjugated antibody was added to each well, after which the plates were incubated in a moist chamber for 60 minutes, and then washed as before. 100 ul of activated substrate was added to each well and colour allowed to develop for 20 minutes before stopping the reaction with 50 ul of sodium fluoride. The plate was incubated in the dark during the enzyme reaction. The absorbance of each well was then read at 410 nM. A typical standard curve is shown in Fig. 7.6. Results were expressed in ng/ml.

Reproducibility.

a) Intra-assay reproducibility. This was assessed by the coefficient of variation of 10 measurements made on the same plasma sample. Results were as follows:

n 10
Mean 508
SD 24.2
CV% 4.8

b) Inter-assay reproducibility. This was assessed by the coefficient of variation of measurements made on aliquots from a single plasma pool on 7 consecutive assay runs. Results were as follows:

n 7
Mean 129
SD 21.1
CV% 16.3

c) Intra-patient reproducibility. Repeated measurements were made on 2 patients over a period of 18 months, during which time drug therapy remained constant. Reproducibility was as follows:

Patient 1. Patient 2.
n 6 6
Mean 139 72
SD 65.5 44.8
CV% 47.0 61.8
6. von Willebrand Factor.

Principle The assay of von Willebrand factor (vWF) is based on the ristocetin cofactor (RiCof) assay described by Weiss et al [236]. The addition of the antibiotic ristocetin to PRP causes the binding of vWF to glycoprotein Ib (GpIb) on the platelet surface, and subsequent agglutination of platelets. Since formalin fixation of platelets does not abolish this reaction, it must be regarded as an agglutination, rather than an aggregation phenomenon. The platelet response to ristocetin depends upon the presence on the platelet surface of (GpIb), and on the presence in plasma of vWF of adequate quantity and multimeric composition to support agglutination. If plasma is added to a suspension of washed platelets, the rate of agglutination in response to a fixed concentration of ristocetin is proportional to the plasma level of vWF activity. vWF may thus be quantitated by reference to the platelet response to standard plasma of known vWF activity.

Sample Preparation. Assays were carried out on PPP samples, obtained and stored as described above.

Materials.

Tyrodes buffer pH 6.5: NaCl 8.0 g
KCl 0.2 g
NaH$_2$PO$_4$. 2H$_2$O 0.065 g
MgCl$_2$. 6H$_2$O 0.415 g
NaHCO$_3$ 1.0 g
Distilled water 1000 ml
Plasma protein fraction 25% (v/v)

Ristocetin: obtained from Lundbeck Ltd (Luton, UK). The contents of a 100 mg vial of ristocetin were dissolved in 3.3 ml of 9g/l NaCl, and stored in 100 ul aliquots at -20°C until required. Ristocetin was further diluted in 9g/l NaCl prior to use to give a final concentration in the assay cuvette of 1.25 mg/ml.

Washed platelets: PRP was obtained as previously described from normal blood anticoagulated with acid citrate dextrose (ACD), and then centrifuged at 640 g and room temperature for 10 minutes in the presence of prostacyclin (100 ng/ml). The platelet pellet was resuspended in tyrodes buffer with prostacyclin (100 ng/ml) and centrifuged again, after which the platelets were resuspended in tyrodes buffer without prostacyclin to give a count of about 200 x
Washed platelets were left at room temperature for at least 60 minutes before being used.

Standard: British standard for coagulation factors was obtained from NIBSC (Potters Bar, UK). The freeze-dried contents of one vial were dissolved in 1 ml of distilled water prior to use, and serial dilutions in tyrodes buffer were used to generate a standard curve.

Aggregometer: Platelet agglutination was measured using a Payton aggregometer (Centronic Sales, New Addington, UK).

Assay Procedure. 175 ul of washed platelets were placed in an aggregometer cuvette together with 50 ul of a dilution of test or standard plasma, and incubated in the aggregometer for 1 minute to reach 37°C. 50 ul of ristocetin (final concentration 1.25 mg/ml) were then added, and the resulting platelet agglutination monitored. The initial rate of agglutination was calculated in the same way as described above for the measurement of aggregation responses. Agglutination rates obtained with serial dilutions from 1/2 to 1/128 were plotted against vWF concentration using a double logarithmic plot. Test samples were diluted in tyrodes buffer to give agglutination rates which fell on the linear portion of the dose-response curve (generally 1/10 and 1/20), and vWF concentration in these samples derived by comparison with the standard curve. A typical standard curve is shown in Fig. 7.7. Results were expressed in U/ml.

Reproducibility of the assay.

a) Intra-assay reproducibility. This was assessed by the coefficient of variation of 8 replicate measurements made on a single plasma sample. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1.39</td>
<td>0.10</td>
<td>6.9 %</td>
</tr>
</tbody>
</table>

b) Intra-patient reproducibility. This was assessed by the coefficient of variation of 5 serial measurements made on one patient over a period of 12 months. Results were as follows:

<table>
<thead>
<tr>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>0.91</td>
<td>0.14</td>
<td>15.9 %</td>
</tr>
</tbody>
</table>
Figure 7.7 Ristocetin cofactor assay for vWF: standard curve.

Figure 7.8 Radioimmunoassay for platelet factor 4: standard curve.

Principle Platelet factor 4 (PF4) was measured by a standard competitive radioimmunoassay in which PF4 in the test plasma samples is allowed to compete with a fixed amount of radioactively labelled PF4 for binding sites on a limited amount of PF4 antiserum. Bound PF4 is then separated from unbound by precipitation with ammonium sulphate, and the radioactivity in the precipitate counted. The amount of radioactivity bound by the antibody and subsequently precipitated is inversely proportional to the amount of non-radioactive PF4 in the test sample. A standard curve may therefore be constructed using standard samples with known concentrations of PF4, and the levels of PF4 in the test samples determined from this standard curve.

Sample preparation. PF4 is a releasable platelet alpha-granule protein, and, if measured values are to reflect platelet activity in vivo, extreme care is required to prevent the release of platelet granule contents during sample preparation. Blood was therefore taken into a mixture of anti-platelet agents, as described by Ludlam and Cash [237], in order to prevent in vitro platelet activation. This mixture consisted of equal volumes of the following:

EDTA 100 g/l in distilled water, pH 7.4
Theophylline 5.4 g/l in distilled water, pH 7.4
Prostaglandin E₁ (PGE₁) 1 ug/ml in aqueous sodium carbonate (0.2 g/l).

3 mls of blood were mixed with 0.3 mls of this mixture, and PPP was prepared and stored as described above.

Materials. All reagents required for the assay were supplied in a commercially available kit (Abbott Diagnostics, Wokingham, UK), and were used in accordance with the manufacturer's instructions.

a) Dilution buffer was 0.01 mol/l Tris buffer with 0.15 mol/l sodium chloride and bovine serum albumin.

b) ¹²⁵I PF4 tracer was supplied diluted in dilution buffer, and had radioactivity of 0.35 uCi/ml.

c) Goat anti-human PF4 antiserum was supplied diluted in dilution buffer.

d) Ammonium sulphate was supplied as a 73% saturated solution in distilled water.

e) PF4 standards with concentrations of 10, 30, 50 and 100 ng/ml were supplied diluted in dilution buffer. Dilution buffer alone was used as
Radioactivity was counted using a gamma counter (LKB, Croydon, UK).

Assay procedure. All tests were performed in duplicate. Assays were performed according to the following protocol (all volumes are in ul):

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$^{125}\text{IPF}_4$</th>
<th>Standards</th>
<th>Samples</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total counts</td>
<td>-</td>
<td>250</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non spec. binding (NSB)</td>
<td>300</td>
<td>250</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zero standard</td>
<td>50</td>
<td>250</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PF4 standards</td>
<td>-</td>
<td>250</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>Samples</td>
<td>-</td>
<td>250</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

After addition of the reagents as outlined above, the assay tubes were vortex mixed and incubated at room temperature for 120 minutes. 1ml of ammonium sulphate was then added to all tubes (except for total counts), the tubes were vortex mixed and allowed to stand for 10 minutes for precipitation of antibody-bound PF4 to reach completion. The precipitate was separated by centrifugation at 1500g for 20 minutes at room temperature and the supernatents decanted by inverting the tubes and allowing them to drain for 5 minutes. The tubes were then counted for 5 minutes each in the gamma counter.

Calculation of results. Average counts per minute (cpm) were calculated for all samples and standards. Percent binding of $^{125}\text{I}$ PF4 was calculated using the following formula:

$$\% \text{ binding} = \frac{\text{cpm of standard or sample} - \text{NSB}}{\text{Total counts}} \times 100$$

A standard curve was constructed by plotting PF4 concentration against % binding, and unknown values derived using this curve. A representative standard curve is shown in Fig. 7.8. Results were expressed in ng/ml.

Reproducibility.

a) Intra-assay reproducibility. This was assessed by the coefficient of variation (CV) of repeated measurements made on a single plasma sample. CV was calculated at a range of concentrations of PF4. Results were as follows:
<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>137</td>
<td>10.2</td>
</tr>
<tr>
<td>SD</td>
<td>2.9</td>
<td>2.1</td>
</tr>
<tr>
<td>CV%</td>
<td>2.1</td>
<td>21.0</td>
</tr>
</tbody>
</table>

b) **Inter-assay reproducibility.** This was assessed by the coefficient of variation of measurements made on aliquots from a plasma pool made on consecutive assay runs. Results were as follows:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>CV%</td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

c) **Intra-patient reproducibility.** Repeated measurements were made in 2 patients over a period of 18 months, during which time drug therapy remained constant. Reproducibility of these measurements was as follows:

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
</tr>
<tr>
<td>Mean</td>
<td>7</td>
</tr>
<tr>
<td>SD</td>
<td>5.4</td>
</tr>
<tr>
<td>CV%</td>
<td>77.7</td>
</tr>
</tbody>
</table>
Measurements of renal function were performed by the Department of Clinical Biochemistry, St. Paul's Hospital. Standard laboratory techniques were used for these estimations, and they will be described here only in outline.

1. Plasma Creatinine
Creatinine was measured in heparinized plasma using the Jaffe Technique, in which a coloured complex is formed between sodium picrate and creatinine under alkaline conditions [238]. The end point of this reaction is measured at 505 nM using a continuous flow analyser (Chemlab Instruments, Essex, UK).

Within-patient reproducibility In order to assess the reproducibility of repeated measurements in individual patients, data from 5 diabetic and 5 non-diabetic patients with stable chronic renal failure was analysed. For each patient a coefficient of variation was calculated for plasma creatinine values obtained over a period of between 3 and 18 months, during which time there was judged to be no progression of renal failure (slope of creatinine clearance against time was zero). Results are shown below:

a) Non-diabetic CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>16</td>
<td>8</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>mean</td>
<td>219</td>
<td>453</td>
<td>220</td>
<td>430</td>
<td>190</td>
</tr>
<tr>
<td>SD</td>
<td>15.1</td>
<td>22.5</td>
<td>10.8</td>
<td>27.2</td>
<td>12.3</td>
</tr>
<tr>
<td>CV%</td>
<td>6.9</td>
<td>5.0</td>
<td>4.9</td>
<td>6.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Mean CV for creatinine measurements in non-diabetic CRF patients was 5.9 %.

b) Diabetic CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>127</td>
<td>158</td>
<td>164</td>
<td>133</td>
<td>113</td>
</tr>
<tr>
<td>SD</td>
<td>19.1</td>
<td>14.1</td>
<td>14.8</td>
<td>18.3</td>
<td>9.1</td>
</tr>
<tr>
<td>CV%</td>
<td>15.1</td>
<td>8.9</td>
<td>9.0</td>
<td>13.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Mean CV for creatinine measurements in diabetic CRF patients was 11.0%.
2. Urinary Protein

Protein was measured in 24 hour collections of urine (containing thymol preservative), by a precipitation method using 12.5% w/v trichloroacetic acid [239]. Results were read at 420 nM using a digital grating spectrophotometer (Cecil Instruments, Cambridge, UK).

Within-patient reproducibility. Coefficients of variation were calculated for results of 24 hour protein excretion in patients with non-diabetic and diabetic CRF, as described above for creatinine values. Results are given below:

a) Non-diabetic CRF.

<table>
<thead>
<tr>
<th>Patient</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1.6</td>
<td>0.67</td>
<td>41.7</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>0.7</td>
<td>0.19</td>
<td>26.7</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1.5</td>
<td>0.21</td>
<td>13.6</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>2.1</td>
<td>0.39</td>
<td>19.0</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>0.7</td>
<td>0.12</td>
<td>16.5</td>
</tr>
</tbody>
</table>

Mean CV for proteinuria in non-diabetic patients was 23.5%.

b) Diabetic CRF.

<table>
<thead>
<tr>
<th>Patient</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>3.7</td>
<td>1.59</td>
<td>43.0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.4</td>
<td>0.1</td>
<td>25.0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>0.3</td>
<td>0.12</td>
<td>34.6</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>1.1</td>
<td>0.55</td>
<td>48.6</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1.2</td>
<td>0.21</td>
<td>17.8</td>
</tr>
</tbody>
</table>

Mean CV for proteinuria in diabetic patients was 33.8%.

3. Calculation of creatinine clearance from plasma creatinine values.

Creatinine clearance (Cr. Cl.) was calculated from the plasma creatinine concentration using the formula described by Hull et al [240]. This formula is as follows:

In men: \[ \text{Cr. Cl.} = \frac{88 \ (145 - \text{age})}{\text{Creatinine (umol/l)}} - 3 \]

In women, values obtained using the formula given above were multiplied by 0.85.

Calculated values of creatinine clearance were used for the estimation of the rate of progression of renal failure. The use of this value for the estimation of the progression of renal failure is not superior to the use of the inverse of plasma creatinine. It was preferred, however,
because it allowed the rate of progression to be expressed in easily understood terms, those of ml/minute/month.

This formula was validated by linear correlation with other established techniques for the estimation of renal function. Correlation of calculated values with conventional creatinine clearance values (derived from plasma (P) and urine (U) creatinine concentrations and urine volume (V) passed per minute, using the formula UV/P), gave a coefficient of correlation of 0.93 (n = 111). The coefficient of correlation obtained with isotopic GFR measurements was 0.91 (n = 79). These correlations are shown in Figs 8.1 and 8.2.

4. Calculation of the rate of progression of renal failure.

The rate of progression of renal failure was assessed as the rate of decline of creatinine clearance, in mls/min/month. For each individual patient, a regression analysis was performed of calculated creatinine clearance values (in mls/minute) against time (in months). The slope of this regression line was taken as the rate of progression. At least 5 calculated creatinine clearance values, measured over at least 12 months, were used to determine this parameter.
Figure 8.1 Correlation between creatinine clearance values calculated from plasma creatinine concentrations and creatinine clearance measured by conventional means.

Figure 8.2 Correlation between creatinine clearance, calculated from plasma creatinine concentrations, and isotopic GFR.
SECTION III  RESULTS
INTRODUCTION
Although the association of uraemia with a bleeding tendency has long been recognised, the mechanism(s) underlying this haemostatic defect are not yet certain. Discrepancies exist in the literature, especially over the role in uraemic bleeding of defects in platelet aggregation, cyclooxygenase activity and platelet / vWF interactions (see introduction).

Many studies of uraemic bleeding have been performed on dialysis patients with end-stage disease, but it is not clear whether the bleeding time is prolonged earlier in the course of renal failure, and if so, at what stage. The aim of the present study was to document the bleeding time at different stages of uraemia, and to identify which determinants of platelet function, if any, became abnormal in parallel with prolongation of the bleeding time.

Platelet function in renal failure may be influenced by active immunological disease [16], diabetes [83], nephrotic syndrome [16] and the haemodialysis procedure [86], all of which are associated with intravascular platelet activation, increased platelet responsiveness, or both. I have therefore chosen in this study to investigate non-dialysed patients with increasing degrees of renal impairment due to essentially urological causes, in order to investigate the effects of increasing uraemia on platelet function, in the absence of such complicating factors.

Aims of study
(1) To document changes in the bleeding time during the early stages of chronic renal failure.
(2) To identify which determinants of platelet function become abnormal in parallel with the bleeding time, so as to assess their contribution to the uraemic bleeding tendency.
PATIENTS AND METHODS

Patients
31 patients with progressive renal failure were studied (20 males, 11 females; mean age 35.1 years, range 17 - 70). Patient details are shown in Tables 9.1 to 9.3. In all cases CRF was due to non-immunological disease, and no patient was suffering from a nephrotic syndrome or diabetes, nor was any undergoing renal replacement therapy.

Patients were classified as suffering from mild (creatinine 120 - 300 umol/l)(n = 10), moderate (300 - 600 umol/l)(n = 14), or severe (>600 umol/l)(n = 7) renal failure on the basis of their plasma creatinine concentration. Results from each group were compared with values obtained from a group of 22 healthy controls (13 males, 9 females; mean age 29.7 years, range 22 - 41), none of whom had taken drugs known to influence platelet function for the 10 days prior to testing.

Methods
Blood was taken after overnight fasting and anticoagulated as described in methods section. Platelet aggregation, collagen-stimulated and serum immunoreactive thromboxane (TxB2), plasma creatinine, fibrinogen, von Willebrand factor (vWF) and C reactive protein (CRP), haematocrit and platelet count were all measured as described in methods section.

Statistics
Statistical comparisons were made using the Mann Whitney test, and correlations were by the Spearman rank method.
Table 9.1 Details of patients with mild CRF.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Creatinine (umol/l)</th>
<th>Diagnosis</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. BC</td>
<td>F</td>
<td>53</td>
<td>123</td>
<td>R</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>2. SH</td>
<td>M</td>
<td>24</td>
<td>212</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>3. BH</td>
<td>M</td>
<td>40</td>
<td>236</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td>4. TJ</td>
<td>F</td>
<td>29</td>
<td>286</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td>5. CM</td>
<td>F</td>
<td>31</td>
<td>137</td>
<td>R</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>6. SH</td>
<td>F</td>
<td>51</td>
<td>285</td>
<td>O</td>
<td>Enalapril</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cephalexin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Allopurinol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frusamide</td>
</tr>
<tr>
<td>7. SH</td>
<td>M</td>
<td>20</td>
<td>205</td>
<td>O</td>
<td>Enalapril</td>
</tr>
<tr>
<td>8. PB</td>
<td>M</td>
<td>17</td>
<td>200</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>9. JD</td>
<td>F</td>
<td>39</td>
<td>135</td>
<td>R</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>10. BH</td>
<td>M</td>
<td>27</td>
<td>150</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>33.1</td>
<td>197</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R = reflux nephropathy; O = obstructive uropathy.
Table 9.2 Details of patients with moderate CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Creatinine (umol/l)</th>
<th>Diagnosis</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CA</td>
<td>F</td>
<td>20</td>
<td>425</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>2. MA</td>
<td>M</td>
<td>21</td>
<td>414</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D</td>
</tr>
<tr>
<td>3. RB</td>
<td>M</td>
<td>44</td>
<td>503</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Allopurinol</td>
</tr>
<tr>
<td>4. AC</td>
<td>F</td>
<td>33</td>
<td>463</td>
<td>O</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>5. GE</td>
<td>M</td>
<td>28</td>
<td>356</td>
<td>O</td>
<td>Nifedipine</td>
</tr>
<tr>
<td>6. FF</td>
<td>M</td>
<td>56</td>
<td>446</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nifedipine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cephalexin</td>
</tr>
<tr>
<td>7. MG</td>
<td>F</td>
<td>32</td>
<td>582</td>
<td>R</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Alucaps</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cephalexin</td>
</tr>
<tr>
<td>8. EG</td>
<td>M</td>
<td>30</td>
<td>440</td>
<td>R</td>
<td>Atenolol</td>
</tr>
<tr>
<td>9. MO</td>
<td>F</td>
<td>53</td>
<td>308</td>
<td>PC</td>
<td>Enalapril</td>
</tr>
<tr>
<td>10.TR</td>
<td>M</td>
<td>19</td>
<td>507</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>11.JT</td>
<td>M</td>
<td>37</td>
<td>373</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td>12.PS</td>
<td>M</td>
<td>37</td>
<td>475</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ranitidine</td>
</tr>
<tr>
<td>13.MH</td>
<td>M</td>
<td>17</td>
<td>475</td>
<td>R</td>
<td>Nifedipine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>14.MH</td>
<td>M</td>
<td>21</td>
<td>410</td>
<td>O</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>32.0</td>
<td>441</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R = reflux nephropathy; O = obstructive uropathy; PC = polycystic kidneys.
### Table 9.3 Details of patients with severe CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Creatinine (umol/l)</th>
<th>Diagnosis</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. JB</td>
<td>F</td>
<td>60</td>
<td>675</td>
<td>O</td>
<td>Atenolol, 1-a vit D, Alucaps</td>
</tr>
<tr>
<td>2. DC</td>
<td>M</td>
<td>70</td>
<td>714</td>
<td>HT</td>
<td>Atenolol, Nifedipine, 1-a vit D, Alucaps</td>
</tr>
<tr>
<td>3. RJ</td>
<td>M</td>
<td>23</td>
<td>654</td>
<td>O</td>
<td>Atenolol, Cotrimoxazole</td>
</tr>
<tr>
<td>4. NJ</td>
<td>M</td>
<td>24</td>
<td>711</td>
<td>O</td>
<td>Atenolol, Nifedipine, 1-a vit D, Alucaps, Sodium bicarbonate</td>
</tr>
<tr>
<td>5. SK</td>
<td>M</td>
<td>18</td>
<td>1038</td>
<td>O</td>
<td>Atenolol, 1-a vit D, Alucaps</td>
</tr>
<tr>
<td>6. AS</td>
<td>M</td>
<td>53</td>
<td>836</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td>7. WW</td>
<td>F</td>
<td>42</td>
<td>678</td>
<td>R</td>
<td>Atenolol, Nifedipine, Alucaps</td>
</tr>
</tbody>
</table>

**Mean**

- Age: 41.4
- Creatinine: 758

R = reflux nephropathy; O = obstructive uropathy; HT = hypertension.
RESULTS

Bleeding time was within the normal range (mean ± 2SD of control values) in the majority of CRF patients (Fig. 9.1). Median bleeding time was slightly longer in mild CRF (median 5.26, range 3.26 - 11.50) than in controls (median 4.86, range 3.25 - 7.17). The markedly prolonged time of 11.50 minutes in one patient may have been an anomalous result, but unfortunately this patient was not available for retesting. In moderate CRF bleeding time was above the normal range in only one patient and median bleeding time was not significantly elevated (median 5.24, range 3.76 - 7.95). In severe CRF median bleeding time was significantly prolonged (median 6.66, range 6.16 - 10.83), though even in this group results were within the normal range in 4 out of 7 patients.

Haematocrit fell with renal function (Fig. 9.2), but in all patient groups platelet counts were not reduced (Fig. 9.3) and ex-vivo aggregation responses to ADP, collagen and ristocetin showed platelet sensitivity to these agents to be normal or increased (Figs 9.4 - 9.6). Spontaneous platelet aggregation in PRP increased progressively with advancing uraemia and was significantly increased in moderate and severe CRF (Fig. 9.7), as were plasma concentrations of fibrinogen, vWF and CRP (Figs 9.8 - 9.10). No platelet aggregation or plasma protein measurement correlated significantly with bleeding time.

The pattern of TxB2 generation by uraemic platelets differed depending on the stimulus. In all three CRF groups collagen-induced TxB2 release was slightly, but not significantly higher than in healthy controls (Fig. 9.11), whereas serum TxB2 concentrations were significantly reduced (Fig. 9.12).

Haematocrit was the only index showing a weak, but significant correlation with bleeding time (rs = -0.43, p = 0.015) (Fig.9.13).

9 patients were taking antibiotic drugs at the time of the study. In order to find out whether these drugs significantly influenced their platelet function, results obtained in these patients were compared with those obtained in the remaining 22 patients who were not taking antibiotics.

The findings are summarised in Table 9.4. No significant differences were noted between the two groups.

In a similar fashion, the influence of antihypertensive therapy on platelet function was investigated. 14 patients were taking
atenolol, 6 nifedipine and 3 enalapril for control of blood pressure; results from these groups were compared with those obtained in 9 patients who were taking no antihypertensive therapy at the time of the study.

The findings are summarised in table 9.5. Platelet function measurements were not significantly different in any of the groups taking antihypertensive agents, from those in patients on no such drugs. The only exception was that ristocetin induced aggregation was higher in the group of patients who were taking nifedipine (p<0.05).
Figure 9.1 Bleeding times in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.

Figure 9.2 Haematocrit values in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.3 Platelet counts in healthy controls and patients with mild, moderate and severe renal failure.

Figure 9.4 ADP threshold values in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.5 Collagen ED50 values in healthy controls and patients with mild, moderate and severe renal failure.

Figure 9.6 Ristocetin-induced platelet aggregation in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.7 Spontaneous platelet aggregation in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.

Figure 9.8 Plasma fibrinogen concentrations in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.9 Plasma concentrations of vWF in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.

Figure 9.10 Serum concentrations of C reactive protein in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.11 Collagen-stimulated thromboxane generation in platelet-rich plasma from healthy controls and patients with mild, moderate and severe renal failure.

Figure 9.12 Serum thromboxane concentrations in healthy controls and patients with mild, moderate and severe renal failure. p values indicate significant differences from controls.
Figure 9.13 Correlation between bleeding time and haematocrit in patients with progressive renal failure.

rs = -0.43
p = 0.015
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Patients on antibiotics n = 9</th>
<th>Patients not on antibiotics n = 22</th>
<th>Mann Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>5.92 (5.17 - 7.95)</td>
<td>5.43 (3.26 - 11.50)</td>
<td>NS</td>
</tr>
<tr>
<td>ADP thresh. (umol/l)</td>
<td>1.40 (0.37 - 2.60)</td>
<td>1.42 (0.75 - 2.75)</td>
<td>NS</td>
</tr>
<tr>
<td>Coll. ED50 (ug/ml)</td>
<td>0.67 (0.40 - 1.04)</td>
<td>0.65 (0.21 - 2.05)</td>
<td>NS</td>
</tr>
<tr>
<td>Ristocetin aggn. (%/min)</td>
<td>72 (59 - 85)</td>
<td>72.5 (16 - 104)</td>
<td>NS</td>
</tr>
<tr>
<td>Spontaneous aggn. (%)</td>
<td>6 (2 - 62)</td>
<td>8.5 (3 - 23)</td>
<td>NS</td>
</tr>
<tr>
<td>Rel. TxB2 (ng/10^8 plat)</td>
<td>720 (501 - 1234)</td>
<td>985 (144 - 1710)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^8 plat)</td>
<td>148 (37 - 296)</td>
<td>160 (17 - 456)</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 9.5 The influence of antihypertensive therapy on platelet function results in CRF patients

Results are median (range). All statistical comparisons have been made against results of the patients on no antihypertensive drugs.

1) Patients on no antihypertensive drugs (n = 9).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>1 ) Patients on no antihypertensive drugs (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>5.32 (3.62 - 6.10)</td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>1.61 (0.76 - 2.60)</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.70 (0.48 - 1.04)</td>
</tr>
<tr>
<td>Ristocetin induced aggn. (%/min)</td>
<td>67 (19 - 78)</td>
</tr>
<tr>
<td>Spontaneous aggn. (%)</td>
<td>6 (2 - 18)</td>
</tr>
<tr>
<td>Released TxB2 (ng/10^9 plats.)</td>
<td>725 (144 - 1710)</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^9 plats.)</td>
<td>127 (49 - 365)</td>
</tr>
</tbody>
</table>

2) Patients on Atenolol (n = 14).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mann Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>5.73 (4.22 - 11.50) NS</td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>1.06 (0.37 - 2.75) NS</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.57 (0.21 - 2.05) NS</td>
</tr>
<tr>
<td>Ristocetin induced aggn. (%/min)</td>
<td>76.5 (16 - 104) NS</td>
</tr>
<tr>
<td>Spontaneous aggn. (%)</td>
<td>9 (3 - 65) NS</td>
</tr>
<tr>
<td>Released TxB2 (ng/10^9 plats.)</td>
<td>1052 (501 - 1466) NS</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^9 plats.)</td>
<td>148 (17 - 308) NS</td>
</tr>
</tbody>
</table>

3) Patients on Nifedipine (n = 6).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mann Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>6.61 (3.76 - 7.95) NS</td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>1.42 (1.03 - 1.85) NS</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.65 (0.50 - 0.82) NS</td>
</tr>
<tr>
<td>Ristocetin induced aggn. (%/min)</td>
<td>75.5 (72 - 90) p&lt;0.05</td>
</tr>
<tr>
<td>Spontaneous aggn. (%)</td>
<td>9.5 (6 - 14) NS</td>
</tr>
<tr>
<td>Released TxB2 (ng/10^9 plats.)</td>
<td>1040 (599 - 1466) NS</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^9 plats.)</td>
<td>192 (66 - 456) NS</td>
</tr>
</tbody>
</table>

4) Patients on Enalapril n = 3.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mann Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>5.55 (5.13 - 5.92) NS</td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>1.75 (0.76 - 2.58) NS</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.52 (0.50 - 0.88) NS</td>
</tr>
<tr>
<td>Ristocetin induced aggn. (%/min)</td>
<td>77 (69 - 103) NS</td>
</tr>
<tr>
<td>Spontaneous aggn. (%)</td>
<td>7 (5 - 10) NS</td>
</tr>
<tr>
<td>Released TxB2 (ng/10^9 plats.)</td>
<td>868 (670 - 985) NS</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^9 plats.)</td>
<td>214 (37 - 238) NS</td>
</tr>
</tbody>
</table>
Platelet aggregation
I found no evidence of reduced platelet aggregation in response to ADP, collagen or ristocetin; on the contrary, responses were often increased compared with healthy controls. It is therefore unlikely that prolongation of the bleeding time in CRF is due to defects in platelet responsiveness to such agonists. Ex vivo platelet aggregation may increase with age but the difference in mean age between controls and patients was not sufficient to influence results significantly. Aggregation results in this study agree with the previous findings of Rylance et al [241], but differ from those of others. Di Minno et al [70] reported threshold aggregating concentrations of collagen, ADP and adrenalin two to three times higher than in controls and Castillo et al [107] described a reduction in ristocetin-induced aggregation in a proportion of their uraemic patients. In these studies, however, patients were receiving dialysis therapy, and were selected because of their prolonged bleeding times and history of haemorrhagic episodes. The patients described in the present study had less advanced disease and no clinical bleeding. Methodological differences may also have contributed to the discrepancy between results. For example, there is no indication in Di Minno's paper that correction of platelet count or of citrate anticoagulant concentration were performed before aggregation was measured, precautions which are essential before comparing responses from different patient groups.

Adhesive proteins
Hyperaggregability in the patients studied may be a consequence of the increase in plasma fibrinogen and vWF, both adhesive proteins which play a major role in platelet aggregation. Meade et al [242] have shown that fibrinogen levels at the high end of the normal range (similar to those in the CRF patients) enhance platelet responses to low doses of agonist. Hyperfibrinogenaemia probably occurs as a non-specific response to disease, and I found a parallel rise in fibrinogen and the acute phase marker protein CRP in progressive CRF. Levels of vWF may rise both through endothelial injury and impaired clearance by the reticulo-endothelial system in uraemia [123]. In the present study, no evidence of a defect in the interaction of platelets with vWF
was observed, and severe CRF was associated with increased plasma vWF and ristocetin-induced platelet aggregation, despite prolonged bleeding times. The increased platelet reactivity I describe in progressive CRF is paradoxical in view of the prolongation of the bleeding time in some patients, but may be of clinical importance, first because CRF patients are at increased risk of atheroma and cardiovascular death [8], and also because platelets have been implicated in the pathological mechanisms underlying the progression of renal failure [180].

Thromboxane generation.

Measurement of thromboxane generation showed wide variation within both patient and control groups (Figs 9.11 and 9.12). In all cases subjects were questioned about their drug history and denied taking drugs known to inhibit thromboxane synthesis prior to venesection. Exclusion of discrepant results was not, therefore, felt to be justified. In contrast to the findings of Remuzzi and colleagues [102], I found no evidence of a cyclooxygenase defect in uraemic platelets. Despite a reduction in the generation of thromboxane during blood clotting (measured as serum TxB₂ concentrations), collagen-induced generation of thromboxane in PRP was slightly higher in than controls in all CRF groups, although this was not statistically significant. My results were therefore similar to those of Bloom et al [105], who suggested the existence of an abnormality in stimulus-response coupling in uraemic platelets, possibly at the level of the thrombin receptor.

Influence of drug therapy.

The possible effect of drug therapy in the CRF group was considered. High concentrations of antibiotic compounds may reduce platelet aggregation responses [243,244] and prolong the bleeding time [245], but in the present study antibiotic treatment was not associated with such changes. Similarly, treatment with atenolol, nifedipine or enalapril did not appear to affect platelet function. Greer et al have previously reported the lack of influence on platelets of atenolol [246], but nifedipine is reported to inhibit platelets in vitro (though only at supra-therapeutic levels) [247], and ex vivo [248]. My results, however, showed no difference in platelet function in patients on nifedipine when compared with patients on no antihypertensive drugs. In addition, a pilot study of my own (unpublished) showed that
administration of nifedipine to volunteers produced no change in bleeding time or in ex-vivo platelet function. These results are consistent with the fact that voltage-operated calcium channels are not involved in platelet activation [249]. It would not, therefore, appear that the pattern of results shown in this study was due to the effects of concomitant drug therapy.

**Anaemia.**
The adverse effect of anaemia on haemostasis in CRF has been emphasised in recent years, and my demonstration of a significant negative correlation between haematocrit and bleeding time supports this hypothesis. Although this correlation does not prove a cause/effect relationship, the correction of bleeding times by red cell transfusions [101] or recombinant erythropoietin [137] argues strongly for a role for anaemia in the uraemic bleeding tendency.

**Conclusion.**
Although prolongation of the bleeding time occurs with increasing frequency in the later stages of progressive CRF, the development of a bleeding tendency is not a consistent observation and may not be directly related to the severity of renal failure. Prolongation of the bleeding time in CRF is not due to reduced platelet responses to ADP, collagen or ristocetin, but appears to be related to anaemia and also possibly to a poorly-defined defect which leads to reduction in serum TxB₂ levels. Vessel wall abnormalities (not tested in this study) may also contribute. The defect is not generally severe, and bleeding time did not exceed 11.5 minutes in any individual. However, a patient might be predisposed to severe bleeding if other factors, such as the accumulation of drugs [139], are superimposed on this mild haemostatic abnormality.
CHAPTER 10 EFFECTS OF RECOMBINANT HUMAN ERYTHROPOIETIN ON URAEMIC HAEMOSTASIS.

INTRODUCTION

The anaemia of CRF is corrected by recombinant human erythropoietin (rHuEPO), which produces a dose-related rise in haemoglobin [250,251], and improvement in the general well-being of uraemic patients [252]. It is reported also to shorten the skin capillary bleeding time [137,138], indicating a correction of the uraemic bleeding tendency [64]. This enhancement of platelet/vessel wall interactions could be due to both elevation of the haematocrit, and to changes in platelet function. Van Geet and colleagues reported that shortening of the uraemic bleeding time following treatment with rHuEPO coincided not only with an increase in the patients' haematocrit values, but also with an increase in the responsiveness of their platelets to aggregating agents [138]. In view of my findings in progressive CRF, that bleeding time correlated only with haematocrit values, and not with other measurements of ex vivo platelet function, correction of the bleeding time by rHuEPO should not require alteration in intrinsic platelet function, but should be explained solely by changes in haematocrit. Reported side effects of rHuEPO include hypertension [253] and thrombosis [254], both of which might be related to increased blood viscosity, and to the enhancement of haemostatic function which shortens the bleeding time. Improved haemostasis might therefore be gained only at a risk of thrombosis.

Aims of study

(1) To document the effect of treatment with rHuEPO on the uraemic bleeding time.

(2) To identify determinants of platelet function which correlate with the correction of the bleeding time following rHuEPO.

(3) To determine whether shortening of the bleeding time by rHuEPO is accompanied by intravascular haemostatic activation.
PATIENTS AND METHODS

Patients
8 patients with end-stage CRF requiring regular haemodialysis were studied (7 males, 1 female; mean age 31.2 years, range 20 - 58). 7 were anephric. All were receiving 10.8 - 19.2 m²hrs per week of haemodialysis, using cuprophane membranes. Details of these patients are given in Table 10.1.

Methods
This was a within-person comparison in which patients acted as their own controls. Haemostatic investigations were carried out before, and 3 months after commencing treatment with rHuEPO. In 4 patients, investigations were repeated after 6 months of rHuEPO.

rHuEPO was administered after dialysis, starting at a dose of 150 U/Kg/week, and increasing the dosage, where necessary, in increments of 75 U/Kg/week until a rise in haemoglobin was observed. Haemoglobin was measured weekly, and treatment controlled to avoid a rate of increase of more than 2g/dl/month.

Venesection Blood was taken between 9 and 11 AM on the day following dialysis, using a 19 gauge needle and minimal stasis. Citrate anticoagulant concentration in blood used for aggregation studies was corrected for the effects of changes in haematocrit, as described in methods section. Platelet rich- and platelet poor plasma were obtained as described in methods.

Investigations Full blood counts, plasma and whole blood viscosity and red cell deformability were measured as described in methods. To investigate platelet function, measurements were made of bleeding time, platelet aggregation, serum and collagen-stimulated thromboxane generation and total platelet nucleotide content. Plasma levels of the adhesive proteins fibrinogen and von Willebrand factor (vWF), the acute phase markers C reactive protein (CRP) and von Willebrand factor antigen (vWFAg), and the haemostatic markers D dimer and platelet factor 4 (PF4) were all measured as described in methods.

Statistics All comparisons were by the Wilcoxon test for matched pairs. Changes in haematocrit were correlated with changes in bleeding time by linear regression analysis. Bleeding times were measured only up to 20 minutes, and values greater than this were taken as being 21 minutes when analysed.
Table 10.1 Patients treated with rHuEPO

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Pre - HD creatinine (umol/l)</th>
<th>Diagnosis</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>VW</td>
<td>M</td>
<td>29</td>
<td>942</td>
<td>DK</td>
<td>Folic acid Alucaps</td>
</tr>
<tr>
<td>LK</td>
<td>F</td>
<td>36</td>
<td>756</td>
<td>HUS</td>
<td>Nifedipine Alucaps Contraceptive pill Iron/folate Triludan</td>
</tr>
<tr>
<td>CD</td>
<td>M</td>
<td>21</td>
<td>875</td>
<td>DK</td>
<td>Calcium carbonate Folate 1-α vit D Desferrioxamine</td>
</tr>
<tr>
<td>AB</td>
<td>M</td>
<td>30</td>
<td>827</td>
<td>GN</td>
<td>Propranolol Nifedipine Folate 1-α vit D Calcium carbonate</td>
</tr>
<tr>
<td>FC</td>
<td>M</td>
<td>58</td>
<td>825</td>
<td>TB</td>
<td>Isoniazid Naproxen Pyridoxine Ranitidine Calcium carbonate</td>
</tr>
<tr>
<td>RO</td>
<td>M</td>
<td>30</td>
<td>1510</td>
<td>O</td>
<td>Alucaps</td>
</tr>
<tr>
<td>SK</td>
<td>M</td>
<td>20</td>
<td>1390</td>
<td>O</td>
<td>Alucaps 1-α vit D</td>
</tr>
<tr>
<td>RD</td>
<td>M</td>
<td>26</td>
<td>1650</td>
<td>R</td>
<td>Alucaps 1-α vit D</td>
</tr>
</tbody>
</table>

DK = dysplastic kidneys; HUS = haemolytic uraemic syndrome; GN = glomerulonephritis; TB = renal tuberculosis; O = obstructive uropathy; R = reflux nephropathy.
RESULTS

Haematological response to rHuEPO Haemoglobin rose from 6.7 g/dl (5.4 - 7.7) (median and range), to 10.8 g/dl (8.6 - 12.3) after three months of rHuEPO. Haematocrit values rose in parallel (Table 10.2 and Fig. 10.1). Platelet counts and white cell counts showed a small, but statistically significant rise following three months treatment, but there was no change in red cell size or mean red cell haemoglobin concentration (Table 10.2).

Blood rheology Whole blood viscosity measured at shear rates of 23 and 230s⁻¹ was significantly elevated after 3 and 6 months of rHuEPO. No change was seen in plasma viscosity or red cell deformability (Table 10.3).

Platelet function Bleeding time showed a significant fall following 3 months of rHuEPO treatment, though it was still markedly prolonged in two patients (Fig. 10.2). One of these patients had stopped rHuEPO for the previous two weeks while on holiday, and also showed only a small rise in haemoglobin at 3 months. When retested at 6 months, her haemoglobin had risen and her bleeding time had corrected (Fig. 10.2). Linear regression analysis of change in haematocrit against change in bleeding time gave a coefficient of correlation of 0.69 (p=0.054, 95% confidence intervals -0.03 to 0.94)(Fig. 10.3).

Platelet aggregation, thromboxane generation and nucleotide content showed no significant changes over the period of treatment (Table 10.4). The only exception was a small drop in collagen-induced thromboxane generation after 3 months of rHuEPO (p = 0.046).

Markers of haemostatic activation There were no significant alterations in measurements of haemostatic activation (D dimer, PF4), adhesive proteins (fibrinogen, vWF), or acute phase markers (C reactive protein, vWF04g) (Table 10.5).

Side effects of rHuEPO No patient suffered thrombosis during the study, but in 3 patients an increase in heparin dose was required during dialysis to prevent the appearance of small clots in the dialyser bubble trap. Pre-dialysis plasma creatinine concentrations did not change significantly after treatment with rHuEPO (mean (SEM) before treatment 1097 (127), after treatment 1150 (101)).
### Table 10.2 Haematological response to rHuEPO

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 4</td>
</tr>
<tr>
<td>White cells (x10^9/l)</td>
<td>5.2</td>
<td>6.3*</td>
<td>6.6</td>
</tr>
<tr>
<td>Red cells (x10^{12}/l)</td>
<td>2.37</td>
<td>3.64***</td>
<td>3.10**</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>6.7</td>
<td>10.8***</td>
<td>9.8***</td>
</tr>
<tr>
<td>Haematocrit (l/l)</td>
<td>(5.4 - 7.7)</td>
<td>(8.6 - 12.3)</td>
<td>(8.3 - 11.0)</td>
</tr>
<tr>
<td></td>
<td>.202</td>
<td>.334***</td>
<td>.297**</td>
</tr>
<tr>
<td></td>
<td>(1.59 - .229)</td>
<td>(.260 - .373)</td>
<td>(.246 - .377)</td>
</tr>
<tr>
<td>MCV</td>
<td>91</td>
<td>92</td>
<td>98</td>
</tr>
<tr>
<td>(fl)</td>
<td>(67 - 98)</td>
<td>(70 - 100)</td>
<td>(95 - 102)</td>
</tr>
<tr>
<td>MCH</td>
<td>30.0</td>
<td>30.4</td>
<td>31.9</td>
</tr>
<tr>
<td>(pg)</td>
<td>(21.7 - 32.7)</td>
<td>(21.7 - 33.9)</td>
<td>(31.3 - 34.4)</td>
</tr>
<tr>
<td>MCHC</td>
<td>33.2</td>
<td>32.6</td>
<td>32.8</td>
</tr>
<tr>
<td>(g/dl)</td>
<td>(32.1 - 34.1)</td>
<td>(31.1 - 34.0)</td>
<td>(32.6 - 33.8)</td>
</tr>
<tr>
<td>Platelets (x10^9/l)</td>
<td>194</td>
<td>260*</td>
<td>244</td>
</tr>
<tr>
<td></td>
<td>(170 - 284)</td>
<td>(202 - 393)</td>
<td>(180 - 354)</td>
</tr>
</tbody>
</table>

Results are median (range). * p<0.05; ** p<0.01; *** p<0.001.

### Table 10.3 Blood Rheology in patients treated with rHuEPO.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 4</td>
</tr>
<tr>
<td>PV (mPas)</td>
<td>1.60</td>
<td>1.60</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>(1.5 - 1.7)</td>
<td>(1.5 - 1.8)</td>
<td>(1.5 - 1.9)</td>
</tr>
<tr>
<td>BV 23s^{-1} (mPas)</td>
<td>4.25</td>
<td>5.95**</td>
<td>5.80*</td>
</tr>
<tr>
<td></td>
<td>(4.0 - 7.3)</td>
<td>(4.5 - 8.5)</td>
<td>(4.0 - 6.4)</td>
</tr>
<tr>
<td>BV 230s^{-1} (mPas)</td>
<td>2.80</td>
<td>3.85**</td>
<td>3.60*</td>
</tr>
<tr>
<td></td>
<td>(2.2 - 3.4)</td>
<td>(3.1 - 4.5)</td>
<td>(3.0 - 3.8)</td>
</tr>
<tr>
<td>RCTT (AU)</td>
<td>11.6</td>
<td>11.5</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>(9.7 - 12.6)</td>
<td>(8.4 - 15.5)</td>
<td>(11.3 - 12.1)</td>
</tr>
<tr>
<td>CR (AU)</td>
<td>1.14</td>
<td>1.28</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>(0.92 - 2.34)</td>
<td>(0.71 - 2.53)</td>
<td>(1.10 - 1.84)</td>
</tr>
</tbody>
</table>

Plasma viscosity (PV) measured at a shear rate of 230s^{-1}, whole blood viscosity (BV) measured at shear rates of 23s^{-1} and 230s^{-1}, red cell transit time (RCTT) and filter clogging rate (CR) in patients before and after 3 and 6 months of rHuEPO. RCTT and CR are expressed in arbitrary units (AU). Results are median (range). * p<0.05; ** p<0.01.
Table 10.4 Bleeding Time and Platelet Indices in rHuEPO Treated Patients.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 4</td>
</tr>
<tr>
<td>Bl. time (mins)</td>
<td>20.27</td>
<td>7.82**</td>
<td>6.62*</td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>2.28</td>
<td>2.10</td>
<td>1.05</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.68</td>
<td>0.72</td>
<td>1.12</td>
</tr>
<tr>
<td>R.I.P.A (%%/min)</td>
<td>26</td>
<td>42</td>
<td>62</td>
</tr>
<tr>
<td>Spont. Aggn. (%)</td>
<td>7.5</td>
<td>4.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10^9 pl.)</td>
<td>208</td>
<td>123</td>
<td>216</td>
</tr>
<tr>
<td>Rel. TxB2 (ng/10^9 pl.)</td>
<td>464</td>
<td>392*</td>
<td>500</td>
</tr>
<tr>
<td>Total ATP (nmol/10^9 pl.)</td>
<td>14.6</td>
<td>14.4</td>
<td>10.2</td>
</tr>
<tr>
<td>Total ADP (nmol/10^9 pl.)</td>
<td>4.4</td>
<td>4.4</td>
<td>4.2</td>
</tr>
<tr>
<td>ATP:ADP ratio</td>
<td>4.5</td>
<td>3.7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Bleeding time (Bl. time), ADP threshold, collagen ED50 (coll. ED50), ristocetin induced platelet aggregation (R.I.P.A.), spontaneous aggregation (spont aggn.), thromboxane production in clotting blood (serum TxB2) and after stimulation with 20 ug/ml collagen (rel. TxB2), and platelet total nucleotide contents in haemodialysis patients before, and after 3 months of treatment with rHuEPO. Results are median (range). * p<0.05; ** p<0.01 v pre-treatment values.

Table 10.5 Plasma proteins and haemostatic markers in patients treated with rHuEPO.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>3 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 8</td>
<td>n = 8</td>
<td>n = 4</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>162</td>
<td>42</td>
<td>92</td>
</tr>
<tr>
<td>PF4 (ng/ml)</td>
<td>26</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.1</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>vWF (U/ml)</td>
<td>1.84</td>
<td>1.64</td>
<td>2.11</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.02</td>
<td>4.15</td>
<td>5.12</td>
</tr>
<tr>
<td>vWF Ag (U/ml)</td>
<td>1.62</td>
<td>1.88</td>
<td>2.44</td>
</tr>
</tbody>
</table>

Results are median (range). PF4 = platelet factor 4; vWF = von Willebrand factor; CRP = C reactive protein; vWF Ag = von Willebrand factor antigen.

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Figure 10.1 Haematocrit values before, and after 3 and 6 months treatment with rHuEPO. p values indicate significant differences from pre-treatment values.

Figure 10.2 Bleeding times before, and after 3 and 6 months of treatment with rHuEPO. p values indicate significant differences from pre-treatment values.
Figure 10.3 Correlation between change in bleeding time and change in haematocrit following treatment with rHuEPO.
DISCUSSION

Effect of rHuEPO on the uraemic bleeding time.
In this study the bleeding time in uraemic patients was markedly
shortened by correction of anaemia with rHuEPO. Moia and colleagues
similarly showed correction of the bleeding time, and of platelet
adhesion to sub-endothelium following rHuEPO treatment, and
postulated that this was due to the haemostatic effects of increased
red cell concentration [137].

Mechanism by which rHuEPO shortens the bleeding time.
Anaemia is a major factor in the pathogenesis of uraemic bleeding. The
bleeding time is shortened by red cell transfusions [101,136], and
significant negative correlations have been demonstrated between
haematocrit and bleeding time. Red cells may improve haemostasis by
a variety of mechanisms. They increase platelet adhesion in flowing
blood by promoting the radial movement of platelets towards the
vessel wall, an effect which is dependent upon haematocrit [255], red
cell size [256] and deformability [257]. Platelet activation may be
initiated or augmented by the release from red cells of ADP [133], and
by the binding and inactivation of prostacyclin [134].
The pathogenesis of defective platelet/vessel wall interactions in
uraemia is, however, multifactorial. In addition to anaemia, there are
abnormalities of platelet aggregation, adhesion, thromboxane
production, granule content, cytoplasmic calcium regulation and
interaction with vWF. Increased vascular prostacyclin production may
also contribute to uraemic bleeding (see introduction).

In the present study, there were no changes in thromboxane production
or platelet nucleotide content which could explain the correction of
the bleeding time, and plasma levels of vWF, as well as platelet
interaction with this adhesive protein (reflected by R.I.P.A.) were
unaltered. Contrary to the findings of van Geet and colleagues [138],
I found no change in ex-vivo platelet aggregation. The small rise
observed in the platelet count, while statistically significant, was also
insufficient to account for the improvement in haemostasis. Although
there was no change in red cell size or deformability, there was a
marked elevation of the haematocrit which coincided with correction
of the bleeding time. van Geet and coworkers in their study reported
a highly significant correlation between haematocrit and the logarithm
of bleeding time \( (r = -0.71, p<0.001) \). This correlation is, however, subject to error due to the use in the regression analysis of both pre-treatment and post-treatment values, which were not, therefore, independent variables. A more correct statistical analysis would have been a correlation of the change in haematocrit versus change in bleeding time. When this analysis was performed on the data reported here, there was a general tendency for change in the haematocrit to parallel change in bleeding time (Fig. 10.3), with a coefficient of correlation of 0.69 (95% confidence intervals -0.03 to 0.94). This correlation did not quite reach statistical significance (p value was 0.054), but this was probably due to the small number of observations, and to the fact that an absolute value was not available for 4 of the 8 pre-treatment bleeding time measurements (recorded as >20 minutes).

**Minor effects of rHuEPO.**

It should be noted that the changes found after 3 months of rHuEPO in white cell count, platelet count and collagen-stimulated thromboxane release were significant only at the 5% level. When a large number of variables are subjected to statistical comparison, as in this study, it is possible for spurious positive results to arise by chance. For this reason changes significant only at the 5% level should be interpreted with caution. The actual p values for the 3 variables listed above were 0.03, 0.04 and 0.046 respectively. These values may allow a better evaluation of the importance of these changes.

**Intravascular haemostatic activation.**

I found no evidence of intravascular haemostatic activation following rHuEPO. Whole blood viscosity increased, but post-treatment values were clustered at the lower end of the normal range. Although heparin requirements during dialysis were increased in 3 patients, there were no episodes of thrombosis, and markers of fibrin breakdown, intravascular platelet activation and the acute phase response were not significantly altered during the study.

**Conclusions.**

In haemodialysis patients, elevation of the haematocrit up to 0.35 with rHuEPO allows correction of the bleeding time, without causing intravascular haemostatic activation. Shortening of the bleeding time with rHuEPO appears to be due to the haemostatic effects of increased red cell numbers, rather than to changes in intrinsic platelet function.
INTRODUCTION
Anaemia is an important determinant of the bleeding tendency of CRF. Prolongation of the bleeding time may occur in non-uraemic patients with severe anaemia [132], though in such cases the bleeding defect is less severe than that seen in uraemia. The relationship between anaemia and prolongation of the bleeding time may be due to the loss of a potentiating influence of red cells on primary haemostasis. Red cells potentiate primary haemostasis by both physical and chemical mechanisms [135]. In flowing blood, red cells travel along the centre of the blood vessel, so that platelets are physically displaced towards the vessel wall. In this way their interaction with the vessel wall is enhanced and platelet adhesion facilitated [255]. Chemical mechanisms by which erythrocytes promote platelet activation include the release of red cell ADP [133], and the binding and inactivation of both prostacyclin [134] and endothelium derived relaxing factor [258]. These chemical interactions might explain the enhancement by red cells of spontaneous platelet aggregation, which has been described under experimental conditions in which conditions of linear flow are absent [259].
In the present study, an impedance whole blood aggregometer has been used to compare the influence of red cells from CRF patients and healthy subjects on platelet aggregation in whole blood (in the absence of linear flow).

Aims of Study
(1) To compare platelet aggregation in matched samples of whole blood and PRP from both uraemic and normal subjects.
(2) To compare the effect of increasing haematocrit on platelet aggregation in both uraemic and normal blood.
(3) To compare directly, by means of a cross-over study, the effect of uraemic and normal washed red cells on platelet aggregation.
PATIENTS AND METHODS

Patients
Six uraemic patients on regular haemodialysis therapy were studied (3 males, 3 females; mean age 37.7 years, range 21 to 50, mean haematocrit .217, range .178 - .256) and six healthy controls (2 males, 4 females; mean age 34.8, range 26 to 47, mean haematocrit .409, range .378 - .444). None of the subjects in the study had taken drugs likely to influence platelet function during the previous two weeks. Details of the patient group are given in Table 11.1.

Methods
Blood was taken from patients prior to dialysis. Venesection, anticoagulation, preparation of PRP and PPP, measurement of platelet counts and haematocrit values and induction of platelet aggregation in the impedance aggregometer were all performed as described in the methods section.

Comparison of platelet responses in whole blood and PRP.
Whole blood and PRP were obtained from both haemodialysis patients and from healthy controls, and the platelet count in PRP adjusted by the addition of PPP to match that in whole blood. Platelet aggregation was then induced simultaneously in the matched samples using the two channels of the aggregometer. The aggregating agent used was collagen at final concentrations of 0.5, 1.0 and 1.5 ug/ml.

Effect of haematocrit alteration on platelet aggregation responses.
Packed red cells were prepared by centrifugation of blood for 15 minutes at 1500g and room temperature, and were then added to autologous PRP in varying proportions to give blood samples with haematocrit values ranging from 0.100 to 0.500 l/l. For example, 0.3 mls packed cells added to 0.7 mls gave a haematocrit of 0.300 l/l. Haematocrits in these artificial samples were measured before use to ensure that they agreed with the assigned values. PRP was also mixed with PPP in the same proportions in order to give control PRP samples with a range of platelet counts matching those in the artificial whole blood samples.

Platelet aggregation was then induced in the matching samples of whole blood and PRP using 1 ug/ml collagen, and compared in order to show the effect of of increasing haematocrit independent of its
dilutional effect on the platelet count.
The experiments were performed using blood from both healthy subjects and haemodialysis patients.

Comparison of the effects of normal and uraemic erythrocytes on platelet aggregation.

In order to assess the respective influence of red cells from haemodialysis patients and healthy subjects on platelet aggregation, washed red cells from the two groups were interchanged prior to the induction of an aggregation response. PRP and packed red cells were obtained from six haemodialysis patients and six healthy volunteers. Following removal of the buffy layer, red cells were washed twice in 9 g/l sodium chloride and then added to PRP to give a haematocrit value of 0.300 1/l. Both autologous red cells and also red cells from one of the other group (with identical ABO blood type) were used. Platelet aggregation was then induced using 1 ug/ml collagen and the effects on the response of the two sets of red cells compared.

Statistics
Statistical comparisons were performed using the Wilcoxon test for matched pairs.
<table>
<thead>
<tr>
<th>Patient Sex</th>
<th>Age (years)</th>
<th>Cr Cl (mls/min)</th>
<th>Hct (l/l)</th>
<th>Cause of CRF</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>F</td>
<td>44</td>
<td>&lt; 5</td>
<td>.256</td>
<td>PN</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D Folate Ranitidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atenolol</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>1-a vit D Folate</td>
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<td>PC</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D Folate</td>
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<td></td>
<td>Atenolol</td>
</tr>
<tr>
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<td>M</td>
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<td>&lt; 5</td>
<td>.233</td>
<td>HT</td>
</tr>
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<td></td>
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<td>Atenolol</td>
</tr>
<tr>
<td>SG</td>
<td>M</td>
<td>21</td>
<td>&lt; 5</td>
<td>.178</td>
<td>O</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D Folate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Atenolol</td>
</tr>
<tr>
<td>JG</td>
<td>M</td>
<td>28</td>
<td>&lt; 5</td>
<td>.178</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1-a vit D Folate</td>
</tr>
</tbody>
</table>

Cr Cl = creatinine clearance; PN = chronic pyelonephritis; HT = hypertension; PC = polycystic kidneys; O = obstructive uropathy.
Comparison of platelet responses in whole blood and PRP.

There was no significant difference between platelet counts in the paired samples of whole blood and PRP from either haemodialysis patients or healthy controls. Values are shown in Table 11.2.

In blood from healthy subjects the rate of platelet aggregation in response to collagen was significantly lower when measured in whole blood than in PRP (Fig.11.1). This trend was not, however, apparent in blood from haemodialysis patients (Fig. 11.2).

Effect of haematocrit on platelet aggregation responses.

Measured haematocrit values in the reconstituted blood samples agreed well with their predicted values (Table 11.3).

Platelet counts measured in the reconstituted blood samples showed a progressive fall as haematocrit increased, due to the dilutional effect of increasing red cell numbers. This fall was matched in the corresponding PRP samples (Figs 11.3 and 11.4).

Rising haematocrit was associated with a fall in platelet aggregation in blood from both healthy subjects and haemodialysis patients, but, when compared with responses in corresponding PRP samples, the presence of red cells was found to have differing effects in controls and patients. In normal blood (Fig. 11.5) red cells caused retardation of the aggregation response, so that, at all haematocrit values, aggregation rate was slower in whole blood than in the corresponding PRP samples. In contrast, aggregation rate in whole blood from haemodialysis patients was slightly raised above that in corresponding PRP samples (Fig. 11.6). This trend was present at all haematocrit values, but was statistically significant only at 0.200 l/l.

When these results are plotted to show aggregation in whole blood as a proportion of aggregation in PRP, it is shown that in blood from healthy controls, increasing haematocrit is associated with a progressive retardation of platelet aggregation, whereas in blood from haemodialysis patients, haematocrit values between 0.100 and 0.200 l/l are associated with a progressive acceleration of platelet aggregation, which subsequently declines with further haematocrit elevation (Fig. 11.7).
Comparison of the effects of normal and uraemic erythrocytes on platelet aggregation.

In the cross-over study in which erythrocyte from healthy subjects and haemodialysis patients were interchanged, haematocrit values and platelet counts were similar in the samples being compared (Table 11.4). In all samples, white cell counts were reduced to less than 0.5 x 10^9/l. There was a trend towards greater platelet responses in the presence of uraemic red cells, but this did not achieve statistical significance (Table 11.4).
Table 11.2 Platelet counts in paired samples of whole blood and PRP.

<table>
<thead>
<tr>
<th></th>
<th>Whole blood</th>
<th>PRP</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Haemodialysis patients</td>
<td>213 (127 - 250)</td>
<td>192 (126 - 287)</td>
<td>NS</td>
</tr>
<tr>
<td>b) Healthy controls</td>
<td>276 (184 - 308)</td>
<td>272 (232 - 293)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are median (range). NS = not significant.
Figure 11.1 Collagen-induced platelet aggregation (mean and SEM) in matched samples of platelet-rich plasma (open circles) and whole blood (closed circles) from healthy controls.

Figure 11.2 Collagen-induced platelet aggregation (mean and SEM) in matched samples of platelet-rich plasma (open circles) and whole blood (closed circles) from haemodialysis patients.
Table 11.3 Haematocrit values in reconstituted blood samples.

a) Healthy controls

<table>
<thead>
<tr>
<th>Predicted haematocrit (l/l)</th>
<th>Measured haematocrit (l/l) (Mean and SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.100</td>
<td>0.101 (0.0021)</td>
</tr>
<tr>
<td>0.200</td>
<td>0.194 (0.0062)</td>
</tr>
<tr>
<td>0.300</td>
<td>0.296 (0.0024)</td>
</tr>
<tr>
<td>0.400</td>
<td>0.394 (0.0092)</td>
</tr>
<tr>
<td>0.500</td>
<td>0.498 (0.0078)</td>
</tr>
</tbody>
</table>

b) Haemodialysis patients

<table>
<thead>
<tr>
<th>Predicted haematocrit (l/l)</th>
<th>Measured haematocrit (l/l) (Mean and SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.100</td>
<td>0.101 (0.0023)</td>
</tr>
<tr>
<td>0.200</td>
<td>0.199 (0.0049)</td>
</tr>
<tr>
<td>0.300</td>
<td>0.295 (0.0067)</td>
</tr>
<tr>
<td>0.400</td>
<td>0.400 (0.0084)</td>
</tr>
<tr>
<td>0.500</td>
<td>0.485 (0.0085)</td>
</tr>
</tbody>
</table>
Figure 11.3 Platelet counts (mean and SEM) in matched samples of platelet-rich plasma (open circles) and reconstituted whole blood (closed circles) from healthy subjects.

Figure 11.4 Platelet counts (mean and SEM) in matched samples of platelet-rich plasma (open circles) and reconstituted whole blood (closed circles) from haemodialysis patients.
Figure 11.5 Comparison of collagen-induced platelet aggregation in matched samples of platelet-rich plasma (open circles) and reconstituted whole blood (closed circles) from healthy subjects, to show the influence of increasing haematocrit. Values are the mean and SEM of 6 experiments.

Figure 11.6 Comparison of collagen-induced platelet aggregation in matched samples of platelet-rich plasma (open circles) and reconstituted whole blood (closed circles) from haemodialysis patients, to show the influence of increasing haematocrit. Values are the mean and SEM of 6 experiments.
Figure 11.7 Platelet aggregation in whole blood as a proportion of that in matched samples of platelet-rich plasma, to show the effect of increasing haematocrit. Open circles = healthy subjects; closed circles = haemodialysis patients.
Table 11.4 Platelet aggregation in the presence of normal and uraemic red cells.

<table>
<thead>
<tr>
<th>Platelets</th>
<th>Red cells</th>
<th>Platelet Haematocrit count</th>
<th>Aggregation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>.321 (0.311 - 0.333) 280</td>
<td>4.5 (3.5 - 6.1)</td>
</tr>
<tr>
<td>Normal</td>
<td>Uraemic</td>
<td>.315 (0.309 - 0.335) 295</td>
<td>5.8 (3.8 - 7.3)</td>
</tr>
<tr>
<td>Wilcoxon test</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Uraemic</td>
<td>Normal</td>
<td>.311 (0.301 - 0.333) 161</td>
<td>1.75 (0.5 - 12.4)</td>
</tr>
<tr>
<td>Uraemic</td>
<td>Uraemic</td>
<td>.317 (0.300 - 0.344) 173</td>
<td>2.8 (0.5 - 10.2)</td>
</tr>
<tr>
<td>Wilcoxon test</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = not significant.
DISCUSSION

Effects of uraemic and normal red cells on platelet aggregation.
This study has shown that the influence of red cells on platelet responses is different when measured in normal blood from that measured in uraemic blood. The most prominent effect of red cells in normal blood was a retardation of the rate of platelet aggregation, compared with the rate of aggregation in matched PRP samples. This retardation increased progressively as haematocrit rose, but was not explained by the dilutional effect of increasing red cell numbers on the platelet count.

In contrast, collagen-induced aggregation in uraemic whole blood was similar to, or greater than, that in PRP. In this case, however, the potentiating effect of uraemic red cells did not show a simple relationship with haematocrit.

Mechanisms by which red cells might influence platelets in uraemic and normal blood.
The explanation of these findings is unclear. In the impedance aggregometer used in this study to measure platelet aggregation, results may be influenced by red cells in a number of ways. On one hand, the contribution of erythrocytes to the size of the aggregate mass would cause an increase in electrical resistance between the detector electrodes, and this, together with the chemical activation of platelets (described in the introduction to this chapter) would serve to increase aggregation responses in whole blood. In contrast, the presence of red cells might also cause physical obstruction to platelet collisions and interactions and thus reduce aggregation responses. The relative importance of these opposing effects may depend upon several factors including the haematocrit, and the metabolic state of the platelets and red cells involved. Conditions in normal blood therefore favour inhibition by red cells when platelet aggregation is measured by the impedance aggregometer, whereas conditions in uraemic blood favour enhancement.

It is possible to envisage a relative increase in the contribution made by red cells to platelet activation in uraemic blood. Intracellular concentrations of ADP are supranormal in uraemic erythrocytes [260,261], whereas intra-platelet levels are subnormal, due to an
acquired storage pool defect [70,89]. Red cell-derived ADP may therefore make a significantly larger contribution to platelet activation in uraemic blood than it does in normal blood, by augmenting the stimulatory effects of collagen to a degree sufficient to overcome the inhibitory influence of red cells on platelet aggregation, so that responses in whole blood matched, or even exceeded, those in collagen-stimulated PRP. It was not possible, however, to prove conclusively that uraemic red cells enhance platelet responses to a greater degree than normal red cells. In the crossover experiments in which red cells from the two groups were interchanged, there was a tendency for aggregation responses to be greater in the presence of uraemic red cells, but this failed to achieve statistical significance.

**Physiological significance.**

These findings might, in part, be artifacts arising from the experimental conditions found in the impedance aggregometer. They might, nevertheless, find a physiological counterpart in areas of the circulation characterised by turbulent flow, such as sites of vessel bifurcation and vascular injury, as well as areas in which blood flow is interrupted by atheromatous plaque or developing thrombus.

**Conclusions.**

In uraemic blood red cells enhanced platelet aggregation, whereas in normal blood they reduced aggregation. The mechanism underlying these different effects was not identified. Red cells therefore play an important and complex role in uraemic haemostasis and thrombosis, not only through their well characterised rheological influence on platelet adhesion, but also through the interactions identified in this study.
CHAPTER 12 BLOOD RHEOLOGY AND ITS RELATIONSHIP TO PROGRESSIVE RENAL FAILURE

INTRODUCTION

The possible role of blood rheological abnormality in the development of focal and segmental glomerulosclerosis (FSGS) has been discussed in the thesis introduction. FSGS is the hallmark of progressive chronic renal failure (CRF) in both diabetic and non-diabetic patients, and in this study blood rheology and its relationship to progressive renal failure in both these patient groups is investigated.

There are few data available on rheology in non-diabetic progressive CRF, but in diabetics, blood flow is impeded by blood hyperviscosity and reduced red cell deformability [262-266]. Rheological abnormalities are more pronounced in diabetics with nephropathy than in those without [267], but no evidence is available to indicate whether the progression of CRF is exacerbated by rheological disturbance.

Proteinuria is a feature of progressive CRF. Impedance of glomerular blood flow caused by hyperviscosity necessitates an increase in glomerular perfusion pressure, thus promoting proteinuria [210]. On the other hand, disturbance of plasma proteins caused by protein loss in the urine promotes hyperviscosity [198]. The relationship between proteinuria and blood rheology is therefore complicated, and must be taken into account when investigating blood rheology in renal failure.

Aims of study.
(1) To identify rheological abnormality in both patients with non-diabetic CRF and diabetic nephropathy.
(2) To investigate the possible role of abnormal rheology in progressive glomerular injury by examining the relationship between rheological indices and the rate of progression of renal failure.
PATIENTS AND METHODS

Patients

a) Non-diabetic CRF patients

39 patients with progressive CRF were studied (26 males, 13 females; mean age 34.6 years, range 17 - 70). Patient details are shown in Table 12.1. In all cases CRF was due to non-immunological causes, and no patient was suffering from a nephrotic syndrome, nor was any undergoing renal replacement therapy. Viscosity and red cell deformability results were not significantly different in those patients who were taking antihypertensive therapy from results in those patients who were not on such therapy.

b) Diabetic nephropathy patients

30 patients were studied (19 males, 11 females; mean age 49.4 years, range 31 - 70). Patient details are shown in Table 12.2. Diabetic nephropathy was diagnosed by the presence of persistent proteinuria of 0.5 g/24 hours or greater. Renal biopsies were performed if the cause of renal failure was not certain to be diabetes. No patient was suffering from a nephrotic syndrome, nor was any undergoing any renal replacement therapy. 20 patients had type I DM, 3 patients with late-onset DM required insulin therapy, and 7 had type II DM. Duration of diabetes ranged from 3 to 34 years with a mean of 17.9 years. Mean level of HbA1 was 10.2% and ranged from 6.3 to 16.3%. Viscosity and red cell deformability results were not significantly different in those patients who were taking antihypertensive therapy from results in patients who were not on such therapy.

Methods

Laboratory investigations

Rheological measurements were made on blood anticoagulated with EDTA (4 mol/l final concentration), and all tests completed within 5 hours of venesection. All patients were studied on 1 to 4 occasions, at intervals of 3 months. For each patient the mean value of all attendances was calculated for each investigation. At every visit the patients brought a 24 hour urine collection, and a midstream urine specimen was cultured. If a patient had a urinary tract infection and
raised C reactive protein on the day of the visit, then the results were excluded from the study.

Plasma viscosity, whole blood viscosity measured at patients' haematocrit and standardized to a haematocrit of 0.450 l/l, red cell deformability, haematocrit, mean red cell volume (MCV) and mean red cell haemoglobin concentration (MCHC) were all measured as described in the methods section.

The following indices of renal function were also measured, as described in the methods section: creatinine clearance, 24 hour protein excretion and rate of progression of renal failure.

Analyses
(1) In order to compare blood rheology in diabetic nephropathy, non-diabetic CRF and healthy controls, 21 patients were selected from the diabetic nephropathy group to match as closely as possible a group of 21 CRF patients in terms of age, sex and degree of renal impairment, and a group of 21 healthy subjects in terms of age and sex. Details of these groups are shown in Table 12.3. Because of some imprecision in the matching, comparison of values from these three groups was performed using Student's t test for unpaired data.

(2) For investigation of the relationship between blood rheology and renal disease, data from all patients were analyzed. The diabetic and non-diabetic CRF groups were analyzed separately. Rheological results were correlated with creatinine clearance, proteinuria and rate of progression of CRF by the Spearman rank correlation method.

(3) The relationships between the three measured indicators of renal disease (creatinine clearance, proteinuria and rate of progression of renal failure) were investigated by Spearman rank correlation.

(4) In order to determine which of the measured indices had significant independent effects on the rate of progression of renal failure, forward stepwise multiple regression analysis was performed with rate of progression as the dependent variable, and proteinuria, creatinine clearance, haematocrit and plasma and whole blood viscosity values as the indicator variables. Values of rate of progression, creatinine clearance and proteinuria were log. transformed to induce a normal distribution, prior to analysis. The CRF and diabetic nephropathy results were analysed separately.
### Table 12.1 Details of patients with non-diabetic CRF.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Cr.Cl. (mls/min)</th>
<th>Cause of CRF</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG</td>
<td>M</td>
<td>31</td>
<td>19.7</td>
<td>R</td>
<td>Atenolol</td>
</tr>
<tr>
<td>TR</td>
<td>M</td>
<td>20</td>
<td>18.0</td>
<td>O</td>
<td>Atenolol, 1-a vit D, Septrin, NaHCO3</td>
</tr>
<tr>
<td>SH</td>
<td>M</td>
<td>27</td>
<td>46.6</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>SH</td>
<td>M</td>
<td>21</td>
<td>48.4</td>
<td>O</td>
<td>Enalapril</td>
</tr>
<tr>
<td>DR</td>
<td>M</td>
<td>38</td>
<td>20.0</td>
<td>O</td>
<td>Enalapril, Atenolol</td>
</tr>
<tr>
<td>GB</td>
<td>F</td>
<td>60</td>
<td>7.0</td>
<td>O</td>
<td>1-a vit D, Alucaps Folate</td>
</tr>
<tr>
<td>FF</td>
<td>M</td>
<td>56</td>
<td>14.2</td>
<td>O</td>
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</tr>
<tr>
<td>NS</td>
<td>M</td>
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<td>79.1</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>BC</td>
<td>F</td>
<td>53</td>
<td>55.9</td>
<td>R</td>
<td>Septrin</td>
</tr>
<tr>
<td>SK</td>
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<td>O</td>
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<td>BH</td>
<td>M</td>
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<td>65.1</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>JD</td>
<td>F</td>
<td>39</td>
<td>57.5</td>
<td>R</td>
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</tr>
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<td>-</td>
</tr>
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<td>F</td>
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<td>Atenolol</td>
</tr>
<tr>
<td>MG</td>
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<td>R</td>
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<td>F</td>
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<td>18.8</td>
<td>O</td>
<td>Enalapril, Frusamide, Allopurinol, Cephalexin</td>
</tr>
<tr>
<td>JT</td>
<td>M</td>
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<td>18.3</td>
<td>O</td>
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<td>O</td>
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<td>M</td>
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<td>O</td>
<td>-</td>
</tr>
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<td>M</td>
<td>29</td>
<td>26.3</td>
<td>O</td>
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</tr>
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<td>29.4</td>
<td>R</td>
<td>Enalapril</td>
</tr>
<tr>
<td>ML</td>
<td>M</td>
<td>20</td>
<td>34.0</td>
<td>O</td>
<td>Enalapril</td>
</tr>
<tr>
<td>RJ</td>
<td>M</td>
<td>23</td>
<td>14.8</td>
<td>O</td>
<td>Atenolol, Hydralazine, Cotrimoxazole</td>
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<td>O</td>
<td>Atenolol, Ranitidine</td>
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<tr>
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<td>M</td>
<td>17</td>
<td>17.6</td>
<td>R</td>
<td>Nifedipine, Septrin</td>
</tr>
<tr>
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<td>F</td>
<td>35</td>
<td>11.6</td>
<td>R</td>
<td>Enalapril</td>
</tr>
<tr>
<td>TJ</td>
<td>F</td>
<td>30</td>
<td>28.1</td>
<td>O</td>
<td>Atenolol</td>
</tr>
<tr>
<td>TS</td>
<td>M</td>
<td>20</td>
<td>62.9</td>
<td>R</td>
<td>Enalapril</td>
</tr>
<tr>
<td>DC</td>
<td>M</td>
<td>70</td>
<td>7.9</td>
<td>HT</td>
<td>Atenolol, Nifedipine, Chlothaladine, Alucaps 1-a vit D</td>
</tr>
<tr>
<td>MO</td>
<td>F</td>
<td>46</td>
<td>14.3</td>
<td>PC</td>
<td>Enalapril, Frusamide</td>
</tr>
<tr>
<td>ML</td>
<td>F</td>
<td>50</td>
<td>10.9</td>
<td>O</td>
<td>Atenolol, 1-a vit D Alucaps</td>
</tr>
<tr>
<td>AS</td>
<td>M</td>
<td>53</td>
<td>6.6</td>
<td>O</td>
<td>Atenolol</td>
</tr>
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</table>
Table 12.2 Details of patients with diabetic nephropathy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Cr.Cl. (mls/min)</th>
<th>Duration (years)</th>
<th>HbAlc (%)</th>
<th>Type</th>
<th>Drugs*</th>
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</thead>
<tbody>
<tr>
<td>GA</td>
<td>M</td>
<td>53</td>
<td>43.5</td>
<td>20</td>
<td>9.5</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td>JL</td>
<td>F</td>
<td>42</td>
<td>41.7</td>
<td>29</td>
<td>11.5</td>
<td>I</td>
<td>Enalapril, Frusamide, Trimethoprim</td>
</tr>
<tr>
<td>NS</td>
<td>M</td>
<td>43</td>
<td>18.0</td>
<td>28</td>
<td>10.4</td>
<td>I</td>
<td>Atenolol, Enalapril, Frusamide</td>
</tr>
<tr>
<td>JS</td>
<td>M</td>
<td>43</td>
<td>23.7</td>
<td>32</td>
<td>10.7</td>
<td>I</td>
<td>Ranitidine</td>
</tr>
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<td>25.5</td>
<td>34</td>
<td>11.8</td>
<td>I</td>
<td>Atenolol, Hydralazine, Trimethoprim</td>
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<td>M</td>
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<td>39.3</td>
<td>27</td>
<td>10.0</td>
<td>I</td>
<td>Captopril, Prazosin, Frusamide</td>
</tr>
<tr>
<td>MP</td>
<td>F</td>
<td>50</td>
<td>57.7</td>
<td>27</td>
<td>12.4</td>
<td>I</td>
<td>Enalapril, Frusamide</td>
</tr>
<tr>
<td>AS</td>
<td>M</td>
<td>43</td>
<td>16.0</td>
<td>23</td>
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<td>I</td>
<td>Atenolol, Enalapril, Frusamide</td>
</tr>
<tr>
<td>DS</td>
<td>M</td>
<td>62</td>
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<td>13</td>
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</tr>
<tr>
<td>JE</td>
<td>M</td>
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<td>103.9</td>
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<td>M</td>
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<td>PC</td>
<td>F</td>
<td>43</td>
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<td>25</td>
<td>11.8</td>
<td>I</td>
<td>Enalapril, Frusamide</td>
</tr>
<tr>
<td>MB</td>
<td>F</td>
<td>62</td>
<td>46.2</td>
<td>9</td>
<td>8.5</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>LP</td>
<td>M</td>
<td>54</td>
<td>33.7</td>
<td>5</td>
<td>8.4</td>
<td>I</td>
<td>Atenolol, Nifedipine, Enalapril</td>
</tr>
<tr>
<td>DG</td>
<td>F</td>
<td>66</td>
<td>25.8</td>
<td>14</td>
<td>16.3</td>
<td>II</td>
<td>Atenolol, Nifedipine, Enalapril</td>
</tr>
<tr>
<td>TH</td>
<td>F</td>
<td>58</td>
<td>26.0</td>
<td>8</td>
<td>9.6</td>
<td>II</td>
<td>Atenolol, Verapamil, Enalapril, Frusamide</td>
</tr>
<tr>
<td>AU</td>
<td>M</td>
<td>64</td>
<td>48.3</td>
<td>3</td>
<td>10.6</td>
<td>II</td>
<td>Atenolapril</td>
</tr>
<tr>
<td>MC</td>
<td>M</td>
<td>50</td>
<td>11.3</td>
<td>24</td>
<td>10.8</td>
<td>I</td>
<td>Atenolol, Minoxidil, Enalapril, Frusamide</td>
</tr>
<tr>
<td>BN</td>
<td>M</td>
<td>31</td>
<td>27.9</td>
<td>10</td>
<td>10.5</td>
<td>II</td>
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</tr>
<tr>
<td>KF</td>
<td>M</td>
<td>32</td>
<td>114.3</td>
<td>14</td>
<td>11.1</td>
<td>I</td>
<td>Enalapril, Thyroxine</td>
</tr>
<tr>
<td>MM</td>
<td>M</td>
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<td>31.8</td>
<td>20</td>
<td>8.2</td>
<td>I</td>
<td>Enalapril</td>
</tr>
<tr>
<td>SD</td>
<td>F</td>
<td>60</td>
<td>29.2</td>
<td>32</td>
<td>10.5</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>CL</td>
<td>M</td>
<td>38</td>
<td>23.3</td>
<td>20</td>
<td>8.2</td>
<td>I</td>
<td>Enalapril, Frusamide</td>
</tr>
<tr>
<td>DC</td>
<td>M</td>
<td>59</td>
<td>56.1</td>
<td>4</td>
<td>7.9</td>
<td>I</td>
<td>Atenolol, Enalapril, Frusamide</td>
</tr>
<tr>
<td>CF</td>
<td>F</td>
<td>55</td>
<td>16.0</td>
<td>15</td>
<td>12.4</td>
<td>I</td>
<td>Atenolol, Enalapril</td>
</tr>
<tr>
<td>JH</td>
<td>F</td>
<td>59</td>
<td>19.7</td>
<td>14</td>
<td>10.0</td>
<td>II</td>
<td>Atenolol, Enalapril</td>
</tr>
<tr>
<td>ED</td>
<td>F</td>
<td>55</td>
<td>96.0</td>
<td>6</td>
<td>9.0</td>
<td>II</td>
<td>Enalapril, Nicardipine</td>
</tr>
</tbody>
</table>

* Insulin and oral hypoglycaemic agents not included in list of drugs.
Table 12.3. Groups for comparison of blood rheology in diabetic nephropathy (DN), CRF and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>DN</th>
<th>CRF*</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>M:F</td>
<td>13:8</td>
<td>13:8</td>
<td>13:8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.4 (31 - 70)</td>
<td>47.6 (30 - 70)</td>
<td>41.2 (31 - 56)</td>
</tr>
<tr>
<td>Cr.Cl.(ml/min)</td>
<td>28.8 (8 - 58)</td>
<td>21.9 (7 - 78)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

* Causes of renal failure: Reflux nephropathy (7), obstructive uropathy (11), hypertensive nephropathy (2), polycystic kidneys (1).
RESULTS

(1) Comparison of blood rheology in diabetic nephropathy, non-diabetic CRF and healthy controls.
Rheological indices were similar in patients with diabetic nephropathy to those in with CRF. Plasma viscosity and standardized whole blood viscosity were higher in both groups of patients than in healthy controls. Both groups of patients were, however, anaemic and uncorrected whole blood viscosity was consequently reduced in patients compared with healthy controls (Table 12.4).
No differences were seen in red cell deformability in the three groups (Table 12.5).

(2) Correlation of blood rheology with indicators of renal disease.
Spearman rank correlations between blood rheological measurements and (a) creatinine clearance, (b) proteinuria and (c) rate of progression of renal disease in the two patient groups are shown in Tables 12.6 and 12.7.
Since red cell deformability values were not abnormal, these measurements were not included in the correlations.
In both patient groups, there was a positive relationship between creatinine clearance and haematocrit, due to increasing anaemia in patients with more advanced renal disease. Uncorrected blood viscosity measurements were also, accordingly, correlated with creatinine clearance.
In both the diabetic and non-diabetic patient groups, proteinuria showed a positive correlation with both fibrinogen (rs 0.40, p = 0.027 and rs 0.32, p = 0.05 respectively) and plasma viscosity (rs 0.45, p = 0.012 and rs 0.48, p = 0.002 respectively)(Figures 12.1 to 12.4).
Rate of progression correlated positively with plasma viscosity (Figure 12.6), fibrinogen and C reactive protein in diabetic patients. In CRF patients these relationships were not evident, though the correlation of rate of progression with plasma viscosity just failed to achieve significance (rs = 0.32; p = 0.058)(Figure 12.5).

(3) Correlations among the different indicators of renal disease.
Spearman rank correlations among the different indicators of renal disease are shown in Table 12.8. In both patient groups, the strongest correlations were between rate of progression and proteinuria (Figs
(4) **Multiple regression analysis.**

In the CRF group, proteinuria was the strongest independent predictor of the rate of progression of renal failure (standardized regression coefficient 0.56, \( p = 0.0000 \)). The only other significant parameter was uncorrected blood viscosity, measured at 230 s\(^{-1} \) (standardized regression coefficient \(-0.36, p = 0.005 \)).

In diabetic nephropathy, proteinuria was once again the index with the strongest influence on rate of progression (standardized regression coefficient 0.52, \( p = 0.0029 \)). In this case, none of the other indices showed a significant independent relationship with rate of progression (Table 12.9).
### Table 12.4. Blood rheology in diabetic nephropathy, CRF and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic nephropathy</th>
<th>CRF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Plasma viscosity (mPas)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncorrected blood viscosity (mPas) 23s⁻¹</td>
<td>5.82b (0.23)</td>
<td>5.81b (0.28)</td>
<td>6.76 (0.20)</td>
</tr>
<tr>
<td>Uncorrected blood viscosity (mPas) 230s⁻¹</td>
<td>3.88b (0.11)</td>
<td>4.00a (0.13)</td>
<td>4.41 (0.12)</td>
</tr>
<tr>
<td>Corrected blood viscosity (mPas) 23s⁻¹</td>
<td>8.50bd (0.32)</td>
<td>7.81b (0.13)</td>
<td>7.03 (0.20)</td>
</tr>
<tr>
<td>Corrected blood viscosity (mPas) 230s⁻¹</td>
<td>4.84a (0.07)</td>
<td>4.79a (0.05)</td>
<td>4.53 (0.13)</td>
</tr>
<tr>
<td>Haematocrit (L/L)</td>
<td>.348c (.010)</td>
<td>.364c (.011)</td>
<td>.435 (.007)</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.43b (0.20)</td>
<td>3.77c (0.26)</td>
<td>2.57 (0.16)</td>
</tr>
<tr>
<td>C reactive protein (mg/l)</td>
<td>1.56b (0.33)</td>
<td>1.48a (0.39)</td>
<td>0.61 (0.06)</td>
</tr>
</tbody>
</table>

Figures are mean (SEM).

a p<0.05, b p<0.01, c p<0.001 v controls. d p<0.05 v CRF group.

### Table 12.5. Red cell indices in diabetic nephropathy (DN), CRF and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic nephropathy</th>
<th>CRF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>RCTT (5 micron) (arbitrary units)</td>
<td>11.9 (0.36)</td>
<td>11.9 (0.20)</td>
<td>11.7 (0.26)</td>
</tr>
<tr>
<td>Clogging rate (5 micron) (arbitrary units)</td>
<td>1.36 (0.15)</td>
<td>1.04 (0.06)</td>
<td>1.08 (0.12)</td>
</tr>
<tr>
<td>RCTT (3 micron) (arbitrary units)</td>
<td>114 (2.0)</td>
<td>119 (3.6)</td>
<td>116 (4.6)</td>
</tr>
<tr>
<td>Clogging rate (3 micron) (arbitrary units)</td>
<td>1.83 (0.09)</td>
<td>1.76 (0.11)</td>
<td>2.24 (0.21)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>88 (1.0)</td>
<td>90 (1.0)</td>
<td>92 (1.0)</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>33.4 (0.15)</td>
<td>33.4 (0.20)</td>
<td>33.3 (0.20)</td>
</tr>
</tbody>
</table>

Figures are mean (SEM).

Measured red cell indices are red cell transit time (RCTT), filter clogging rate, mean red cell volume (MCV) and mean red cell haemoglobin concentration (MCHC).
Table 12.6 Correlations between blood rheological values and indicators of renal disease in non-diabetic CRF.

<table>
<thead>
<tr>
<th>Rheological measurement</th>
<th>Creatinine clearance</th>
<th>Proteinuria</th>
<th>Rate of progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>rs</td>
</tr>
<tr>
<td>Plasma viscosity</td>
<td>-0.42</td>
<td>0.006</td>
<td>0.48</td>
</tr>
<tr>
<td>Uncorrected blood</td>
<td>0.52</td>
<td>&lt;0.001</td>
<td>-0.05</td>
</tr>
<tr>
<td>viscosity (23 s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncorrected blood</td>
<td>0.57</td>
<td>&lt;0.001</td>
<td>-0.16</td>
</tr>
<tr>
<td>viscosity (230 s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected blood</td>
<td>-0.28</td>
<td>0.088</td>
<td>0.05</td>
</tr>
<tr>
<td>viscosity (23 s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected blood</td>
<td>-0.20</td>
<td>0.233</td>
<td>0.10</td>
</tr>
<tr>
<td>viscosity (230 s⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.72</td>
<td>&lt;0.001</td>
<td>-0.24</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.60</td>
<td>&lt;0.001</td>
<td>0.32</td>
</tr>
<tr>
<td>C reactive protein</td>
<td>-0.28</td>
<td>0.083</td>
<td>0.24</td>
</tr>
<tr>
<td>Rheological measurement</td>
<td>Creatinine clearance</td>
<td>Proteinuria</td>
<td>Rate of progression</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------</td>
<td>-------------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>rs</td>
</tr>
<tr>
<td>Plasma viscosity</td>
<td>0.02</td>
<td>0.926</td>
<td>0.45</td>
</tr>
<tr>
<td>Uncorrected blood viscosity (23 s(^{-1}))</td>
<td>0.60</td>
<td>&lt;0.001</td>
<td>-0.30</td>
</tr>
<tr>
<td>Uncorrected blood viscosity (230 s(^{-1}))</td>
<td>0.57</td>
<td>0.001</td>
<td>-0.32</td>
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<tr>
<td>Corrected blood viscosity (23 s(^{-1}))</td>
<td>-0.06</td>
<td>0.740</td>
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<tr>
<td>Corrected blood viscosity (230 s(^{-1}))</td>
<td>-0.05</td>
<td>0.792</td>
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<tr>
<td>Haematocrit</td>
<td>0.68</td>
<td>&lt;0.001</td>
<td>-0.44</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.22</td>
<td>0.246</td>
<td>0.40</td>
</tr>
<tr>
<td>C reactive protein</td>
<td>0.00</td>
<td>0.978</td>
<td>0.07</td>
</tr>
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</table>
Table 12.8 Correlations among indicators of renal disease

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Non-diabetic CRF</th>
<th>Diabetic nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
</tr>
<tr>
<td>Creatinine clearance v proteinuria</td>
<td>-0.32</td>
<td>0.050</td>
</tr>
<tr>
<td>Creatinine clearance v rate of progression</td>
<td>-0.21</td>
<td>0.204</td>
</tr>
<tr>
<td>Proteinuria v rate of progression</td>
<td>0.50</td>
<td>0.001</td>
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</table>
Table 12.9 Results of multiple regression analysis with rate of progression as the dependent variable.

<table>
<thead>
<tr>
<th>CRF (n = 39)</th>
<th>Diabetic nephropathy (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All variables</td>
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<tr>
<td>Multiple R</td>
<td>R²</td>
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<td>0.74</td>
<td>0.55</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Beta</th>
<th>p</th>
<th>Beta</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria</td>
<td>0.56</td>
<td>0.001</td>
<td>0.52</td>
<td>0.003</td>
</tr>
<tr>
<td>Uncorrected blood viscosity (230s⁻¹)</td>
<td>-0.36</td>
<td>0.005</td>
<td>-0.20</td>
<td>0.218</td>
</tr>
</tbody>
</table>
Figure 12.1 Correlation between proteinuria and plasma viscosity in non-diabetic patients with progressive renal failure.

Figure 12.2 Correlation between proteinuria and plasma fibrinogen in non-diabetic patients with progressive renal failure.
Figure 12.3 Correlation between proteinuria and plasma viscosity in patients with diabetic nephropathy.

Figure 12.4 Correlation between proteinuria and plasma fibrinogen in patients with diabetic nephropathy.
Figure 12.5 Correlation between rate of progression of renal failure and plasma viscosity in non-diabetic patients.

Figure 12.6 Correlation between rate of progression of renal failure and plasma viscosity in patients with diabetic nephropathy.
Figure 12.7 Correlation between rate of progression of renal failure and proteinuria in non-diabetic patients.

Figure 12.8 Correlation between rate of progression of renal failure and proteinuria in patients with diabetic nephropathy.
DISCUSSION

Blood viscosity and red cell deformability in non-diabetic CRF and diabetic nephropathy.
Patients showed increased plasma viscosity and standardized whole blood viscosity when compared with healthy control subjects. This pattern of rheological abnormality was similar in nature and degree in both diabetics and non-diabetics with a similar degree of renal insufficiency. Thus the superimposition of diabetes on CRF did not appear to cause any additional rheological derangement.

Red cell deformability was normal in both diabetic and non-diabetic patients, in contrast to some previous studies [202,266,267]. Red cell filtration has generally been assessed in whole blood, or in patients' plasma, but Stuart and colleagues showed that this measurement is influenced by the presence of plasma proteins and leucocytes, and that filtration results are normal in DM if the effects of these extrinsic variables are removed [268]. Similarly, red cell deformability in non-dialysed CRF patients has been found by some authors to be normal [203]. Our findings of normal filtrability using washed, leucocyte-depleted suspensions of red cells in buffer, are consistent with these reports.

Role of plasma proteins.
Plasma hyperviscosity may be explained by changes in levels of plasma proteins, in particular of those which are raised as a consequence of an acute phase response. Acute phase proteins have high intrinsic viscosity due to their molecular shape and size, and elevated levels of these proteins contribute to the rise in serum viscosity which is seen in DM [269]. Consistent with this, plasma concentrations of fibrinogen and C reactive protein, both acute phase proteins, were significantly elevated in both patient groups.

Inter-relationships among rheology, proteinuria and the rate of progression of renal failure.
It was also found that plasma viscosity was significantly correlated with the rate of progression of renal failure in diabetic nephropathy (rs = 0.50, p <0.005), and in non-diabetic CRF, though here the correlation was not quite significant (rs = 0.32, p = 0.058). Was hyperviscosity the cause or the effect of progression? Multiple
regression analysis showed proteinuria to be the strongest independent predictor of the rate of progression, and the link between plasma viscosity and progression might therefore be due to the influence of increasing proteinuria. Hepatic production of proteins such as fibrinogen is stimulated in response to protein loss [270], leading to alterations in plasma protein levels which might induce hyperviscosity. Consistent with this, both plasma viscosity and fibrinogen showed a positive correlation with proteinuria in the two patient groups studied here. In diabetic nephropathy, plasma levels of both fibrinogen and C reactive protein also correlated with progression, and it is therefore likely that, in this group, the relationship between viscosity and the rate of decline of renal function reflected not only proteinuria, but also an acute phase response in patients with a more aggressive disease. Nevertheless, the question still remain whether hyperviscosity, once established, may exacerbate progressive glomerular injury.

Influence of blood rheology on renal function.

Is there evidence that increased viscosity causes or accelerates renal failure? Published evidence which is reviewed in the thesis introduction shows that blood rheology certainly influences glomerular haemodynamics. Elevation of either plasma or whole blood viscosity results in renal vasodilatation in order to maintain renal blood flow [209], and a direct relationship exists between haematocrit, a major determinant of blood viscosity [58], and both glomerular transcapillary hydraulic pressure and filtration fraction [206]. Since intraglomerular hydraulic pressure is thought to promote proteinuria [163] and initiate endothelial injury and glomerular sclerosis [159], a feasible mechanism exists by which rheological disturbance may promote glomerular injury. These considerations led Garcia and colleagues to postulate that the anaemia of renal failure is a haemodynamically favorable adaptation in that, by reducing blood viscosity, it limits glomerular hypertension and glomerular injury [214]. In the present study, uncorrected blood viscosity was lower than normal in the patients, due to anaemia. Although this might be expected to protect against glomerular hypertension, we found that both haematocrit and uncorrected blood viscosity measured at 230 s⁻¹ correlated negatively with the rate of progression of renal failure, a result inconsistent with a causative relationship between haematocrit and glomerular injury. Observations
of CRF patients receiving recombinant human erythropoietin have also
failed to confirm a harmful role for rising haematocrit [215].
Furthermore, Ditzel found no correlation between glomerular filtration
rate and viscosity in insulin-dependent diabetics with hyperfiltration
(but without overt nephropathy) and interpreted this as evidence
against a viscosity-induced increase in intraglomerular pressure [271].
In view of the discrepancy which exists between the haematocrit (and
consequently the viscosity) of blood perfusing the microcirculation and
that of blood flowing in the larger vessels [61], it may be misleading
to use apparent viscosity values measured in venous blood to predict
events occurring in the capillaries, such as those of the glomerulus.
In these vessels, plasma viscosity may be a more relevant rheological
measurement than blood viscosity measured at venous haematocrit
[56].
Simpson has proposed that rheological disturbance in CRF and diabetic
nephropathy leads to an exaggerated rise in efferent arteriolar blood
viscosity following loss of filtrate along the glomerular capillary, and
that hyperviscosity thus produced promotes glomerular capillary
hypertension [211,272]. The correlation of plasma viscosity with the
rate of progression of renal failure found in this study is consistent
with Simpson's hypothesis, but does not constitute proof of it.

Conclusions.

The rate of progression of renal failure has been found to correlate
with plasma viscosity and with proteinuria. Increased plasma viscosity
is probably a consequence of increased acute phase proteins, due to
both proteinuria and glomerular injury. Even so, hyperviscosity might
act in a vicious cycle to worsen glomerular injury. Further work is
required to define the role of blood rheology in the progression of
renal failure.
INTRODUCTION

A number of observations suggest that the haemostatic system may participate in the progression of CRF, as outlined in the thesis introduction. This might be detectable by measuring plasma concentrations of sensitive haemostatic markers. When investigating patients with renal failure the interpretation of haemostatic markers may be complicated by the fact that the kidney participates in their clearance from the circulation. Plasma concentrations of the platelet α-granule protein β-thromboglobulin (BTG) are elevated for this reason and its usefulness is therefore limited [273]. On the other hand, platelet factor 4 (PF4) is not retained and is a reliable marker of platelet activation in renal failure [274]. Similarly, a number of sensitive markers of fibrin turnover are unreliable in patients with renal failure, due to reduced clearance by the kidney. This is true of fibrinopeptide A (FpA) [275], FgE Ag [275] and the beta 15-42 fragment [276]. Monoclonal antibodies have recently become available to breakdown products of cross-linked fibrin, such as D dimer [235]. Immunoassays using these antibodies might allow a more reliable assessment of fibrin breakdown in renal failure.

Aims of study

(1) To assess the usefulness of plasma D dimer as a marker of fibrin breakdown in renal failure.

(2) To look for haemostatic activation in diabetic and non-diabetic progressive renal failure. Since haemostasis is altered in patients with a nephrotic syndrome [277,278], as well as in those with immunologically mediated glomerular injury [16,279], such patients were excluded.

(3) To investigate the role of haemostatic activation in progressive glomerular injury by examining its relationship to the rate of progression of renal failure.
PATIENTS AND METHODS

Patients
39 patients with non-diabetic CRF, and 30 patients with diabetic nephropathy were studied. These are the same patients used for the study of blood rheology in progressive CRF, and their details are given in chapter 12.
No patient in either group was undergoing dialysis treatment. All had some degree of proteinuria, but none was suffering from a nephrotic syndrome. Comparison of haemostatic indices in patients on antihypertensives with values in those who were not on such therapy showed no statistical differences.

Methods
Venesection Blood was taken after overnight fasting, using a 19 gauge needle and minimal stasis, and anticoagulated as described in the methods section. Plasma and serum samples were obtained by centrifugation at 4°C for 30 minutes at 1500g, and stored at -40°C until assayed. Platelet rich plasma (PRP) for platelet aggregation studies was obtained as described in the methods section.
Haemostatic markers The following measurements were made in order to reflect different aspects of haemostatic activation;
(1) D dimer was used as a measure of the formation and subsequent breakdown of crosslinked fibrin.
(2) PF4 was used as a measure of intravascular platelet activation since it is a reliable haemostatic marker in renal failure [274]. Platelet reactivity was further characterized by measurements of spontaneous aggregation and of serum TxB₂ levels.
(3) Plasma levels of the endothelium-derived protein vWFAg were measured as an indicator of vascular injury [122,280].
(4) Coagulation factor VII (FVIIc) were measured as a risk factor for atheroma-related cardiovascular disease [281].
Assay techniques D dimer, PF4, serum TxB₂, spontaneous platelet aggregation, vWFAg, and FVIIc were all measured as described in the
methods section. Serum FgE Ag measurements were kindly performed by Drs D.A.Lane and H.Ireland in the Department of Haematology, Charing Cross Hospital, using a radioimmunoassay technique [275].

Renal function Plasma creatinine, urinary protein, creatinine clearance and the rate of progression of renal failure (measured as rate of decline in creatinine clearance) were all measured as described in the methods section.

Analyses
(1) In order to confirm the validity of D dimer measurements in renal disease, plasma D dimer concentrations were correlated against creatinine clearance values from 91 patients with renal disease and varying degrees of renal dysfunction. By comparison, a similar correlation was performed between fibrinogen fragment E antigen (FgE Ag), a fibrinogen derivative which is known to be cleared in part by the kidney, and creatinine clearance values from 28 patients with renal disease. D dimer and FgE Ag values were log. transformed prior to correlation with creatinine clearance by linear regression analysis.

(2) In order to detect haemostatic abnormality in non-diabetic CRF and diabetic nephropathy, haemostatic indices were compared in 21 patients selected from each of the two patient groups to match one another in terms of age, sex and degree of renal impairment, and in 21 healthy controls matched for age and sex. Details of these groups are given in table 12.3. Statistical comparisons were by the Mann Whitney test.

(3) The relationship between haemostatic indices and the severity of renal disease were investigated by Spearman rank correlation of these indices with creatinine clearance, proteinuria and rate of progression of renal failure.

(4) In order to determine which factors had a significant independent effect on the rate progression of renal failure, stepwise multiple regression analysis was performed with rate of progression as the dependent variable, and the different haemostatic measurements as the indicator variables. As in the analysis of blood rheological indices (see previous chapter), proteinuria, creatinine clearance, and C reactive protein (CRP) values were also included as indicator variables in order
to determine whether any relationships shown between haemostatic factors and the rate of progression were secondary to proteinuria, loss of renal function, or an acute phase response. Values of proteinuria and creatinine clearance were log. transformed prior to analysis. The non-diabetic CRF and diabetic nephropathy groups were analysed separately, using data from all patients studied in each group.

RESULTS

(1) Correlation of D dimer and FgE Ag with creatinine clearance.
There was no significant correlation between creatinine clearance and D dimer in 91 patients with renal disease (r = -0.14, p > 0.05)(Fig. 13.1), whereas FgE Ag showed a significant negative correlation with creatinine clearance (r = -0.57, p < 0.002)(Fig. 13.2). A close correlation was seen between D dimer and FgE Ag (r = 0.71, p <0.001).

(2) Comparison of haemostatic indices in CRF, diabetic nephropathy and healthy controls.
Spontaneous platelet aggregation, and plasma concentrations of D dimer, vWFAg, and FVIIc were all elevated to a similar degree in both CRF and diabetic nephropathy (Table 13.1). In contrast, serum TxB₂ was reduced. PF4 was higher than controls in both patient groups, but this was not statistically significant.

(3) Correlation of haemostatic indices with indicators of the severity of renal disease.
Correlations performed by the Spearman rank technique showed a number of significant relationships.

(a) Correlations with creatinine clearance.
In non-diabetic CRF, creatinine clearance showed significant inverse correlations with vWFAg (rs = -0.37, p = 0.02) and spontaneous platelet aggregation (rs = -0.35, p = 0.029). There were no significant correlations in diabetic patients between creatinine clearance and haemostatic parameters.
(b) Correlations with proteinuria.
None of the haemostatic factors measured in this study showed a significant correlation with proteinuria.

(c) Correlations with the rate of progression of renal failure.
In non-diabetic CRF, the rate of progression of renal failure correlated positively with proteinuria (rs = 0.50, p = 0.001), vWFAg (rs = 0.37, p = 0.021) and serum TxB₂ (rs = 0.46, p = 0.003) (Fig. 13.3).
In diabetic nephropathy, rate of progression showed a negative correlation with creatinine clearance (rs = -0.45, p = 0.012) and a positive correlation with proteinuria (rs = 0.52, p = 0.003) and vWFAg (rs = 0.38, p = 0.042) (Fig. 13.4).

(4) Multiple regression analysis
The factors which showed a significant independent effect on the rate of progression of renal failure were proteinuria and serum TxB₂ in non-diabetic CRF, and proteinuria and vWFAg in diabetic nephropathy (Table 13.2).
Figure 13.1 Correlation between plasma concentrations of D dimer and creatinine clearance values in 91 patients with renal disease.

Figure 13.2 Correlation between serum concentratons of FgE Ag and creatinine clearance in 28 patients with renal disease.
Table 13.1 Haemostatic markers in non-diabetic progressive CRF and diabetic nephropathy.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls</th>
<th>CRF</th>
<th>Diabetic Nephropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>D dimer (ng/ml)</td>
<td>52 (27 - 260)</td>
<td>220&lt;sup&gt;c&lt;/sup&gt; (42 - 1020)</td>
<td>280&lt;sup&gt;c&lt;/sup&gt; (34 - 740)</td>
</tr>
<tr>
<td>vWFAg (U/ml)</td>
<td>1.00 (0.67 - 1.60)</td>
<td>1.58&lt;sup&gt;c&lt;/sup&gt; (0.91 - 2.69)</td>
<td>1.40&lt;sup&gt;c&lt;/sup&gt; (0.91 - 2.77)</td>
</tr>
<tr>
<td>PF4 (ng/ml)</td>
<td>10 (2 - 26)</td>
<td>12 (6 - 26)</td>
<td>16 (2 - 97)</td>
</tr>
<tr>
<td>Serum TxB2 (ng/10&lt;sup&gt;9&lt;/sup&gt; plats)</td>
<td>288 (41 - 1043)</td>
<td>140&lt;sup&gt;c&lt;/sup&gt; (17 - 524)</td>
<td>173&lt;sup&gt;b&lt;/sup&gt; (35 - 1169)</td>
</tr>
<tr>
<td>Spont. Aggn. (%)</td>
<td>3 (1 - 11)</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVIIc (U/ml)</td>
<td>0.96 (0.54 - 1.60)</td>
<td>1.09&lt;sup&gt;a&lt;/sup&gt; (0.40 - 1.43)</td>
<td>1.08 (0.25 - 2.69)</td>
</tr>
</tbody>
</table>

D dimer, von Willebrand factor antigen (vWFAg), platelet factor 4 (PF4), serum thromboxane (TxB<sub>2</sub>), spontaneous platelet aggregation (spont. aggn.), and factor FVII clotting activity (FVIIc) in non-diabetic progressive renal failure (CRF), diabetic nephropathy, and matched healthy controls. Figures are median (range). <sup>a</sup> p<0.05; <sup>b</sup> p<0.01; <sup>c</sup> p<0.001.
Figure 13.3 Correlation between rate of progression of renal failure and serum thromboxane in non-diabetic patients.

rs = 0.46
p = 0.003

Figure 13.4 Correlation between rate of progression of renal failure and plasma vWFAg in patients with diabetic nephropathy.

rs = 0.38
p = 0.042
Table 13.2 Results of multiple regression analysis with rate of progression as dependent variable.

<table>
<thead>
<tr>
<th></th>
<th>CRF (n = 39)</th>
<th>Diabetic nephropathy (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Variables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple R</td>
<td>0.75</td>
<td>0.70</td>
</tr>
<tr>
<td>R2</td>
<td>0.57</td>
<td>0.48</td>
</tr>
<tr>
<td>p</td>
<td>0.008</td>
<td>0.014</td>
</tr>
<tr>
<td><strong>Independent Variables</strong></td>
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<td></td>
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<tr>
<td>Beta</td>
<td>Beta</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Proteinuria</td>
<td>0.56</td>
<td>0.45</td>
</tr>
<tr>
<td>Serum TxB2</td>
<td>0.29</td>
<td>0.45</td>
</tr>
<tr>
<td>vWFAg</td>
<td>0.18</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.195</td>
<td>0.533</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Multiple correlation coefficients (R), and standardized regression coefficients (beta) of proteinuria, serum thromboxane (TxB2) and von Willebrand factor antigen (vWFAg) against rate of progression of renal failure (as the dependent variable) in non-diabetic progressive chronic renal failure (CRF) and diabetic nephropathy.
DISCUSSION

**D dimer as a haemostatic marker in renal failure.**

The preliminary study of the relationship between plasma D dimer and creatinine clearance confirmed that D dimer is a useful marker of fibrin breakdown in patients with renal failure. The correlation observed between FgE Ag and D dimer suggests that they were both derived from the breakdown of cross-linked fibrin. FgE (molecular weight 50,000) is, however, partially cleared by the kidney [275], and this is reflected by the fact that it showed a negative correlation with creatinine clearance. Studies in mice have shown that D dimer is eliminated primarily by the liver, although there is slight uptake by the kidney [282]. D dimer (molecular weight 190,000) is too large to be filtered at the glomerulus, and it failed to show a significant correlation with renal function, implying that any role played by the kidney in the elimination of D dimer is very limited, and therefore that elevated levels of D dimer in renal failure will reflect a genuine increase in the breakdown of cross-linked fibrin, rather than reduced elimination of D dimer.

**Haemostatic activation in progressive renal failure.**

I have demonstrated haemostatic activation in both non-diabetic CRF and diabetic nephropathy. This is consistent with published evidence of intraglomerular deposition of platelets and fibrin in human chronic renal disease [194], and supports the hypothesis that glomerular vascular injury and microthrombosis contribute to the development of FGS. R² values of 0.57 and 0.48 respectively were found in multiple regression analysis of data from non-diabetic CRF and diabetic nephropathy, suggesting that roughly half the variation in the rate of progression of renal failure could be explained by the indicator variables included in the analysis. Other variables, such as blood pressure or dietary protein intake, which were not included in the analysis, are also clearly important in determining the rate of progression.

van Hulstelijn and colleagues reported activation of haemostasis in some patients with CRF on the basis of elevated plasma concentrations of fibrinopeptide A (FpA) and PF4 [283]. Raised levels of both FpA and of
the platelet α-granule protein β-thromboglobulin were also reported by Lane et al, but, since plasma PF4 was not raised, the authors concluded that FpA elevation in CRF was due, at least in part, to decreased elimination, rather than to haemostatic activation [275]. My demonstration of raised plasma concentrations of D dimer, which is a reliable haemostatic marker in renal failure, suggests a true increase in fibrin turnover in CRF. This is consistent with the shortening of fibrinogen half-life reported in primary FGS [19,284], and might reflect the glomerular fibrin deposition which is a feature of FGS both in experimental animals [155] and in human patients [147,178]. The observed levels (200 to 300 ng/ml) are lower than those in patients with overt venous thrombosis [235], but would be consistent with the scale of the FGS lesion. It is important to note, however, that there was no correlation between the rate of progression of renal failure and plasma D dimer, and it is therefore possible that I have detected thrombosis occurring at sites unrelated to the kidney, especially since the incidence of atheroma-related cardiovascular disease is increased in these patients. Risk factors for atheroma.

Both FVIIc and fibrinogen have been identified by the Northwick Park heart study as risk factors for cardiovascular mortality [281], and raised plasma concentrations of FVIIc, as well as of fibrinogen (see previous chapter), were present in both patient groups. Since FGS is in many ways analogous to atherosclerosis [159], this haemostatic imbalance in progressive renal failure may be important in the promotion of glomerular injury, as well as of cardiovascular morbidity.

vWFAg and endothelial injury.

The value of vWFAg as a marker of endothelial injury in CRF is uncertain, firstly because of reduced reticulo-endothelial clearance of vWFAg in renal failure [123], and secondly because plasma vWFAg behaves as an acute phase reactant [124]. Plasma concentrations of vWFAg were raised in this study, as previously reported by others [119,120,285]. This was interpreted by Wegmuller et al [120] and Turney et al [119] as a reflection of vascular injury, either due to glomerular damage or to atherosclerosis. In the latter study, disparity between the plasma
concentrations vWF\text{Ag} and factor VIII coagulant (FVIII\text{c}) suggested intravascular coagulation. In the present study, acute phase changes were evident in both patient groups, as shown by a small but significant elevation of CRP (see previous chapter), and this might therefore explain the increase in vWF\text{Ag}. Nevertheless, vWF\text{Ag}, but not CRP, correlated independently with the rate of progression of renal failure in the diabetic nephropathy group; plasma vWF\text{Ag} may therefore be a true reflection of glomerular vascular injury in these patients.

**Platelet activation.**

Platelet activation promotes renal injury in glomerulonephritis [194], but in the present study plasma PF4 showed only a small elevation in the two patient groups, which was not statistically significant. PF4 lacks some sensitivity as a marker of intravascular platelet activation [286], and is cleared from the circulation very rapidly [287]; a chronic, microthrombotic lesion such as FGS might therefore be too subtle to be detected by measurement of this marker in peripheral blood. Platelets in both progressive CRF and diabetic nephropathy showed a greater tendency to aggregate spontaneously than in healthy controls, and this in spite of the fact that the generation of TxB\textsubscript{2} in clotting blood was reduced. It is uncertain whether spontaneous aggregation reflects prior intravascular activation of platelets, or whether increased aggregation in uraemic PRP results simply from alterations in the plasma environment. It nevertheless suggests an increased tendency of the platelets to aggregate when stimulated within the body. Recent evidence has shown a close relationship between spontaneous platelet aggregation and coronary mortality in survivors of myocardial infarction, suggesting that spontaneous aggregation may be a useful biological marker of atherosclerotic vascular damage [288].

Reduction in serum TxB\textsubscript{2} is a manifestation of the uraemic platelet defect [102], although its exact mechanism is uncertain [105] (see thesis introduction). I have previously shown this defect to be present even in the early stages of progressive CRF (see chapter 9 of thesis). Despite being lower than in healthy controls, serum TxB\textsubscript{2} showed a weak, but significant independent relationship to the rate of progression of renal
failure in non-diabetics. This finding is of interest since thromboxane synthetase inhibitors protect the kidney in both the renal ablation [180] and the Dahl-S rat models of progressive CRF [181]. In the former study, amelioration of progressive CRF could not be explained by reduction in blood pressure, renal hypertrophy or glomerular hyperfiltration (which, if anything, was increased). The authors concluded that inhibition of platelet aggregation and intraglomerular thrombosis was an important mechanism by which benefit was achieved. My findings raise the intriguing possibility that, at least in non-diabetics, the platelet defect associated with renal failure protects against further progression of glomerular injury. Platelet-derived thromboxane might promote renal injury not only by reinforcing platelet activation within the glomerulus, but also by causing renal vasoconstriction [289] and mesangial contraction [197]. It is not yet clear, however, whether thromboxane plays such a direct role in the development of FSGS, since GR 32191, a thromboxane receptor antagonist with only limited anti-platelet activity, failed to protect experimental rats from the consequences of a reduction in renal mass [182].

Proteinuria.

The correlation between proteinuria and the rate of progression of diabetic nephropathy in both non-diabetic and diabetic renal failure was highlighted in the previous chapter. In this study proteinuria was again shown to be more strongly related to the rate of progression than any of the haemostatic factors which were measured. These results confirm those of Williams and colleagues, who found significant correlations between proteinuria and the rate of progression in CRF due to glomerulonephritis and pyelonephritis, and a suggestive, though not statistically significant, correlation in diabetic nephropathy [145]. I would support their conclusion that proteinuria may be an important prognostic index in established renal failure. The relationship between proteinuria and the rate of progression of CRF may be due to the fact that the amount of proteinuria reflects the level of intraglomerular hydraulic pressure, which is both the driving force for the passage of protein across the glomerular basement membrane, and a factor in the
initiation and development of FGS [163].

Conclusions.

I have found haemostatic changes which may reflect the microthrombotic component of FGS. Proteinuria, vWFAg and serum TxB$_2$ were related to the rate of progression of renal failure, and might therefore have prognostic value.
CHAPTER 14 ANTI-PLATELET THERAPY IN DIABETIC AND NON-DIABETIC PROGRESSIVE RENAL FAILURE

INTRODUCTION

The histopathological, experimental and clinical evidence for the involvement of the haemostatic mechanism in the development of FSGS, and hence in the progression of CRF, has been detailed in the thesis introduction. In my previous two chapters, evidence has been given of rheological and haemostatic changes which might promote, or reflect, a microthrombotic process within the kidney in patients with progressive CRF. On this basis a clinical trial of anti-thrombotic therapy in progressive CRF was performed, with the aims of slowing the rate of progression of CRF and reducing proteinuria. The study was performed in both non-diabetic and diabetic patients with progressive CRF, but the results obtained from these two groups were analysed separately.

The treatment chosen was a combination of dipyridamole, aspirin and maxEPA, each of which possesses anti-platelet activity which, together, might afford an additive beneficial effect. The anti-platelet action of dipyridamole is achieved by three main mechanisms [290], which may be summarized as follows: (1) prevention of the cellular uptake of adenosine, a substance with potent platelet-inhibitory activity; (2) inhibition of platelet phosphodiesterase, and thus of the breakdown of intra-platelet cAMP; (3) stimulation of vascular prostacyclin release.

Aspirin irreversibly acetylates the cyclo-oxygenase enzyme, thus preventing the conversion of arachidonic acid into endoperoxide intermediates and, in the platelet, the synthesis of thromboxane A₂ [291]. Acetylation of vascular cyclo-oxygenase results in a reduction in prostacyclin synthesis, which, theoretically, might be counter-productive to the anti-thrombotic effect of aspirin. It has been proposed that the use of low doses of aspirin (50 - 100 mg/day) should allow complete inhibition of platelet thromboxane synthesis, while preserving the capacity of the endothelium to produce prostacyclin [292].
MaxEPA is a fish oil concentrate which is rich in Ω3 series polyunsaturated fatty acids (PUFA), in particular eicosapentaenoic acid (18% v/v) and docosahexaenoic acid (12% v/v). The actions of maxEPA are not fully understood, but are explained in part by the enrichment of platelet and other cellular membranes with Ω3 PUFA, leading to an increase in membrane fluidity and an alteration in the balance between eicosanoid derivatives of Ω6 series PUFA (for example arachidonic acid) and Ω3 series PUFA (for example eicosapentaenoic acid) [293].

In previous clinical studies, dipyridamole reduced proteinuria [189,294], and in combination with aspirin [186], warfarin [185] or heparin [284] reduced haemostatic activation and stabilized renal function in CRF patients. The administration of maxEPA to CRF patients corrected lipid abnormality, and reduced both blood pressure and platelet responsiveness [295]. MaxEPA also reduced blood viscosity by causing erythrocytes to become more deformable [296].

Aims of study
(1) To investigate, in both diabetic and non-diabetic nephropathy, the influence of anti-platelet therapy on:
   a) the rate of progression of renal failure.
   b) the level of proteinuria.
   c) haemostatic indices.

PATIENTS AND METHODS

CRF Patients without diabetes
44 non-diabetic patients with chronic renal failure, not receiving any renal replacement therapy, were recruited.
Of these, 10 patients progressed to end-stage renal failure requiring dialysis during the run-in phase of the study, and a further 3 during the early part of the treatment phase.
11 patients were excluded because their rate of progression of CRF was too slow to be measureable.
6 other patients were lost from the study: 2 due to intercurrent illness (myocardial infarction in one, and infectious mononucleosis leading to acute renal failure in the other), 2 due to non-compliance, and 2 because they went abroad. The results presented are therefore based on the 14 patients who completed the study.

This group included 11 males and 3 females, with a mean age of 31.4 years (range 19 - 52). 13 patients were being treated for hypertension by the time that the treatment phase of the study commenced; the aim was to maintain supine blood pressure at a level of 135/90 or less.

Details of these patients are given in Table 14.1.

Diabetic nephropathy patients
Diabetic nephropathy was diagnosed by the presence of persistent proteinuria of 0.5 g/24 hours or greater. 21 insulin-dependent diabetics were recruited into the study. Of these, 4 came to require dialysis while still in the run-in phase, 5 were excluded because their rate of progression was unmeasureable and 4 were lost to follow-up for other reasons. The remaining 8 patients completed the study.

In this group there were 6 males and 2 females; mean age was 43.1 years (range 33 - 59). Duration of diabetes ranged from 5 - 28 years with a mean of 22.6 years. All 8 patients were suffering from retinopathy, and 4 also from neuropathy. All were being treated for hypertension. Details of these patients are given in Table 14.2.
Table 14.1 Details of patients with non-diabetic CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Cause of CRF</th>
<th>Mean run-in Cr.Cl.</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>M</td>
<td>20</td>
<td>R</td>
<td>62.8</td>
<td>Enalapril</td>
</tr>
<tr>
<td>SB</td>
<td>M</td>
<td>19</td>
<td>R</td>
<td>29.4</td>
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<tr>
<td>BH</td>
<td>M</td>
<td>41</td>
<td>O</td>
<td>39.0</td>
<td>Atenolol</td>
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<tr>
<td>GE</td>
<td>M</td>
<td>29</td>
<td>O</td>
<td>27.1</td>
<td>Enalapril, Nifedipine</td>
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<tr>
<td>RB</td>
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<td>45</td>
<td>O</td>
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<td>Atenolol, Allopurinol, 1α vit D</td>
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<tr>
<td>MO</td>
<td>F</td>
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<td>PC</td>
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<td>Enalapril, Frusamide</td>
</tr>
<tr>
<td>JT</td>
<td>M</td>
<td>38</td>
<td>O</td>
<td>18.0</td>
<td>Atenolol</td>
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<tr>
<td>HR</td>
<td>F</td>
<td>23</td>
<td>GN</td>
<td>29.9</td>
<td>Atenolol</td>
</tr>
<tr>
<td>NJ</td>
<td>M</td>
<td>25</td>
<td>O</td>
<td>12.2</td>
<td>Atenolol, Nifedipine, 1α vit D, NaHCO₃</td>
</tr>
<tr>
<td>MH</td>
<td>M</td>
<td>22</td>
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<tr>
<td>DR</td>
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<td>38</td>
<td>O</td>
<td>19.6</td>
<td>Enalapril, Atenolol</td>
</tr>
</tbody>
</table>

R = reflux nephropathy.
O = surgically corrected obstructive uropathy in 9 patients (caused by post urethral valves (4), stones (2), solitary kidney with obstruction of contralateral ureter (2) and bladder extrophy (1)).
PC = polycystic kidneys.
GN = chronic familial glomerulonephritis.

Table 14.2 Details of patients with diabetic nephropathy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>HBAlc</th>
<th>Mean run-in Cr.Cl.</th>
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<td>MP</td>
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<td>M</td>
<td>33</td>
<td>10.0</td>
<td>39.5</td>
<td>Enalapril, Frusamide, Prazosin</td>
</tr>
<tr>
<td>NS</td>
<td>M</td>
<td>43</td>
<td>10.4</td>
<td>19.4</td>
<td>Enalapril, Atenolol, Frusamide</td>
</tr>
<tr>
<td>DH</td>
<td>M</td>
<td>38</td>
<td>10.0</td>
<td>21.4</td>
<td>Enalapril, Frusamide, Thyroxine, Ranitidine</td>
</tr>
<tr>
<td>DC</td>
<td>M</td>
<td>59</td>
<td>7.9</td>
<td>55.2</td>
<td>Enalapril, Atenolol, Frusamide</td>
</tr>
</tbody>
</table>
Study Protocol

Patients were formally assessed at 3 monthly intervals during the study, which consisted of a run-in period, a treatment period and a wash-out period.

a) Run-in period: patients were interviewed by a dietician at the start of the run-in period of the study, and their dietary protein intake adjusted so that it did not exceed one gram /kg body weight /day. During the run-in period of the study, which lasted at least 9 months (mean 16.7 months), patients' dietary protein intake and blood pressure were carefully controlled. At each attendance, blood samples were taken for the measurement of plasma creatinine, urea and other standard biochemical indices. Proteinuria and haemostatic indices were measured at 3 monthly intervals during the last 6 months of the run-in period.

b) Treatment period: treatment consisted of dipyridamole (300 mg/day) for 3 months, followed by the addition of aspirin (75 mg/day) and, after a further 3 months, MaxEPA (10 ml/day). The drugs were introduced in this stepwise manner so that the effect of each on proteinuria and haemostasis could be measured. Patients then continued on these 3 anti-platelet drugs for a further 6 months, making a treatment period of 12 months in all. Anti-platelet therapy was then stopped. Other drug therapy remained constant throughout this period, and proteinuria and haemostatic indices were measured 3 months after the addition of each anti-platelet agent.

c) Wash-out period: patients were assessed 3 months after stopping anti-platelet therapy, and measurements were made of proteinuria and haemostasis. Plasma creatinine measurements taken during a period of at least 6 months (mean 14.9 months) after stopping anti-platelet therapy were used to assess the rate of progression of CRF during this wash-out period.

In order to assess the risk of bleeding caused by anti-platelet therapy, skin capillary bleeding time was measured in 12 patients (3 diabetics and 9 non-diabetics) at 3 points in the study: (1) during the run-in period, (2) during the period in which patients were receiving a combination of dipyridamole and aspirin and (3) during the period in which patients were receiving a combination of dipyridamole, aspirin and MaxEPA.
Measurements of renal function
Plasma creatinine, urinary protein excretion, creatinine clearance and rate of progression of renal failure were all measured as described in the methods section of the thesis.

Haemostatic measurements
Blood viscosity, bleeding time, plasma concentrations of platelet factor 4 (PF4), D dimer and von Willebrand factor antigen (vWFAg), and serum concentrations of C reactive protein and immunoreactive thromboxane were all measured as described in the methods section of the thesis.

Comparisons between patients who responded and did not respond to treatment.
Mean values obtained during the run-in period of mean arterial pressure, creatinine clearance, proteinuria, rate of progression and haemostatic indices were compared between patients whose rate of progression was retarded during anti-platelet therapy (responders) and those whose rate of progression was unchanged or accelerated (non-responders). Mean arterial pressures during the treatment period were also compared between the two groups.

Statistics
All statistical comparisons were performed by the Wilcoxon test for matched pairs. Run-in values of proteinuria and haemostatic indices were examined for trends by comparing values at the beginning and end of the run-in period. If no trend was seen, results obtained during treatment were compared in each patient with the mean value obtained from that patient during the run-in period. If there was a trend, then treatment values were compared with measurements obtained at the end of the run-in period.

RESULTS

(1) Blood pressure
Blood pressure fell during the run-in period, but there was no further significant change in blood pressure during either the treatment or wash-out periods (Fig. 14.1).
(2) Proteinuria

Proteinuria fell during the run-in period, and continued to fall during the treatment period. Compared with values at the end of the run-in period, the reduction during treatment was significant only in diabetic patients receiving a combination of dipyridamole and aspirin, or of dipyridamole, aspirin and Maxepa. When the anti-platelet therapy was withdrawn, proteinuria increased again towards pre-treatment levels (Fig. 14.2).

(3) Rate of progression of renal failure

a) Non-diabetic CRF:

7 out of the 14 patients studied showed a retardation of the rate of progression of renal failure during the treatment period, 3 patients showed no change, and 4 patients showed an acceleration of their renal failure. In two of these latter patients, a sharp impairment of renal function associated with Maxepa therapy contributed to the acceleration of renal failure. The mean fall in the rate of progression over the whole group was 0.30 ml/min/month (95% confidence intervals -0.10 to 0.70 ml/min/month). This was not statistically significant (p = 0.198).

During the wash-out period, the rate of progression was slower than during the run-in period in 9 out of 14 patients, unchanged in two and faster in three. There was a mean fall in the rate of progression of 0.06 ml/min/month (95% confidence intervals -0.26 to 0.38 ml/min/month)(p = 0.414) (Fig. 14.3) (Table 14.3).

b) Diabetic nephropathy:

Progression of renal failure was slower during the treatment period than during the run-in period in 5 out of the 8 patients studied; in 2 patients the rate of progression was unchanged, and in one it was accelerated. The mean fall in the rate of progression was 0.14 ml/min/month (95% confidence intervals -0.03 to 0.31 ml/min/month). This was not statistically significant (p = 0.142).

During the wash-out period, the rate of progression was slower than during the run-in period in 4 patients, unchanged in two and faster in one. One patient suffered an episode of acute renal failure during this period (associated with the administration of X ray contrast medium), and his rate of progression could not, therefore, be assessed. Compared with the run-in period, there was a mean fall in the rate of
progression of 0.31 ml/min/month (95% confidence intervals -0.09 to 0.71 ml/min/month) (p = 0.181) (Fig. 14.4) (Table 14.3).

(4) Haemostatic indices
In both diabetic and non-diabetic patients, there were no significant alterations over the course of the study in circulating levels of PF4, vWF Ag or of CRP (Figs 14.5 to 14.7). Blood viscosity measured at both 23 s^{-1} (data not shown) and 230 s^{-1} (Fig. 14.8) was significantly reduced in both diabetics and non-diabetics during the period in which patients were receiving maxEPA. In non-diabetics blood viscosity was also reduced during treatment with dipyridamole alone (Fig. 14.8). Plasma D dimer fell in both patient groups during both the treatment and wash-out periods, although this reduction was significant only during the treatment period when patients were receiving dipyridamole alone (Fig. 14.9). Serum TxB2 showed a marked reduction during the period of the study during which patients were receiving aspirin (Fig. 14.10).

Bleeding time was 5.08 (0.38) (mean and SEM) during the run-in period. This increased to 10.00 (1.08) during treatment with a combination of dipyridamole and aspirin (p <0.005), and further to 11.78 (1.70) when patients were treated with dipyridamole, aspirin and maxEPA (p <0.002).

(5) Comparisons between responders and non-responders.
In the non-diabetic group there were 7 patients in each category (Table 14.4). There were no significant differences, although there was a tendency for rate of progression, proteinuria and D dimer to be higher in responders. Blood pressure was similar between the two groups during both the run-in period (responders 99 ± 4.3; non-responders 98 ± 2.2 mmHg), and the treatment period (responders 98 ± 1.8 mmHg, non-responders 96 ± 2.5 mmHg).

In diabetic nephropathy (5 responders and 3 non-responders) (Table 14.5), creatinine clearance was significantly lower, and rate of progression significantly higher in responders. Mean arterial pressure was significantly higher during the run-in period in non-responders than in responders (112 ± 3.7 v 103 ± 0.6 mmHg, p <0.05), but during the treatment period values were not significantly different (103 ± 3.5 v 101 ± 2.5 mmHg).

(7) Side effects of treatment
Three patients could not tolerate the full dose of dipyridamole; this
was due to gastrointestinal symptoms (one patient), and headache and muscle pains (two patients). All three patients were, however, able to continue on a reduced dose of dipyridamole, two on 150 mg/day and one on 200 mg/day.

Treatment with Maxepa had to be reduced or stopped in 7 patients, due to unpalatability (3), exacerbation of anginal pain (1), eyesight disturbance (1), and acute reduction in renal function (2). Three of these patients were able to continue on a reduced dose of Maxepa (4 - 6 capsules per day). One patient suffered vaginal bleeding after 9 months of anti-platelet therapy, and treatment was stopped at this time. In this patient the bleeding time had risen from 5.54 minutes during the run-in period, to 12.6 minutes when bleeding occurred. No other patient suffered significant haemorrhagic complications.
Figure 14.1 Mean arterial pressure (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles).

Figure 14.2 Proteinuria (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles). p values indicate significant changes from values at the end of the run-in period.
Figure 14.3 Decline in creatinine clearance during the three phases of the study in 14 non-diabetic patients.

Figure 14.4 Decline in creatinine clearance during the three phases of the study in 8 patients with diabetic nephropathy.
Table 14.3 Rate of progression of renal failure (mls/min/month) during the three periods of the study.

a) Non-diabetic CRF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Run-in</th>
<th>Treatment</th>
<th>Wash-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.77</td>
<td>0.79</td>
<td>2.13</td>
</tr>
<tr>
<td>2</td>
<td>1.11</td>
<td>0.66</td>
<td>0.92</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>1.11</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>0.92</td>
<td>0.24</td>
<td>0.54</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>0.07</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>0.52</td>
<td>0.57</td>
<td>0.28</td>
</tr>
<tr>
<td>7</td>
<td>0.38</td>
<td>0.93</td>
<td>0.26</td>
</tr>
<tr>
<td>8</td>
<td>0.90</td>
<td>0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>9</td>
<td>0.12</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>10</td>
<td>0.13</td>
<td>0.32</td>
<td>0.14</td>
</tr>
<tr>
<td>11</td>
<td>0.58</td>
<td>0.36</td>
<td>0.12</td>
</tr>
<tr>
<td>12</td>
<td>0.96</td>
<td>0.30</td>
<td>0.94</td>
</tr>
<tr>
<td>13</td>
<td>0.44</td>
<td>0.67</td>
<td>0.88</td>
</tr>
<tr>
<td>14</td>
<td>0.52</td>
<td>0.22</td>
<td>0.24</td>
</tr>
<tr>
<td>Mean</td>
<td>0.58</td>
<td>0.46</td>
<td>0.55</td>
</tr>
<tr>
<td>SEM</td>
<td>0.08</td>
<td>0.09</td>
<td>0.15</td>
</tr>
</tbody>
</table>

b) Diabetic Nephropathy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Run-in</th>
<th>Treatment</th>
<th>Wash-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.18</td>
<td>0.17</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>0.48</td>
<td>-0.60</td>
</tr>
<tr>
<td>3</td>
<td>0.61</td>
<td>0.50</td>
<td>0.39</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>-0.30</td>
<td>0.13</td>
</tr>
<tr>
<td>5</td>
<td>0.13</td>
<td>0.37</td>
<td>0.23</td>
</tr>
<tr>
<td>6</td>
<td>0.44</td>
<td>0.22</td>
<td>-0.03</td>
</tr>
<tr>
<td>7</td>
<td>0.14</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.46</td>
<td>0.47</td>
<td>0.44</td>
</tr>
<tr>
<td>Mean</td>
<td>0.38</td>
<td>0.24</td>
<td>0.11</td>
</tr>
<tr>
<td>SEM</td>
<td>0.09</td>
<td>0.10</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Values of rate of progression of renal failure are in mls/min/month. A negative value indicates an improvement in renal function. In both non-diabetic CRF and diabetic nephropathy, statistical comparison of the groups, using the Wilcoxon test for matched pairs, showed no significant differences.
Figure 14.5 Platelet factor 4 (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles).

Figure 14.6 vWFAg (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles).
Figure 14.7 C reactive protein (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles).

Figure 14.8 Blood viscosity (mean and SEM) measured at a shear rate of 230 s\(^{-1}\) in non-diabetic (open circles) and diabetic patients (closed circles). p values indicate significant changes from mean values obtained during the run-in period.
Figure 14.9 D dimer (mean and SEM) in non-diabetic (open circles) and diabetic patients (closed circles). p values indicate significant changes from mean values obtained during the run-in period.

Figure 14.10 Serum thromboxane in non-diabetic (open circles) and diabetic patients (closed circles). p values indicate significant changes from mean values obtained during the run-in period.
Table 14.4. Comparison of renal and haemostatic indices between responders and non-responders in non-diabetic CRF patients

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>99 (4.3)</td>
<td>98 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Proteinuria (g/24 hours)</td>
<td>3.72 (0.76)</td>
<td>1.84 (0.46)</td>
<td>NS</td>
</tr>
<tr>
<td>Rate of progression (ml/min/month)</td>
<td>0.75 (0.24)</td>
<td>0.41 (0.09)</td>
<td>NS</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>239 (52.2)</td>
<td>108 (24.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet factor 4 (ng/ml)</td>
<td>13.3 (3.1)</td>
<td>13.5 (2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>von Willebrand factor antigen (U/ml)</td>
<td>1.79 (0.19)</td>
<td>1.38 (0.16)</td>
<td>NS</td>
</tr>
<tr>
<td>C reactive protein (mg/l)</td>
<td>1.13 (0.46)</td>
<td>1.43 (0.46)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum thromboxane (ng/10^9 platelets)</td>
<td>170 (49.2)</td>
<td>225 (67.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figures are mean (SEM). Statistical comparisons were performed using the Mann-Whitney U test.
Table 14.5. Comparison of renal and haemostatic indices between responders and non-responders in diabetic nephropathy.

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>103 (0.6)</td>
<td>112 (3.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proteinuria (g/24 hours)</td>
<td>2.4 (0.66)</td>
<td>2.9 (0.47)</td>
<td>NS</td>
</tr>
<tr>
<td>Rate of progression (ml/min/month)</td>
<td>0.46 (0.13)</td>
<td>0.26 (0.10)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>291 (114.7)</td>
<td>158 (12.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet factor 4 (ng/ml)</td>
<td>21.4 (7.9)</td>
<td>20.0 (7.0)</td>
<td>NS</td>
</tr>
<tr>
<td>von Willebrand factor antigen (U/ml)</td>
<td>1.55 (0.21)</td>
<td>1.42 (0.27)</td>
<td>NS</td>
</tr>
<tr>
<td>C reactive protein (mg/l)</td>
<td>0.86 (0.26)</td>
<td>2.02 (1.03)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum thromboxane (ng/10^9 platelets)</td>
<td>258 (55.7)</td>
<td>214 (80.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figures are mean (SEM). Statistical comparisons were performed using the Mann-Whitney U test.
Effect of anti-platelet therapy on the rate of progression of renal failure.

There is good evidence for the participation of the haemostatic system in immune complex-mediated glomerular injury [297], and antithrombotic therapy has been used in forms of chronic renal disease which appear to have an immune basis such as membranoproliferative glomerulonephritis [185,186], and IgA nephropathy [188]. In contrast, the present study was designed to investigate, in human patients, the role of platelets in chronic non-immune glomerular injury. The majority of the patients studied were suffering from chronic renal diseases in which immune mechanisms are not considered to play a major pathogenetic role. The haemostatic changes and clinical outcome which followed anti-platelet therapy were essentially similar in both non-diabetic CRF and diabetic nephropathy.

In both groups, roughly half the patients showed a slowing of the rate of progression of renal failure with treatment. In each group however, the overall mean retardation was not statistically significant. This was due not only to the variation in response, but also to the small number of recruited patients who completed the study. The reasons for this unavoidable drop-out rate are detailed above.

Differences between responders and non-responders.

Analysis of clinical outcome in each group as a whole might overlook the existence of sub-groups of patients who are more likely than others to respond to anti-platelet therapy. Comparison of responders with non-responders suggested that retardation of CRF was more likely in those patients with a higher rate of progression during the run-in phase of the study. Rather than delineating a sub-group of CRF patients who respond to anti-platelet therapy, this is more likely to reflect the fact that, over the relatively short duration of the study, it was easier to show improvement in rapidly progressive than in slowly progressive disease. This simple fact is important when analysing treatment results. In a controlled study of 38 patients with IgA nephropathy lasting a mean of 33.2 months, Chan and coworkers concluded that anti-platelet therapy did not favourably modify the course of the disease [188]. In their control group, however, mean
creatinine clearance only fell from 73 to 72 ml/min over the study period. Renal function was, therefore, declining at such a slow rate that it would have been impossible to show any modification. It should be noted that in my study all the diabetic patients and 60% of non-diabetics were receiving an angiotensin converting enzyme inhibitor, a drug which may itself slow the decline of renal function and reduce proteinuria.

In the present study, comparison between responders and non-responders in the diabetic group showed significantly higher mean arterial pressures in non-responders. This might suggest that, unless blood pressure is adequately controlled in these patients, other forms of treatment have little chance of success. It is, however, difficult to draw conclusions from such small numbers, especially as the difference in blood pressure in the two groups was not significant during the treatment period.

Effect of anti-platelet therapy on proteinuria.

Platelets contain a variety of vasoactive and cationic mediators capable of influencing the permeability of the glomerular basement membrane [298] and anti-platelet therapy might therefore reduce proteinuria by preventing the release of such mediators at sites of glomerular injury. Alternatively, proteinuria might be reduced by drug-induced changes in glomerular haemodynamics [190]. My findings of a reduction in proteinuria in diabetic nephropathy agree with previous reports in which patients with this condition have been treated with either a combination of aspirin and dipyridamole [299], or with dipyridamole alone [294]. Proteinuria was reduced to a greater extent with a combination of aspirin and dipyridamole, than with the latter drug alone, but maxepa appeared to confer no further benefit and was poorly tolerated. Dipyridamole has also been shown to reduce proteinuria in non-diabetic renal disease [189], but the fall I observed in proteinuria in non-diabetic CRF was not statistically significant.

Influence of anti-platelet therapy on haemostatic indices.

In nephrotic children with primary FGS, treatment with heparin and dipyridamole preserved renal function, and corrected the previously shortened half-life of both platelets and fibrinogen [284]. In the present study, anti-platelet therapy reduced serum thromboxane levels, but in spite of this there were no significant alterations in levels of
PF4 (a marker of intravascular platelet activation), vWF Ag (an endothelium-derived protein which might reflect vascular injury) or of CRP (a marker of the acute phase response). There was, however, a reduction during treatment in plasma D dimer, a breakdown product of crosslinked fibrin. In patients with progressive renal failure, but without nephrotic syndrome, circulating markers of haemostatic activation show only a moderate elevation, which would be consistent with the microscopic scale of the FGS lesion (see previous chapter). This might explain the failure to detect marked haemostatic changes during the treatment period of this study.

Conclusions.
Anti-platelet therapy had a favorable influence on the course of progressive CRF in 50% of the patients studied, and in diabetics there was a significant fall in urinary protein excretion. Although suggestive of benefit, the results were not statistically significant, and further long-term studies with larger numbers of patients will be required finally to determine the value anti-platelet therapy in progressive CRF. The difficulties which might be involved in this are highlighted both by the recent re-evaluation of one such project [300], and, in the work reported here, by the small proportion of recruited patients who finally completed the study.
SECTION IV CONCLUSIONS
CHAPTER 15 CONCLUSIONS

1) The bleeding tendency of uraemia

Haematological abnormalities which have been reported in uraemia include those of platelets, red cells, circulating haemostatic proteins and the vessel wall. The relative impact of these different factors on primary haemostasis may vary between patients, and this might explain some of the discrepancies which exist in the literature.

The work described in this thesis has shown that prolongation of the skin capillary bleeding time does not necessarily develop in parallel with the progression of chronic renal failure, and this indicates that the uraemic bleeding tendency is unlikely to be due simply to the influence of retained metabolites on the haemostatic system. The fact that the haematocrit was the only measured index which showed a significant (negative) correlation with the bleeding time, and that rHuEPO shortened the uraemic bleeding time without altering either platelet responsiveness or storage granule content, suggests that anaemia is a more important determinant of uraemic bleeding than any intrinsic platelet abnormality. My study of platelet aggregation in whole blood shows that the capacity of red cells to support platelet aggregation is enhanced in uraemia, although the mechanism involved remains uncertain.

Areas for future study. Despite exhaustive study of the uraemic platelet, questions remain concerning the pathogenesis and treatment of uraemic bleeding. Effective therapies include DDAVP, cryoprecipitate, conjugated oestrogens, and the correction of anaemia (either by red cell transfusion or by rHuEPO), but only in the latter case is the mode of action understood. It is likely that new experimental approaches will be required in order to resolve the outstanding questions, and vascular function in renal failure is one area which merits further research. Reduced adhesion of platelets to the vessel wall in uraemia might involve not only the rheological deficit caused by anaemia, but also a vascular abnormality such as the loss of sub-endothelial receptors for either vWF or platelets, or an imbalance in the endothelial production of nitric oxide, a potent anti-adhesive molecule [301].

A further study which is required is a comparison of haemostatic
function in uraemic patients who suffer from haemorrhage, with that in a group of matched patients with a similar degree of renal functional impairment, but without haemorrhagic symptoms. This might allow pathogenetic haemostatic abnormalities to be discriminated from those which arise simply as a consequence of renal failure.

2) The role of haemostasis in the progression of CRF.

The histopathological features of FSGS clearly indicate that glomerular vascular injury and microthrombosis occur in progressive CRF, and the amelioration of experimental CRF by anti-thrombotic drugs suggests that in these animal models, the haemostatic system is directly involved in progressive glomerular injury. In the studies reported in this thesis I have examined whether the same may be true of human CRF. Haemostatic and rheological disturbance which might promote, or reflect, glomerular microthrombosis was detected in patients with both diabetic and non-diabetic nephropathy. Although some of these laboratory abnormalities correlated with the rate of progression of CRF, there was no direct evidence that they were of pathogenetic relevance.

The use of anti-platelet therapy in progressive CRF patients allowed the role of haemostasis in this condition to be assessed more directly. This study is important in being one of only a very small number to address this question in human subjects. The results suggested that, in a proportion of patients, platelet inhibition might be a useful adjunct to anti-hypertensive therapy in retarding the progression of CRF. Due to the small number of patients who completed the study, it was difficult to identify those characteristics which distinguished the sub-group of patients who benefited from anti-platelet therapy from those whose disease was unaffected. The failure of some patients to respond might simply have been due to the fact that their rate of progression was too slow for a measureable benefit to be detected over the study period. The pattern of response to therapy was similar in both diabetic and non-diabetic patients.

Future studies. The results described here are sufficiently encouraging to warrant further investigations, ideally studying a larger number of patients with rapidly progressive CRF, and assessing the rate of progression by serial measurements of isotopic GFR.
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PUBLICATIONS


Reprints of these papers are enclosed.
RECOMBINANT HUMAN ERYTHROPOIETIN SHORTENS THE URAEMIC BLEEDING
TIME WITHOUT CAUSING INTRAVASCULAR HAEMOSTATIC ACTIVATION.

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G.H. Neild.

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ABSTRACT
Blood rheology and haemostasis have been investigated in 8 haemodialysis patients during treatment with recombinant human erythropoietin (rHuEPO). The aim was to elucidate the mechanism by which rHuEPO improves haemostasis, and to determine whether rHuEPO promotes intravascular coagulation. Investigations were performed before, and after 3 months of treatment. Haemoglobin and haematocrit rose significantly after rHuEPO (p<0.001) and there was a concurrent shortening of the bleeding time. No significant changes were observed in platelet aggregation, thromboxane generation, or platelet nucleotide content during the treatment period. Whole blood viscosity increased following rHuEPO (p<0.01), but plasma viscosity and red cell deformability were unchanged, as were markers of intravascular platelet activation and plasma levels of cross-linked fibrin derivatives. No patient suffered from thrombosis during the study period, and elevation of the haematocrit in uraemic patients up to 0.35 with rHuEPO did not appear to lead to intravascular coagulation. Shortening of the prolonged bleeding time in haemodialyzed patients following rHuEPO appeared to be due to the increase in circulating red cells, rather than to changes in platelet reactivity.

INTRODUCTION
The anaemia of chronic renal failure (CRF) is corrected by recombinant human erythropoietin (rHuEPO). rHuEPO produces a dose-related rise in haemoglobin (1,2), and improvement in general well-being in uraemic patients (3). It is reported also

Key words: Erythropoietin, uraemia, bleeding time, haemostasis.
to shorten the skin capillary bleeding time (4,5), indicating a
correction of the uraemic bleeding tendency (6).
The benefits of rHuEPO can, however, be offset by detrimental
side effects. These include hypertension (7), and thrombosis (8),
both of which may be related to an increase in blood viscosity
comitant with the rise in haemoglobin. Concern has been
expressed that sustained treatment with rHuEPO might increase
cardiovasacular morbidity (9).
Shortening of the bleeding time after treatment with rHuEPO
indicates an enhancement of platelet/vessel wall interactions,
which could be due to both elevation of the haematocrit, and
changes in platelet function. The increased incidence of
thrombosis after rHuEPO may reflect the combined effects of
increased blood viscosity and enhanced haemostatic function.
In order to study the mechanisms by which rHuEPO improves
haemostatic function, and to determine whether it promotes
intravascular coagulation, we have measured blood rheological
indices, platelet function and markers of haemostatic activation,
in a group of uraemic patients before and after correction of
anaemia with rHuEPO. For the purpose of this study we define
intravascular coagulation as a state characterized by an increase
in circulating markers of haemostatic activation.

PATIENTS AND METHODS

Patients 8 patients with end-stage CRF requiring regular
haemodialysis were studied (7 males, 1 female; mean age 31.2
years, range 20 - 58). Causes of renal failure were obstructive
uropathy (2), reflux nephropathy (1), glomerulonephritis (1),
dysplastic kidneys (2), renal tuberculosis (1) and haemolytic
uraemic syndrome (1). 7 of the patients were anephric. All eight
patients were anaemic before starting rHuEPO (pre-treatment
haemoglobin concentration ranged from 5.4 - 7.7 g/dl).
Patients were receiving from 10.8 to 19.2 m²hrs of haemodialysis
per week, using cuprophan membranes. Drug therapy in the patient
group included nifedipine (2 patients), propranolol (1 patient),
and naproxen (1 patient). No other drugs known to affect platelet
function were taken, and drug therapy remained constant
throughout the period of the study.
Haemostatic indices were also measured on healthy subjects who
had not taken any drugs known to influence haemostasis for the
previous 10 days. The normal ranges quoted for the various tests
were obtained from between 18 and 50 individuals. Healthy control
data is given for reference only, and no formal comparison was
made between results from patients and healthy controls.

Methods This was a within-person comparison in which patients
acted as their own controls. Haemostatic investigations were
carried out before, and 3 months after commencing treatment with
rHuEPO. In four patients, investigations were repeated after 6
months of rHuEPO.
rHuEPO was administered after dialysis, starting at a dose of 150
U/Kg/week, and increasing the dosage, where necessary, in
increments of 75 U/Kg/week until a rise in haemoglobin was
observed. Haemoglobin was measured weekly, and treatment
controlled to avoid a rate of increase of more than 2g/dl/month.

Venesection Venesection was performed between 9 and 11 AM on the
day following dialysis, using a 19 gauge needle and minimal stasis. Citrate anticoagulant concentration in blood used for aggregation studies was corrected for the effects of changes in haematocrit (10).

Platelet aggregation studies were completed within 2 hours, and blood rheological investigations within 5 hours of venesection. Plasma samples for measurement of coagulation factors and haemostatic markers were obtained by centrifugation at 4°C for 30 minutes at 1500g, and stored at -40°C until assayed.

Blood counts Full blood counts were performed on blood anticoagulated with EDTA (4mmol/l final concentration), using an automated electronic counter (Coulter Electronics, Luton, UK).

Blood rheology Whole blood and plasma viscosity were measured using a Wells-Brookfield Cone and plate viscometer, and red cell deformability was assessed by filtration of a washed, leucocyte-depleted suspension of red cells through polycarbonate filters of 5 micron pore size, as previously described (11). Measured parameters were the red cell transit time (RCTT), reflecting the deformability of the red cell suspension as a whole, and the filter clogging rate (CR), which allows detection of subpopulations of rigid cells (12).

Platelet function studies Bleeding time was measured using a Simplate II device (Organon Teknika, Turnhout, Belgium). Platelet aggregation in response to ADP, collagen and ristocetin, and generation of immunoreactive thromboxane (TxB₂) were measured as previously described (13).

Measured aggregation indices were ADP threshold (minimum dose of ADP giving secondary aggregation), collagen ED50 (collagen dose giving 50% of maximal initial rate of aggregation - maximal defined as that rate of aggregation obtained in response to 20 ug/ml collagen), ristocetin-induced platelet aggregation (R.I.P.A) (initial rate of aggregation in response to 1.25 mg/ml ristocetin), and spontaneous aggregation (degree of aggregation obtained after stirring platelet-rich plasma (PRP) in an aggregometer for 15 minutes at 37°C). The latter two parameters were expressed in terms of percent aggregation, where 100% was the difference in light transmission between platelet rich- and platelet-poor plasma.

Serum thromboxane was measured after allowing blood to clot for 60 minutes at 37°C and released thromboxane (rel. TxB₂) was measured in stirred PRP after 4 minutes stimulation with 20 ug/ml collagen.

Platelet nucleotides were measured by luminometry using firefly luciferase (14), after lysis of platelets with triton X 100 (3.5 % final concentration), and extraction of proteins with ethanol. Extracts were stored diluted in tris/EDTA buffer (pH 7.5) at -40°C until assayed.

Coagulation factors and haemostatic markers. Fibrinogen, von Willebrand factor, (vWVF), von Willebrand factor antigen (vWFAg), C reactive protein (CRP), D dimer and platelet factor 4 (PF4) were measured as previously described (13,15). D dimer and PF4 were chosen as haemostatic markers because their plasma levels are not influenced by loss of renal excretory function (15,16). Standard techniques were used for the measurement of platelet aggregate ratio (P.A.R.) (17) and euglobulin clot lysis time.
Statistics All comparisons were by the Wilcoxon test for matched pairs. Changes in haematocrit were correlated with changes in bleeding time by linear regression analysis. Bleeding time values greater than 20 minutes were taken as being 21 minutes when analysed.

RESULTS

Haematological response to rHuEPO Haemoglobin rose from 6.7 g/dl (5.4 - 7.7) (median and range), to 10.8 g/dl (8.6 - 12.3) after three months of rHuEPO. Haematocrit values rose in parallel (table I). During the study period there was no change in the patients' weight after haemodialysis. In three patients in whom detailed radio-isotopic studies were performed, red cell volume increased from a mean of 13.99 ml/kg before treatment to 25.97 ml/kg after 3 months of rHuEPO, while mean plasma volume diminished from 58.19 to 47.28 ml/kg. The ratio of whole body haematocrit to venous haematocrit remained unchanged over the treatment period (mean values were 0.991 pre-treatment and 1.005 post-treatment). This, together with the patients' stable weight, indicate that there was no change in patients' state of hydration during the study.

Platelet counts and white cell counts showed a small, but statistically significant rise following 3 months of rHuEPO (p = 0.04 and 0.03 respectively) (table I).

Side Effects of rHuEPO No patient suffered thrombosis during the study, but in 3 patients an increase in heparin dose was required during dialysis to prevent the appearance of small clots in the dialyser bubble trap. Mean pre-dialysis plasma creatinine concentrations did not change significantly after treatment with rHuEPO.

Blood rheology Whole blood viscosity measured at shear rates of 23 and 230s⁻¹ was significantly elevated after 3 months treatment with rHuEPO. No change was seen in plasma viscosity or indices of red cell deformability (table II).

Platelet Function Bleeding time showed a significant fall following 3 months of rHuEPO treatment, though it was still markedly prolonged in two patients (fig. 1.). One of these patients had stopped rHuEPO for the previous two weeks while on holiday, and also showed only a small rise in haemoglobin at 3 months. When re-tested at 6 months, her haemoglobin had risen and her bleeding time had corrected (fig. 1.). Linear regression analysis of change in haematocrit against change in bleeding time gave a coefficient of correlation of 0.69 (p = 0.054, 95% confidence intervals -0.03 to 0.94) (fig. 2). No significant changes in platelet aggregation, thromboxane generation or nucleotide content were seen over the treatment period, except for a drop in collagen-induced thromboxane release after 3 months of rHuEPO (p = 0.046)(table III).
Fig. 1. Bleeding times in haemodialysis patients before and after 3 and 6 months treatment with rHuEPO. Results greater than 20 minutes are taken as 21 minutes.

Fig. 2. Change in bleeding time v change in haematocrit in 8 patients treated with rHuEPO. \( r = 0.69, p = 0.054 \).
### Table I

Haematological Response to rHuEPO

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>White cells (x10^9/l)</td>
<td>5.2</td>
<td>6.3*</td>
</tr>
<tr>
<td>Red cells</td>
<td>2.37</td>
<td>3.64**</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>6.7</td>
<td>10.8**</td>
</tr>
<tr>
<td>Haematocrit (l/l)</td>
<td>202</td>
<td>.334**</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>30.0</td>
<td>30.4</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>33.2</td>
<td>32.6</td>
</tr>
<tr>
<td>Platelets (x10^9/l)</td>
<td>194</td>
<td>260*</td>
</tr>
</tbody>
</table>

Haematological indices (median and range) before and after 3 months of rHuEPO. *p<0.05; **p<0.001 v pre-treatment values.

### Table II

Blood Rheology in rHuEPO Treated Patients

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>3 months</th>
<th>Normals</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV 230s^-1 (mPas)</td>
<td>1.60</td>
<td>1.60</td>
<td>1.3 - 1.8</td>
</tr>
<tr>
<td>BV 23s^-1 (mPas)</td>
<td>4.25</td>
<td>5.95*</td>
<td>4.8 - 8.4</td>
</tr>
<tr>
<td>BV 230s^-1 (mPas)</td>
<td>2.80</td>
<td>3.85*</td>
<td>3.3 - 5.2</td>
</tr>
<tr>
<td>RCTT (AU)</td>
<td>11.6</td>
<td>11.5</td>
<td>9.9 - 13.9</td>
</tr>
<tr>
<td>CR (AU)</td>
<td>1.14</td>
<td>1.28</td>
<td>0.18 - 2.02</td>
</tr>
</tbody>
</table>

Plasma viscosity (PV) measured at a shear rate of 230s^-1, whole blood viscosity (BV) measured at shear rates of 23s^-1 and 230s^-1, red cell transit time (RCTT) and filter clogging rate (CR) in patients before and after 3 months treatment with rHuEPO. RCTT and CR are expressed in arbitrary units (AU). Results are median (range).

* p<0.01 v pre-treatment values.
Table III.

Bleeding Time and Platelet Indices in rHuEPO Treated Patients.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>3 months</th>
<th>Normals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl. time (mins)</td>
<td>20.27</td>
<td>7.82**</td>
<td>3 - 7</td>
</tr>
<tr>
<td>(mins)</td>
<td>(9.18 - 21.00)</td>
<td>(4.67 - 17.46)</td>
<td></td>
</tr>
<tr>
<td>ADP threshold (umol/l)</td>
<td>2.28</td>
<td>2.10</td>
<td>&lt; 4.3</td>
</tr>
<tr>
<td>Collagen ED50 (ug/ml)</td>
<td>0.68</td>
<td>0.72</td>
<td>&lt; 1.53</td>
</tr>
<tr>
<td>R.I.P.A (%/min)</td>
<td>26</td>
<td>42</td>
<td>13 - 109</td>
</tr>
<tr>
<td>Spont. Aggn. (%)</td>
<td>7.5</td>
<td>4.5</td>
<td>&lt; 11</td>
</tr>
<tr>
<td>Serum TxB₂ (ng/10⁶ pl.)</td>
<td>208</td>
<td>123</td>
<td>103 - 440</td>
</tr>
<tr>
<td>Rel. TxB₂ (ng/10⁶ pl.)</td>
<td>464</td>
<td>392*</td>
<td>254 - 1443</td>
</tr>
<tr>
<td>Total ATP (nmol/10⁹ pl.)</td>
<td>14.6</td>
<td>14.4</td>
<td>11.2 - 32.2</td>
</tr>
<tr>
<td>Total ADP (nmol/10⁹ pl.)</td>
<td>4.4</td>
<td>4.4</td>
<td>5.0 - 13.7</td>
</tr>
<tr>
<td>ATP:ADP ratio</td>
<td>4.5</td>
<td>3.7</td>
<td>&lt; 4.3</td>
</tr>
<tr>
<td></td>
<td>(1.5 - 6.8)</td>
<td>(2.1 - 7.8)</td>
<td></td>
</tr>
</tbody>
</table>

Bleeding time (Bl. time), ADP threshold, collagen ED50 (coll. ED50), ristocetin induced platelet aggregation (R.I.P.A.), spontaneous aggregation (spont aggn.), thromboxane production in clotting blood (serum TxB₂) and after stimulation with 20 ug/ml collagen (rel. TxB₂), and platelet total nucleotide contents in haemodialysis patients before, and after 3 months of treatment with rHuEPO. Results are median (range). * p<0.05; ** p<0.01 v pre- treatment values.

Markers of haemostatic activation. There were no significant alterations in measurements of haemostatic activation (D dimer, PF4, P.A.R.), adhesive proteins (fibrinogen, vWF), acute phase markers (C reactive protein, vWFAg) or fibrinolysis (ECLT) (table IV).
Table IV

Markers of Haemostatic Activation and Other Measurements.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>3 months</th>
<th>Normals</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.A.R</td>
<td>0.96</td>
<td>0.95</td>
<td>0.77 - 1.09</td>
</tr>
<tr>
<td>(0.84 - 0.98)</td>
<td>(0.87 - 1.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF4</td>
<td>26</td>
<td>28</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(6 - 55)</td>
<td>(9 - 72)</td>
<td></td>
</tr>
<tr>
<td>D dimer</td>
<td>162</td>
<td>42</td>
<td>&lt; 150</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>(23 - 370)</td>
<td>(22 - 438)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>3.1</td>
<td>3.3</td>
<td>1.3 - 3.8</td>
</tr>
<tr>
<td>(g/l)</td>
<td>(2.3 - 3.7)</td>
<td>(2.5 - 4.2)</td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>1.84</td>
<td>1.64</td>
<td>0.36 - 1.65</td>
</tr>
<tr>
<td>(u/ml)</td>
<td>(0.78 - 4.98)</td>
<td>(0.58 - 4.90)</td>
<td></td>
</tr>
<tr>
<td>vWFAg</td>
<td>1.62</td>
<td>1.88</td>
<td>0.45 - 1.53</td>
</tr>
<tr>
<td>(U/ml)</td>
<td>(0.86 - 3.40)</td>
<td>(0.78 - 4.40)</td>
<td></td>
</tr>
<tr>
<td>ECLT</td>
<td>208</td>
<td>240</td>
<td>&lt; 320</td>
</tr>
<tr>
<td>(mins)</td>
<td>(104 - 381)</td>
<td>(130 - 301)</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>1.02</td>
<td>4.15</td>
<td>&lt; 1.36</td>
</tr>
<tr>
<td>(mg/l)</td>
<td>(0.65 - 7.20)</td>
<td>(0.60 - 15.6)</td>
<td></td>
</tr>
</tbody>
</table>

Platelet aggregate ratio (P.A.R.), platelet factor 4 (PF4), D dimer, von Willebrand factor (vWF), von Willebrand factor antigen (vWFAg), euglobulin clot lysis time (ECLT) and C reactive protein (CRP) in haemodialysis patients before, and 3 months after treatment with rHuEPO. Results are median (range).

Results of studies carried out at 6 months. Results from the 4 patients analysed after 6 months of treatment (using paired non-parametric statistics) showed a pattern similar to that obtained after 3 months. Haemoglobin was 9.8 (8.3 - 11.0) g/dl (median and range)(p<0.001 compared with pre-treatment values), and whole blood viscosity was significantly elevated at shear rates of both 23s⁻¹ and 230s⁻¹ (p<0.05). Bleeding times were again significantly shorter than pre-treatment values (fig. 1.)(p<0.05).
Other tests of blood rheology, platelet function and haemostatic markers showed no statistically significant changes.

DISCUSSION

In this study the bleeding time in uraemic patients was markedly shortened by correction of anaemia with rHuEPO. Moia and colleagues similarly showed correction of the bleeding time, and of platelet adhesion to sub-endothelium following rHuEPO treatment, and postulated that this was due to the haemostatic effects of increased red cell concentration (4).
Anaemia is a major factor in the pathogenesis of uraemic bleeding. The bleeding time is shortened by red cell transfusions (19,20), and significant negative correlations have been demonstrated between haematocrit and bleeding time in progressive
CRF (13), and in haemodialysis patients (19,20). Red cells may improve haemostasis by a variety of mechanisms. They increase platelet adhesion in flowing blood by promoting the radial movement of platelets towards the vessel wall, an effect which is dependent upon haematocrit (21), red cell size (22) and deformability (23). Platelet activation may be initiated or augmented by the release from red cells of ADP (24), and by the binding and inactivation of prostacyclin (25). A study of impedance aggregometry in uraemic whole blood showed an erythrocyte-induced enhancement of platelet aggregation that was not evident in blood from healthy controls (26). The haemostatic role of red cells may therefore be more prominent in uraemic patients than in healthy subjects.

The pathogenesis of defective platelet/vessel wall interactions in uraemia is, however, multifactorial. In addition to anaemia, abnormalities of platelet aggregation, adhesion, thromboxane production, granule content, and interaction with vWF have all been reported (27). Abnormal platelet cytoplasmic calcium regulation (28) and increased vascular prostacyclin production (29) may also contribute to uraemic bleeding.

In the present study, there were no changes in thromboxane production or platelet nucleotide content which could explain the correction of the bleeding time, and plasma levels of vWF, as well as platelet interaction with this adhesive protein (reflected by R.I.P.A.) were unaltered. Contrary to the findings of van Geet and colleagues (5), we found no change in ex-vivo platelet aggregation. The small rise we observed in the platelet count, while statistically significant, was also insufficient to account for the improvement in haemostasis. In contrast, marked elevation of the haematocrit coincided with correction of the bleeding time. There was a general tendency for change in the haematocrit to parallel change in bleeding time (fig. 2). This correlation did not quite reach statistical significance (p = 0.054), but this was probably due to the small number of observations, and to the fact that an absolute value was not available for 4 of the 8 pre-treatment bleeding time measurements (recorded as >20 minutes). The changes found after 3 months of rHuEPO in white cell count, platelet count and collagen-stimulated thromboxane release were significant only at the 5% level, and should be interpreted cautiously in view of the large number of statistical comparisons performed.

In our study we found no evidence of intravascular haemostatic activation following rHuEPO. Whole blood viscosity increased, but post-treatment values were clustered at the lower end of the normal range. Although heparin requirements during dialysis were increased in 3 patients, there were no episodes of thrombosis, and markers of fibrin breakdown, intravascular platelet activation and the acute phase response were not significantly altered during the study.

In conclusion, our findings suggest that, in haemodialysis patients, elevation of the haematocrit up to 0.35 with rHuEPO allows correction of the bleeding time, without causing intravascular haemostatic activation. Shortening of the bleeding time with rHuEPO appears to be due to the haemostatic effects of increased red cell numbers, rather than to changes in intrinsic platelet 'function.
ACKNOWLEDGEMENTS

We thank the St. Peter's Trust and the Sir Halley Stewart Trust for supporting this project, and Ortho Cilag (UK) for providing rHuEPO.

REFERENCES


Plasma D Dimer: A Useful Marker of Fibrin Breakdown in Renal Failure

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Key words
Renal failure – D dimer – Fibrin – Fibrinolysis

Summary
D dimer and other large fragments produced during the breakdown of crosslinked fibrin may be measured by enzyme immunoassay using monoclonal antibodies. In 91 patients with renal disease and varying degrees of renal dysfunction, plasma D dimer showed no correlation with renal function, whereas FgE antigen, a fibrinogen derivative which is known to be cleared in part by the kidney, showed a significant negative correlation with creatinine clearance. Plasma concentrations of D dimer were, however, increased in patients with chronic renal failure (244 ± 31 ng/ml) (mean ± SEM) and diabetic nephropathy (308 ± 74 ng/ml), when compared with healthy controls (96 ± 13 ng/ml), and grossly elevated in patients with acute renal failure (2,451 ± 1,007 ng/ml). The results indicate an increase in fibrin formation and lysis, and not simply reduced elimination of D dimer by the kidneys, and are further evidence of activated coagulation in renal disease. D dimer appears to be a useful marker of fibrin breakdown in renal failure.

Introduction
Immunological assays of fibrin and fibrinogen degradation products (FDPs) in serum have been widely used for the detection of intravascular fibrin formation and its subsequent lysis (1). However, the polyclonal antibodies used show cross-reactivity between derivatives of fibrin and fibrinogen, and inaccuracy may arise when FDPs are measured in serum since some of the material detected may be produced during preparation of the sample (2, 3). More sensitive and specific immunoassays are now available for fibrinogen fragments D and E (4), fibrinopeptide A (FpA), the first peptide cleaved from fibrinogen by thrombin (5), β 15-42 antigen (B15-42), produced by the action of plasmin on fibrin and fibrinogen (6), and for fragments produced during the lysis of crosslinked fibrin (7, 8), such as X oligomer (9) and D dimer (10).

When investigating patients with renal failure the interpretation of markers of haemostatic activation may be complicated by the fact that the kidney participates in the clearance of these peptides and fragments from the circulation. Plasma concentrations of the platelet α-granule protein β-thromboglobulin (BTG) (M. W. 35,400) are elevated for this reason and its usefulness is therefore limited (11). On the other hand another platelet α-granule protein, platelet factor 4 (PF4) (M. W. 30,800), is not retained and is a reliable marker of platelet activation in uraemic patients (12). The fibrinogen derivatives FpA (M. W. 1,500), fragment E (FgE) (M. W. 50,000) and B15-42 (M. W. 3,000) are all increased in renal failure, but this is in part due to reduced renal elimination (13–15).

Thrombosis in the glomerular microcirculation may play an important role in the pathogenesis of the glomerulosclerosis which is associated with progressive renal failure (16). We have measured plasma concentrations of D dimer (M. W. 190,000), as well as PF4 and FgE, in a group of patients with various degrees of uraemia to look for evidence of intravascular coagulation and to determine the usefulness of D dimer as a marker of fibrin formation in renal failure.

Patients and Methods

Patients
D dimer was measured in 91 patients with chronic renal disease of various aetiologies and degrees of severity, and in 24 healthy hospital personnel. 17 of the patients were diabetic. No patient was taking anticoagulant drugs or undergoing any form of renal dialysis therapy. Creatinine clearance in the patient group ranged from 6–160 ml/min, with a mean of 34.6 ml/min, and was less than 76 ml/min in all but four cases. The four patients with normal renal function were diabetics who had elevated urinary protein excretion, indicating the presence of diabetic nephropathy.

In addition, 9 patients with acute renal failure (ARF) due to sepsis (2 cases), haemolytic uraemic syndrome (1 case), rhabdomyolysis (1 case) and major surgery (5 cases) were studied. FgE was measured in 28 of the 91 patients with chronic renal disease. Mean creatinine clearance in this group was 35.3 ml/min and ranged from 6–120 ml/min.

PF4 was measured in 13 patients with diabetic nephropathy, 24 patients with non-diabetic chronic renal failure, and 24 healthy controls.

Venesection
Blood for D dimer measurement was collected with minimal stasis using a 19 gauge needle and anticoagulated with EDTA (4 mmol/l final concentration). Plasma was obtained by centrifugation at 4°C for 15 minutes at 1,500 x g and stored at –40°C until assayed. For measurement of platelet factor 4 (PF4), plasma was obtained from blood anticoagulated with a mixture of EDTA, theophylline and PGEi, as described by Ludlam and Cash (17). FgE was measured in blood collected into tubes containing final concentrations of 10 U/ml thrombin and 1800 U/ml soybean trypsin inhibitor. Serum was obtained by incubation at room temperature for 30 minutes followed by centrifugation as described above. Fibrinogen was measured in blood collected into a ⅔ volume of 3.13% w/v trisodium citrate.

Assays
D dimer was measured by enzyme immunoassay as described by Whitaker et al. (18), using reagents and microtitre plates precoated with monoclonal antibody to D dimer supplied by MAbCo (Springwood, Australia). The coating antibody used (DD-3B6/22) reacts with all derivatives of cross-linked fibrin, but has greatest affinity for D dimer (18). The tag antibody is panspecific for fibrinogen and its derivatives. A calibration curve was produced using D dimer standard and plasma samples diluted where necessary to give measurements on the linear part
of the curve. Values for healthy volunteers and some patients had to be read from the lower region of the curve. PF4 was measured by radioimmunoassay using a commercial kit (Abbott, Wokingham UK). FgE was measured by radioimmunoassay as previously described (13). Fibrinogen was measured in citrated plasma by a clotting method (19).

Plasma and 24 hour urine creatinine were measured by standard laboratory techniques, and creatinine clearance calculated using the formula UV/P, where U is urinary creatinine concentration, V is volume of urine produced per minute and P is plasma creatinine concentration.

Detection of Haemostatic Activation in Renal Failure

Plasma D dimer and PF4 in 24 healthy controls were compared with values in (1) 24 non-diabetic patients with chronic renal failure (CRF) matched for age and sex, (2) a group of 13 patients with diabetic nephropathy of similar age range to controls, and (3) 9 patients with ARF.

Effect of Abnormal Renal Function on Plasma D Dimer

Plasma D dimer in 91 patients with chronic renal disease was correlated with creatinine clearance in order to determine whether reduction in renal function was directly related to circulating levels of D dimer. A similar analysis was performed on FgE measurements from 28 of the patients.

Correlation of Fibrinogen with D Dimer

Plasma levels of D dimer and fibrinogen were correlated using data from 67 of the patients studied.

Statistics

For all analyses values of D dimer and of FgE were subjected to logarithmic transformation prior to statistical testing. Comparison of patient and control groups was by Student's t test and correlations were by linear regression analysis.

Results

Calibration curves using D dimer standard were linear over the range 100–2,000 ng/ml. Coefficient of variation was 7.8% (intra-assay), and 9.1% (inter-assay). Analysis of data from control subjects showed a correlation coefficient between plasma D dimer and age of subject of 0.16, which was not statistically significant. No sex-related difference was seen on comparison of plasma D dimer in male and female controls. Our data suggest a "normal range" of <220 ng/ml. Plasma fibrinogen was elevated in patients with renal impairment (Table 1) and showed a weak, but statistically significant correlation with log D dimer (r = 0.34, p <0.005).

Concentrations of D dimer were significantly higher in both patients with non-diabetic CRF and in those with diabetic nephropathy when compared with controls. Plasma PF4 was also increased in these two groups (Table 1). All nine patients with ARF had markedly elevated levels of D dimer. There was wide scatter in levels of D dimer in each of the patient groups studied (Fig. 1).

There was no significant correlation between renal function (measured by creatinine clearance) and D dimer (r = -0.14) in the 91 patients with renal impairment (Fig. 2), but FgE showed a significant negative correlation with creatinine clearance (r = -0.57, p <0.002) (Fig. 3). A close correlation was seen between FgE and D dimer (r = -0.71, p <0.001).

Discussion

The availability of monoclonal antibodies to D dimer has allowed the development of a simple enzyme immunoassay which is specific for derivatives of cross-linked fibrin. EDTA was used as anticoagulant in order to remove calcium ions and eliminate factor XIII activity, thus minimising cross-linking of soluble fibrin during the collection and processing of blood samples. The assay was non-linear at D dimer concentrations below 100 ng/ml, which is the range characteristic of most normal subjects. It was therefore difficult to measure normal levels of D dimer reliably, especially since sample dilution is required prior to the performance of the assay, and also difficult to discriminate patients with mildly elevated plasma D dimer levels from healthy controls. Values reported in patients suffering from thrombotic disorders are considerably higher than this range (18), therefore pathological samples are reliably detected. We found no evidence of age- or sex-related differences in plasma D dimer concentration in our

Table 1 Plasma concentrations of D dimer, platelet factor 4 and fibrinogen in healthy controls and patients with renal failure

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRF*</th>
<th>Diabetic nephropathy</th>
<th>ARF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>24</td>
<td>24</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>M:F</td>
<td>14:10</td>
<td>14:10</td>
<td>10:3</td>
<td>5:4</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30 (22–41)</td>
<td>30 (20–44)</td>
<td>38 (33–46)</td>
<td>52 (24–77)</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>96 (12.7)</td>
<td>244 (30.6)b</td>
<td>308 (74.5)c</td>
<td>2451 (1007)c</td>
</tr>
<tr>
<td>PF4 (ng/ml)</td>
<td>6.8 (1.0)</td>
<td>9.2 (1.0)b</td>
<td>15.1 (2.6)a</td>
<td>Not done</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>2.9 (0.11)</td>
<td>3.7 (0.18)b</td>
<td>3.7 (0.33)a</td>
<td>4.5 (0.32)b</td>
</tr>
</tbody>
</table>

* Causes of chronic renal failure (CRF) were glomerulonephritis (4), reflux nephropathy (10) and corrected urinary tract obstruction (10)
Figures quoted in the table are mean (SEM), except for age which is mean (range). a  p <0.05; b  p <0.005; c p <0.001.
control group, though this would need to be confirmed in a larger population.

The correlation observed in this study between circulating levels of FgE and D dimer suggested that they were both derived from the breakdown of cross-linked fibrin, however, differences in the clearance of these derivatives must also be taken into account. The elimination of FgE from the circulation occurs, at least in part, via the kidney (14), and consistent with this we found a significant negative correlation between plasma FgE and creatinine clearance. FgE levels in renal failure are therefore determined by reduced elimination, as well as increased production. The kidney is involved in the elimination of a number of low molecular weight proteins. This process involves filtration at the glomerulus followed by reabsorption and metabolism by tubular epithelial cells (20), and sometimes uptake by renal parenchymal cells from the peritubular circulation (21). Studies in mice have shown that D dimer is eliminated primarily by the liver, although there is slight uptake by the kidney (22). D dimer is too large to be filtered at the glomerulus, and in the present study it failed to show any correlation with renal function, implying that any role played by the kidney in the elimination of D dimer is very limited. The elevated plasma levels found in CRF and ARF therefore reflect a genuine increase in the formation and subsequent breakdown of cross-linked fibrin, rather than reduced clearance of D dimer.

Fibrin and fibrinogen degradation products have been found in the serum (23) and urine (24) of patients with various types of renal disease. Intra-glomerular cross-linked fibrin can be demonstrated by immunohistochemical means (25), and cross-linked derivatives have been shown in urine by both electrophoretic (26), and monoclonal antibody techniques (27).

In chronic renal failure, progressive decline in kidney function is thought to occur by non-immunological mechanisms (28), and is associated with a progressive focal glomerular sclerosis (FGS). Experimental work has shown that vascular injury and thrombosis occur within the glomerulus leading to FGS (16). In patients with both non-diabetic CRF and diabetic nephropathy, we found not only raised levels of D dimer, but also raised plasma PF4, indicative of intravascular platelet activation. These results suggest an activation of haemostasis in these patients, which is compatible with the concept of a role for the haemostatic system in the progression of chronic renal failure.

All nine patients with ARF had greatly increased levels of D dimer. Intravascular coagulation is a frequent or even universal feature of ARF (29), although its pathophysiological role in this condition is not certain. It is likely that elevation of D dimer was due to the primary events leading to ARF, and not necessarily to thrombosis occurring within the kidney itself.

Our finding of a weak, but significant correlation between plasma fibrinogen and D dimer levels might indicate that increased concentrations of fibrin degradation products are stimulating fibrinogen synthesis and increasing fibrinogen levels (30, 31). Alternatively, plasma containing high concentrations of fibrinogen may also contain increased amounts of cross-linked fibrin derivatives.

Plasma D dimer appears to be a sensitive marker of fibrin breakdown which is not affected by the degree of renal impairment. It is therefore particularly suitable for use in the investigation of haemostasis in renal disease and other disorders complicated by renal failure.

Acknowledgements

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References

**Abnormal Blood Rheology in Progressive Renal Failure: A Factor in Non-immune Glomerular Injury?**

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**Abstract.** Chronic renal insufficiency progresses by a final common pathway of glomerular damage characterised by microvascular injury and glomerulosclerosis. In order to investigate the possible role of blood rheology in this process, rheological indices were compared between healthy controls and a group of patients with progressive renal failure due to renal diseases that were not considered to be immunologically mediated. Plasma viscosity was significantly increased in the renal insufficiency group \( P < 0.005 \), and correlated with raised plasma concentrations of fibrinogen \( r = 0.63; P < 0.005 \). Whole-blood viscosity corrected to a standard haematocrit of 0.45 was also raised. A weak but significant correlation was seen between plasma viscosity and 24-h urinary protein excretion \( r = 0.50; P < 0.005 \).

Our data show that in chronic renal insufficiency, rheology is abnormal. Proteinuria correlates with plasma viscosity, which is consistent with the hypothesis that raised plasma viscosity leads to an increase in glomerular capillary pressure and thence glomerular permeability. Correction of rheological abnormalities might help to preserve kidney function and reduce proteinuria in these patients.

**Key words:** Blood viscosity; Proteinuria; Renal failure; Rheology

**Introduction**

Kidney disease may be initiated by a variety of pathological processes, but once renal function has fallen to a critical low level, progression of renal insufficiency is usually inexorable. The final common pathway is characterised haemodynamically by high glomerular plasma flow and hydraulic pressure [1], clinically by hypertension and proteinuria [2], and histologically by endothelial injury, glomerular thrombosis and sclerosis [3]. Abnormal blood rheology may contribute to this progressive ischaemic injury to the glomerulus.

Clinical studies of blood rheology have demonstrated its importance in other diseases in which impairment of blood flow leads to ischaemia and thrombosis [4–6]. There are very few data on rheology in non-uraemic renal insufficiency. Blood and plasma viscosity are raised in the nephrotic syndrome [7] and in experimental acute renal failure [8], probably as a result of the increased plasma concentrations of fibrinogen, a major determinant of viscosity. Fibrinogen levels are raised in severe chronic renal failure [9], and the deformability of red cells is reported to be reduced in end-stage renal failure [10].

We have studied blood rheology in a homogeneous group of patients with progressive renal failure due to non-immunologically mediated renal diseases in order to characterise rheological abnormalities in the earlier stages of renal insufficiency and to investigate the possible role of blood rheology in progressive glomerular injury.
Patients

Thirty-two patients with progressive renal failure (20 males, 12 females; mean age 34.6 years, range 17–70 years) were studied, and results compared with those from a group of 25 healthy controls (14 males, 11 females; mean age 30.2 years, range 22–41 years). The underlying renal diseases are shown in Table 1. In all cases renal insufficiency was due to non-immunological disease.

<table>
<thead>
<tr>
<th>Table 1. Underlying renal diseases in 32 patients with progressive renal insufficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgically corrected urinary-tract obstruction</td>
</tr>
<tr>
<td>Reflux nephropathy</td>
</tr>
<tr>
<td>Hypertensive nephropathy</td>
</tr>
<tr>
<td>Polycystic kidneys</td>
</tr>
</tbody>
</table>

*Bladder extrophy (2); urethral valves (6); solitary kidney (1); megaureters (1); horseshoe kidney (1); tuberculosis of bladder (1); neuropathic bladder (2); stones (3).

All patients were studied on 2-4 occasions at intervals of 3 months, during a period not exceeding 12 months. At the start of the study period patients were seen by the dietician, and protein intake was adjusted to 1 g protein/kg body weight per day. At every visit the patient brought a 24-h urine collection, and a midstream urine specimen was cultured. If the patient had a urinary-tract infection and raised C-reactive protein on the day of the visit, then results were excluded from the study.

Renal insufficiency varied in severity both in degree and in rate of progression. Plasma creatinine ranged from 121 to 1050 μmol/l, with a mean level of 438 μmol/l. Creatinine clearance was less than 76 ml/min in all patients. Urinary protein excretion ranged from 0.2 to 7.4 g/24 h but none of the patients had a nephrotic syndrome. Hypotensive therapy was adjusted to maintain supine blood pressure at 140/90 or less. Twenty-two patients were taking antihypertensive therapy at the time of study. Drugs used were nifedipine (five patients), atenolol (ten patients), propranolol (three patients) and enalapril (four patients). Six patients were on prophylactic antibiotics. Other drugs used were sodium bicarbonate, vitamin D, allopurinol and aluminium hydroxide capsules.

Methods

Every 3 months blood for rheological investigations was taken after overnight fasting, using a 19-gauge needle and plastic syringe, and anticoagulated with dipotassium EDTA (4 mmol/l final concentration). Samples were kept at room temperature and measurements were performed within 5 h of venesection. For each patient the mean values from all attendances were calculated for each investigation. Intraindividual variation in the various measured indices is shown in Table 2.

| Table 2. Intraindividual coefficients of variation in measured indices (n=32) |
|-----------------------------|--------|
| Index | Mean CV (%) |
| Plasma viscosity | 6.2 |
| Whole-blood viscosity (uncorrected haematocrit) 23 s^{-1} | 9.3 |
| Whole-blood viscosity (uncorrected haematocrit) 230 s^{-1} | 7.0 |
| Whole-blood viscosity (standard haematocrit) 23 s^{-1} | 7.7 |
| Whole-blood viscosity (standard haematocrit) 230 s^{-1} | 5.0 |
| RCTT (5-μm filters) | 7.4 |
| RCTT (3-μm filters) | 8.3 |
| Fibrinogen | 12.4 |
| 24-h urinary protein excretion | 19.0 |

Whole-Blood and Plasma Viscosity

Viscosity was measured using a Wells–Brookfield cone and plate viscometer at shear rates of 23 s^{-1} and 230 s^{-1}, and at a temperature of 37°C. The gap between the cone and plate was calibrated before each set of measurements and the calibration checked using a mineral oil standard (Wells–Brookfield). Viscosity was measured after equilibration for 1 min, on 1.5 ml samples of either whole blood, or plasma obtained after centrifugation for 10 min at 1500 g. Whole-blood samples were thoroughly mixed immediately before testing. The results of whole-blood viscosity were reported both at the patient’s haematocrit, and corrected to a standard haematocrit value of 0.45. Correction was performed by means of a regression line relating blood viscosity to haematocrit, obtained from viscosity measurements on blood from 20 healthy subjects following progressive dilution of red cells with autologous plasma to give a range of haematocrit values from 0.10 to 0.60. The use of this regression line was validated by analysis of data from CRF patients, showing that at the shear rates used in this study the relationship between viscosity and haematocrit was similar in patients and healthy controls.

Red-Cell Deformability

Red-cell deformability was assessed by filtration of a washed, leucocyte-depleted red cell suspension through polycarbonate filters of 3 μm and 5 μm pore size (Nucleopore Corporation, Pleasanton, USA). Filters
were taken from the same batch and discarded after a single use. Red-cell deformability was assessed using a St Georges Filtrometer (CarriMed Ltd, Dorking), which measured initial rate of flow through the filter, and from this derived the red-cell transit time (RCTT), an index reflecting the deformability of the sample as a whole. In addition, the rate of clogging of filter pores was measured, allowing the detection of subpopulations of rigid cells [11].

Red cells were separated from plasma by centrifugation at 1500 g for 10 min at room temperature, then washed twice in phosphate-buffered saline (PBS) of pH 7.4 ± 0.05 and osmolarity 300 ± 2 mOsm/kg. White cells were removed by discarding the buffy layer after each washing step, and 1.5 ml of packed red cells then obtained from the centre of the red-cell column and diluted in PBS for deformability measurement. For filtration through 5-μm filters, red-cell count was adjusted to 1.2 × 10¹²/l, and white cells depleted to less than 0.25 × 10⁹/l. For 3-μm filters a red-cell count of 0.12 × 10¹²/l was used with white cells depleted to less than 0.025 × 10⁹/l.

Other Laboratory Investigations

Haematocrit and red-cell indices were measured using a Coulter counter model S Senior. Fibrinogen was measured in citrated plasma by a clotting assay (Clauss), C-reactive protein (CRP) in serum by enzyme immunoassay using commercial polyclonal antibodies (Dako), and immunoglobulins in serum by radial immunodiffusion. Each time blood rheology was measured, 24-h urine volumes were collected for estimation of urinary creatinine and protein excretion. Mean values of all attendances were used for analysis of data. Creatinine concentrations in urine and plasma were performed by the Jaffe technique, using an autoanalyzer. Urinary protein was measured turbidimetrically, following precipitation with trichloroacetic acid, and plasma albumin by immunoelectrophoresis using the Laurell technique. Creatinine clearance was calculated using the formula UV/P, where U is the urinary creatinine concentration, V is the volume of urine passed per minute, and P is the plasma creatinine concentration. Rate of progression of renal insufficiency was assessed as change in creatinine clearance with time (ml/min per year). Renal failure was considered to be slowly progressive if the decline in creatinine clearance was less than 3 ml/min per year, and more rapidly progressive if greater than this. In the group studied, the two categories each contained 16 patients.

Statistics

Statistical analysis was by Students t test and linear regression analysis. For some analyses, patients were divided into three groups according to degree of renal impairment: mild CRF (creatinine < 300 μmol/l) (n = 11); moderate CRF (creatinine 300–600 μmol/l) (n = 14); and severe CRF (creatinine > 600 μmol/l) (n = 7).

Results

Plasma viscosity was increased in CRF patients compared with healthy subjects (Table 3 and Fig. 1). Plasma concentrations of fibrinogen were also raised in the CRF group (Table 3) and correlated with the logarithm of plasma creatinine (r = 0.47, P < 0.01) and with plasma viscosity (r = 0.63, P < 0.005). CRP was also increased in the patient group (Table 3) and correlated with the logarithm of plasma creatinine (r = 0.48, P < 0.005). Serum IgG was above the normal range in three patients, and IgM in ten patients. Serum concentrations of IgA were normal. There was no correlation between serum IgM and plasma viscosity.

Haematocrit, a major determinant of whole-blood viscosity, was reduced in the CRF group (Table 3). Thus, whole-blood viscosity was lower than in healthy controls. However, whole-blood viscosity corrected for the effects of haematocrit was higher in patients than in controls (Table 3), and rose progressively with increasing renal insufficiency (Fig. 1).

Although some CRF patients had values of red-cell deformability outside the normal range, mean values of red-cell deformability were similar in the two groups (Table 4). Red-cell transit time (RCTT) was increased in three out of 32 patients (9%) using 3-μm filters, and in seven patients (22%) clogging rate was increased. Using 5-μm filters, RCTT was increased in only one out of 32 patients (3%) and clogging rate in three patients (9%). Mean cell volume and other red-cell indices were similar in patient and control groups (Table 4). A weak correlation was observed between plasma creatinine and red-cell transit time (5-μm filters) (r = 0.37; P < 0.05) and clogging rate (5-μm filters) (r = 0.36; P < 0.05). However, no correlation was seen with results of filtration through 3-μm filters.

Twenty-four-hour urinary protein excretion rose with increasing plasma viscosity (r = 0.50; P < 0.005) (Fig. 2), fibrinogen (r = 0.36, P < 0.05) and whole-blood viscosity corrected for haematocrit at low (r = 0.42, P < 0.02), and high (r = 0.50; P < 0.005) shear rate. No relationship was seen between proteinuria and plasma creatinine concentration. Plasma albumin was not reduced in any patient (mean 38 g/ml, range 30–50 g/ml) and showed no correlation with proteinuria or with plasma fibrinogen or viscosity. Plasma creatinine was higher in patients with more rapidly progressive renal failure than in those with more stable renal function (mean values 489 and 331 μmol/l respectively; P < 0.05). Plasma viscosity was the only rheological index which was significantly higher in those
Table 3. Viscosity measurements in CRF patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>CRF (n=32)</th>
<th>Controls (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td></td>
</tr>
<tr>
<td>Plasma viscosity (mPas)</td>
<td>1.62 (0.02)</td>
<td>1.52 (0.03)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Whole-blood viscosity (mPas) (uncorrected haematocrit) 23 s⁻¹</td>
<td>5.82 (0.18)</td>
<td>6.49 (0.20)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Whole-blood viscosity (mPas) (uncorrected haematocrit) 230 s⁻¹</td>
<td>4.09 (0.10)</td>
<td>4.23 (0.10)</td>
<td>NS</td>
</tr>
<tr>
<td>Whole-blood viscosity (mPas) (standard haematocrit) 23 s⁻¹</td>
<td>7.64 (0.10)</td>
<td>7.00 (0.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Whole-blood viscosity (mPas) (standard haematocrit) 230 s⁻¹</td>
<td>4.78 (0.05)</td>
<td>4.43 (0.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma fibrinogen (g/l)</td>
<td>3.88 (0.16)</td>
<td>2.78 (0.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)</td>
<td>1.35 (0.24)</td>
<td>0.55 (0.15)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>36.9 (0.8)</td>
<td>43.7 (0.6)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Standard haematocrit = haematocrit standardised to 0.45 (45%).

Table 4. Erythrocyte deformability in CRF patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>CRF (n=32)</th>
<th>Controls (n=25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td></td>
</tr>
<tr>
<td>RCTT (3-μm filters)</td>
<td>116 (2.4)</td>
<td>115 (2.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Clogging rate</td>
<td>1.77 (0.10)</td>
<td>1.64 (0.05)</td>
<td>NS</td>
</tr>
<tr>
<td>RCTT (5-μm filters)</td>
<td>11.9 (0.16)</td>
<td>11.6 (0.23)</td>
<td>NS</td>
</tr>
<tr>
<td>Clogging rate</td>
<td>1.20 (0.07)</td>
<td>1.11 (0.06)</td>
<td>NS</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>90 (0.6)</td>
<td>91 (0.7)</td>
<td>NS</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>33.8 (0.14)</td>
<td>34.0 (0.26)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fig. 1. Plasma and blood viscosity (standard haematocrit) (Std Hct) at two shear rates (23 s⁻¹ and 230 s⁻¹) in healthy controls and patients with different degrees of renal insufficiency (mean ± SEM). *P < 0.05; **P < 0.02; ***P < 0.005; ****P < 0.001.

patients progressing more rapidly (mean values 1.68 and 1.59 mPas respectively; P < 0.05).

Discussion

Our study group was composed entirely of patients with non-immunological renal disease, so that the results obtained reflect the relationship between blood rheology and renal insufficiency in the absence of underlying immunological disease.

Fig. 2. Plasma viscosity vs 24-h urinary protein excretion in 32 patients with progressive renal failure.
We have demonstrated elevated plasma viscosity and whole-blood viscosity (at standard haematocrit) even when renal insufficiency is mild, and in a minority of patients, abnormally rigid red cells. Fibrinogen is a major determinant of plasma viscosity, and as expected we found a close correlation between them both. Fibrinogen and C-reactive protein rose progressively as renal failure advanced. Both are acute-phase reactants and this elevation may represent a non-specific acute-phase protein response to chronic renal disease. Increased fibrinogen synthesis also occurs in response to urinary protein loss [12], and this may also in part explain the elevated plasma concentrations of this protein. However, plasma albumin showed no correlation with either plasma fibrinogen or viscosity. Furthermore, immunoglobulins were elevated in a proportion of our patients, and together with other proteins may contribute to plasma viscosity.

Previous studies of erythrocyte rheology in end-stage renal failure have demonstrated a loss of both deformability and membrane fluidity [10,13]. We rarely found an abnormal degree of red-cell deformability, and only a weak correlation between progressive loss of deformability and deteriorating renal function. Our results may have been influenced by antihypertensive therapy, since beta blockers may lower calcium levels in red cells [14], and calcium antagonists have been reported to improve red-cell deformability [15]. We found no difference, however, between results in patients taking these drugs and those who were not on any therapy.

Whole-blood viscosity is determined not only by haematocrit and plasma viscosity but also by both red-cell deformability and aggregation. Because of anaemia in the CRF group, whole-blood viscosity measured at uncorrected haematocrit was lower than that in controls. However the corrected whole-blood viscosity was elevated even with mild renal insufficiency. This probably reflects the sum effect of abnormal plasma viscosity, red-cell deformability, and aggregation. In the capillary circulation there is a progressive fall in haematocrit and the major rheological determinants of blood flow are plasma viscosity and red-cell deformability [16]. For this reason, we believe that the corrected whole-blood viscosity is relevant to capillary rheology.

Simpson has proposed an hypothesis that basement membranes are thixotropic gels, displaying pressure-dependent permeability [17]. He argues that viscosity-related resistance to glomerular blood flow causes a compensatory elevation in glomerular hydrostatic pressure, which then leads to an increase in protein permeability across the glomerular basement membrane [18]. Furthermore, the increased viscosity may lead to stasis, and subsequently capillary thrombosis and glomerulosclerosis [3,18]:

Consistent with Simpson's hypothesis, we found that urinary protein excretion correlated significantly with both plasma viscosity and whole-blood viscosity measured at standard haematocrit and high shear rate (a measurement largely determined by plasma viscosity), though this does not prove a causal relationship. The coefficient of correlation was low ($r=0.50$), indicating that although viscosity may play a role in promoting proteinuria, other factors, of course, are involved.

We can therefore put forward, in accord with Simpson, the testable hypothesis that the increased plasma viscosity which we describe in CRF may represent a factor in the pathogenesis of progressive glomerular injury, by retarding blood flow and promoting both proteinuria and glomerular thrombosis with subsequent sclerosis. Reduction in the deformability of red cells in some of these patients may also contribute to this. There exists at present no adequate therapeutic measure for the long-term reduction of plasma viscosity, but red-cell deformability may be reduced by drugs such as pentoxifylline [19], or by dietary supplementation with fish oils containing high concentrations of eicosapentaenoic acid [20]. It will be of great importance to see whether rheological therapy will lead to reduction in proteinuria and a retardation of renal insufficiency.

Acknowledgement. We are very grateful to the St Peter's trust for funding this work.

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Platelet Function and the Bleeding Time in Progressive Renal Failure

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Key words
Platelets – Bleeding time – Renal failure

Summary

Bleeding time and platelet function tests were performed on 31 patients with progressive chronic renal failure (CRF) due to non-immunological (urological) causes, and compared with 22 healthy controls. Patients were classified as mild (plasma creatinine <300 μmol/l), moderate (300–600 μmol/l) or severe renal failure (>600 μmol/l). Bleeding time was rarely prolonged in mild and moderate CRF and mean bleeding time significantly elevated only in severe CRF (p <0.005). Haematocrit was the only index which correlated with bleeding time (r = −0.40). Platelet counts, collagen-stimulated thromboxane generation, and platelet aggregation responses to ADP, collagen and ristocetin were all either normal or increased in all three CRF groups, but thromboxane production in clotted blood was reduced. Plasma fibrinogen, C reactive protein and von Willebrand factor (vWF) were elevated in proportion to CRF. We found no evidence that defects in platelet aggregation or platelet interaction with vWF prolong the bleeding time in patients with progressive CRF.

Introduction

It is well recognised that uraemia may be associated with a bleeding tendency. The mechanism, however, remains unknown. Unfortunately most studies have been on patients established on dialysis, and haematological studies on patients with less severe renal failure have often been confounded by the underlying diseases such as immune complex mediated glomerulonephritis or diabetes. For this reason we have deliberately studied patients with urological causes of renal failure, in which immune mechanisms are not thought to play a role. Prolongation of the skin capillary bleeding time is the best indicator of a bleeding tendency (1). Abnormalities of platelet function which have been reported in uraemia include defective aggregation (2), reduction in cyclooxygenase activity (3) and impaired interaction with von Willebrand factor (vWF) (4). However, these abnormalities have not been found consistently (5–7). Vessel wall dysfunction, including excessive production of prostacyclin (PGI2) (8), and reduced binding of vWF and platelets to subendothelium (9) may also impair haemostasis.

The prolonged bleeding time in patients with severe chronic renal failure (CRF) may be shortened by transfusions of red cells, which suggests that anaemia is a major determining factor of uraemic bleeding (10). Although many studies of uraemic haemostasis have been performed on dialysis patients with end-stage renal failure, it is unclear whether the bleeding time is prolonged earlier in the course of renal disease.

We have therefore studied a group of patients with progressive renal failure but different degrees of renal function, to determine the stage at which a bleeding tendency becomes apparent, and to investigate the mechanisms involved.

Patients, Materials and Methods

Patients

31 patients with progressive renal failure were studied (20 males, 11 females; mean age 35.1 years, range 17–70). Causes of renal failure are shown in Table 1, and in all cases were due to non-immunological disease. No patient was suffering from a nephrotic syndrome or diabetes, nor was any one undergoing renal replacement therapy. Eleven patients were taking atenolol and five nifedipine for control of blood pressure, but no other drugs known to influence platelet function had been taken for 10 days prior to testing.

Patients were classified as suffering from mild (creatinine 120–300 μmol/l) (n = 10), moderate (300–600 μmol/l) (n = 14) or severe (>600 μmol/l) (n = 7) renal failure on the basis of their plasma creatinine concentration. Results from each group were compared with values obtained from a group of 22 healthy controls (Table 2).

Methods

Blood was taken after overnight fasting using a 19 gauge needle and minimal stasis. Samples for platelet aggregation were anticoagulated using 3.13% w/v trisodium citrate. We have previously shown that correction of citrate concentration is important when studying platelet aggregation in uraemic patients with low haematocrit values (11). Citrate concentration was therefore corrected by the method of Kelton et al. (12) to that which would be present in blood with an haematocrit of 0.55. Blood for measurement of haematocrit and platelet count was taken into EDTA (4 mmol/l final concentration) and for measurement of fibrinogen and vWF into a 1/10 volume of 3.13% w/v trisodium citrate buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (5 g/dl).

Bleeding time. A Simplate II device (General Diagnostics) was used, making a vertical incision on the lateral aspect of the anterior surface of the forearm, with a torniquet pressure maintained at 40 mmHg. All tests were performed by the same investigator.

Platelet aggregation. Platelet-rich plasma (PRP) was prepared by centrifugation for 10 min at 150 g and room temperature, and platelet poor plasma (PPP) by centrifugation at 1,500 g for a further 10 min. Platelet counts in PRP were corrected to 200 x 10⁹/l by the addition of poor plasma (PPP) by centrifugation at 1,500 g for a further 10 min. Platelet counts in PRP were corrected to 200 x 10⁹/l by the addition of PPP, prior to the measurement of aggregation responses using a turbidimetric aggregometer (Payton).

The following indices were measured:
1. ADP threshold: minimum concentration of ADP (Sigma chemicals) giving a secondary wave of aggregation.
2. Collagen ED50: Concentration of collagen (Hormon Chemie) giving 50% maximal rate of aggregation. (Maximal rate defined as that obtained using 20 μg/ml collagen.)
3. Ristocetin aggregation rate: Initial rate of aggregation obtained after the addition of 1.25 mg/ml ristocetin (Lundbeck).
4. Spontaneous aggregation: Degree of aggregation obtained after stirring PRP for 15 min in the absence of any aggregating agent.

Thromboxane generation. Generation of immunoreactive thromboxane (TXB₂) was measured (i) after clotting of native whole blood at 37° C for 60 min and (ii) in stirred PRP stimulated with 20 μg/ml collagen at 37° C, the reaction being stopped after 4 min with EDTA (13.4 mmol/l).
Table 1
Causes of renal failure in the 31 patients studied

| Corrected urinary tract obstruction | 16* |
| Reflux nephropathy          | 14  |
| Hypertension                | 1   |

* Bladder extrophy (2); urethral valves (6); solitary kidney (1); megareters (1); horseshoe kidney (1); neuropathic bladder (2); stones (3).

Table 2
Patient groups

<table>
<thead>
<tr>
<th>M:F</th>
<th>Age (yrs) mean (range)</th>
<th>Mean creatinine (μmol/l)</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13:9</td>
<td>29.7 (22-41)</td>
<td>&lt;120</td>
</tr>
<tr>
<td>Mild CRF</td>
<td>5:5</td>
<td>33.3 (17-53)</td>
<td>197</td>
</tr>
<tr>
<td>Moderate CRF</td>
<td>10:4</td>
<td>32.0 (17-56)</td>
<td>441</td>
</tr>
<tr>
<td>Severe CRF</td>
<td>5:2</td>
<td>41.4 (18-70)</td>
<td>758</td>
</tr>
</tbody>
</table>

(At = Atenolol; Nif = Nifedipine)
(Mild CRF creatinine <300 μmol/l; moderate CRF 300-600 μmol/l; severe CRF >600 μmol/l)

Results

Bleeding time was within the normal range (mean ± 2SD of control values) in the majority of CRF patients (Fig. 1). Mean bleeding time was slightly longer in mild CRF (mean 5.71, range 3.26–11.50 min) than in controls (mean 4.79, range 3.25–7.17 min), but this was due to a grossly prolonged time in one patient, which may have been an anomalous result. Unfortunately this patient was not available for retesting. In moderate CRF bleeding time was above the normal range in only one patient and mean bleeding time was not significantly elevated (mean 5.17, range 3.85–7.95 min). In severe CRF mean bleeding time was significantly prolonged (mean 7.31, range 5.00–10.83 min, p <0.005), though even in this group results were within the normal range in 4 out of 7 patients.

Haematocrit fell with loss of renal function (Fig. 2), but in all patient groups platelet counts were not reduced and ex vivo aggregation responses to ADP, collagen and ristocetin showed platelet sensitivity to these agonists to be normal or increased. Spontaneous platelet aggregation in PRP increased progressively with advancing uraemia and was significantly raised in moderate...
Table 3 Platelet counts, platelet aggregation and plasma concentrations of fibrinogen, CRP and vWF in healthy controls and CRF patients (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF</td>
<td>n = 22</td>
<td>n = 10</td>
<td>n = 14</td>
<td>n = 7</td>
</tr>
<tr>
<td>Platelets (x10^9/l)</td>
<td>205 (15.8)</td>
<td>244 (13.5)</td>
<td>254 (11.9)</td>
<td>234 (13.7)</td>
</tr>
<tr>
<td>ADP threshold (μmol/l)</td>
<td>2.17 (0.24)</td>
<td>1.58 (0.20)</td>
<td>1.47 (0.17)</td>
<td>1.25 (0.23)</td>
</tr>
<tr>
<td>Collagen ED50 (μg/ml)</td>
<td>0.87 (0.06)</td>
<td>0.68 (0.06)</td>
<td>0.65 (0.05)</td>
<td>0.82 (0.21)</td>
</tr>
<tr>
<td>Ristocetin (%) (%/min)</td>
<td>64 (4.2)</td>
<td>74 (6.1)</td>
<td>64 (5.9)</td>
<td>85 (4.5)</td>
</tr>
<tr>
<td>Spontaneous (%)</td>
<td>4.1 (0.62)</td>
<td>5.3 (1.22)</td>
<td>8.4 (0.14)</td>
<td>10.5 (1.4)</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>2.65 (0.20)</td>
<td>3.50 (0.31)</td>
<td>3.85 (0.25)</td>
<td>4.60 (0.27)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.55 (0.15)</td>
<td>0.79 (0.19)</td>
<td>1.08 (0.20)</td>
<td>2.20 (0.57)</td>
</tr>
<tr>
<td>vWF (U/ml)</td>
<td>1.07 (0.08)</td>
<td>1.48 (0.12)</td>
<td>1.40 (0.10)</td>
<td>1.66 (0.14)</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.02; *** p < 0.005; **** p < 0.001.

The pattern of TxB2 generation by uraemic platelets differed depending on the stimulus. In all three CRF groups collagen-induced TxB2 production was slightly, but not significantly higher than in healthy controls (Fig. 3), whereas serum TxB2 concentrations were significantly reduced (mild CRF p<0.02, moderate and severe CRF p<0.05) (Fig. 4).

Plasma concentrations of fibrinogen and vWF, and the acute phase protein CRP were elevated, most markedly so in severe CRF (p <0.001) (Table 3). Plasma albumin was within the normal range in all patients.

Haematocrit was the only index showing a weak, but significant negative correlation with bleeding time (r = -0.40; p <0.05).

Discussion

Platelet function in renal failure may be influenced by active immunological disease (15), diabetes (16), nephrotic syndrome (15) and the haemodialysis procedure (17), all of which are associated with intravascular platelet activation, increased platelet responsiveness, or both. Our choice of patients with essentially urological causes of renal failure has enabled us to study the effects of increasing uraemia on platelet function in the absence of these complicating factors.

We found no evidence of reduced platelet aggregation in response to ADP, collagen or ristocetin; on the contrary responses were often increased compared with normal controls. It is therefore unlikely that prolongation of the bleeding time in CRF...
is related to defects in platelet responsiveness to such agonists. In vitro platelet aggregation may increase with age but the difference in mean age between controls and patients is not sufficient to influence results significantly. Aggregation results in this study agree with our own previous findings (7), but differ from those of others. Di Minno et al. (2) reported threshold aggregating concentrations of collagen, ADP and adrenaline two to three times higher in CRF patients than in controls and Castillo et al. (9) described a reduction in ristocetin-induced aggregation in a proportion of their uraemic patients. However, in these studies patients were receiving dialysis treatment, and were selected because of their prolonged bleeding times and history of haemorrhagic episodes. Our patients had less advanced disease and no clinical bleeding. Methodological differences may also have contributed to the discrepancy between results. There is no indication in Di Minno’s paper that correction of platelet count or citrate anticoagulant concentration were performed before aggregation was measured, precautions we, and others, consider essential before comparing responses from different patient groups.

The possible effect of antihypertensive therapy in some patients is uncertain. Atenolol does not affect platelet function (18) and, although nifedipine has been shown to have an inhibitory effect on platelets in vitro, this is seen only at concentrations of the drug vastly in excess of therapeutic levels (19), due to the absence in platelets of voltage operated calcium channels (20). Reduction in ex vivo platelet function has been reported following nifedipine administration (21), but in a pilot study of our own (unpublished), administration of nifedipine to volunteers produced no change in bleeding time or ex vivo platelet aggregation, and in the present study we found no difference between results in patients taking these drugs and those on no antihypertensive therapy. Thus, the effects of nifedipine on platelets are not yet clearly established.

Our findings of hyperaggregability may be a consequence of the increase in plasma fibrinogen and vWF, both adhesive proteins which play a major role in platelet aggregation. Meade et al. (22) have shown that fibrinogen levels at the high end of the normal range (similar to those in our patients) enhance platelet responses to low doses of agonist. Hyperfibrinogenaemia probably occurs as a non-specific response to disease, and we found a parallel rise of fibrinogen and the acute phase protein CRP in progressive CRF. Levels of v-WF may rise both through endothelial damage and impaired clearance by the reticulo-endothelial system in uraemia (23). The increased platelet reactivity which we describe in progressive renal failure is paradoxical in view of the prolongation of the bleeding time in some patients, but may be of clinical importance, first because CRF patients are at increased risk of atheroma and cardiovascular death (24), and also because platelets have been implicated in the pathological mechanisms underlying the progression of renal failure (25).

In contrast to the findings of Remuzzi et al. (3), we found no evidence of a cyclooxygenase defect in uraemic platelets. Despite a reduction in the generation of thromboxane during blood clotting (measured as serum TxB2 concentrations), collagen-induced generation of thromboxane in PRP was slightly higher than controls in all CRF groups, although this was not statistically significant. Our results are therefore similar to those of Bloom et al. (6), who suggested the existence of an abnormality in stimulus-response coupling in uraemic platelets, possibly at the level of the thrombin receptor.

Uraemic bleeding can be prevented by cryoprecipitate (26) or DDAVP (27), perhaps by alteration in the quantity or quality of circulating vWF. However, no pretreatment deficiency or abnormality has been consistently demonstrated. Conjugated oestro- gens also correct the bleeding time in uraemia, but without changing levels of the factor VIII/vWF complex (28). The mechanism by which these agents act on uraemic haemostasis is therefore unclear. In the present study no evidence of a defect in platelet/vWF interactions was observed and severe CRF was associated with increased ristocetin-induced platelet aggregation and plasma vWF despite prolonged bleeding times.

The adverse effect of anaemia on haemostasis in CRF has been emphasised in recent years, and our demonstration of a significant correlation between haematocrit and bleeding time supports this hypothesis. Although this correlation does not prove a cause-effect relationship, the correction of bleeding times by red cell transfusions (10) argues strongly for a role for anaemia in the uraemic bleeding tendency.

In conclusion, although prolongation of the bleeding time occurs with increasing frequency in the later stages of progressive CRF, the development of a bleeding tendency is not a consistent observation and may not be directly related to the severity of renal failure. Increase in the bleeding time in CRF is not due to reduced platelet responses to ADP, collagen or ristocetin, but appears to be related to anaemia and also possibly to a poorly-defined platelet defect which leads to reduction in serum TxB2 levels. Vessel wall abnormalities, (not tested in this study), may also contribute. The defect is not generally severe and in our study bleeding time did not exceed 11.5 min in any individual. However, a patient may be predisposed to severe bleeding if other factors, such as the accumulation of drugs (29), are superimposed on this mild haemostatic abnormality.

Acknowledgements

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References

Blood Hyperviscosity and Its Relationship to Progressive Renal Failure in Patients with Diabetic Nephropathy


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Blood rheology was investigated in patients with diabetic nephropathy and progressive renal insufficiency, and compared with similar non-diabetic patients and healthy control subjects. Plasma viscosity and whole blood viscosity at standardized haematocrit were elevated to a comparable degree in the two patient groups, but erythrocyte deformability was normal. In diabetic patients, the rate of progression of renal failure showed weak, but significant, correlations with plasma viscosity \( r_s = 0.50, p = 0.005 \), standardized whole blood viscosity \( r_s = 0.41, p = 0.021 \), plasma fibrinogen \( r_s = 0.46, p = 0.010 \), C reactive protein \( r_s = 0.40, p = 0.023 \), and proteinuria \( r_s = 0.52, p = 0.003 \).

Both plasma viscosity and plasma fibrinogen correlated significantly with proteinuria \( r_s = 0.50, p = 0.012 \) and \( 0.40, p = 0.027 \), respectively. Rheological abnormality is probably a manifestation of increased acute phase proteins, but it remains to be determined whether these are the cause or the effect of the renal injury. Abnormal blood rheology may be a risk factor for the progression of renal failure in diabetic nephropathy.

Introduction

Diabetic nephropathy is a major cause of death in people with diabetes. The factors underlying the progression of renal failure in diabetic nephropathy are not fully understood. Control of blood pressure has been shown to slow the rate of decline of renal function in established nephropathy,\(^1\) but control of hyperglycaemia is not associated with this benefit.\(^2\)

Focal and segmental glomerulosclerosis is the hallmark of progressive renal failure in both diabetic and non-diabetic patients, and its development shares similarities with atherosclerosis.\(^3\) In diabetic nephropathy, fibrin deposition within the glomerulus is a frequent finding,\(^4\) and is probably a consequence of vascular injury and microthrombosis of the glomerular capillaries. Haematological abnormalities in diabetes, which may be relevant to vascular disease, include activation of both the coagulation system\(^5\) and platelets,\(^6\) and reduction in the release of fibrinolytic activity from the endothelium.\(^7\) It is not clear, however, whether these haemostatic changes represent cause or effect.

The rheological properties of blood are important in determining blood flow, and there are correlations between rheological abnormalities and vascular disease.\(^8\) Increased aggregation of erythrocytes occurs in diabetes, and may impede blood flow in large and small vessels.\(^9,10\)

In addition, both hyperviscosity and reduced erythrocyte deformability have been reported in diabetes.\(^11-13\) Abnormalities of these have been found to be more pronounced in patients with diabetic nephropathy than in those without,\(^14\) and may be important in the development of nephropathy.\(^15\)

It has previously been shown that both plasma, and whole blood viscosity at standardized haematocrit, are elevated in non-diabetic progressive renal failure, and that plasma viscosity is related to the degree of renal functional impairment, rate of progression of renal failure, and degree of proteinuria.\(^16\) In the present study we examine whether similar rheological abnormalities occur with diabetic nephropathy, and investigate the possible role of blood rheology in this condition.

Patients and Methods

Patients

Rheological measurements were made with informed consent in 30 patients with diabetic nephropathy (19 males, 11 females; age 49 (range 31–70) years). Diabetic nephropathy was diagnosed by the presence of persistent proteinuria of 0.50 g 24-h\(^{-1}\) or greater. Renal biopsies were performed if the cause of the renal disease was not...
In order to compare blood rheology in diabetic nephropathy, non-diabetic chronic renal failure and healthy control subjects, 21 patients were selected from the diabetic nephropathy group to match as closely as possible a group of 21 chronic renal failure patients in terms of age, sex, and degree of renal impairment, and a group of 21 healthy subjects in terms of age and sex. Details of these groups are given in Table 1. Because of some imprecision in the matching, unpaired statistics were used for comparison of the data from these three groups.

For investigation of the relationship between blood rheology and diabetic renal disease data from all 30 diabetic patients were analysed. Rheological results were correlated with creatinine clearance, proteinuria, and rate of progression. Correlations were also performed.
Table 1. Characteristics of the groups studied for comparison of blood rheology in diabetic nephropathy, chronic renal failure and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic nephropathy</th>
<th>Chronic renal failure</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>M : F</td>
<td>13 : 8</td>
<td>13 : 8</td>
<td>13 : 8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.4 (31 - 70)</td>
<td>47.6 (30 - 70)</td>
<td>41.2 (31 - 56)</td>
</tr>
<tr>
<td>Creatinine clearance (ml min⁻¹ 70-kg⁻¹)</td>
<td>28.8 (8 - 58)</td>
<td>21.9 (7 - 78)</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Number, or mean (range).

*Causes of renal failure: Reflux nephropathy (7), obstructive uropathy (11), hypertensive nephropathy (2), polycystic kidneys (1).

between blood rheological values and HbA₁ values, to investigate the effects of diabetic control.

**Statistical Analysis**

Comparison of rheological values in the different patient groups was performed using Student's t-test for unpaired data. C reactive protein and erythrocyte clogging rate results were subjected to log transformation prior to statistical analysis. Values of creatinine clearance, proteinuria, and rate of progression of renal failure were not normally distributed and correlations between rheological values and these measurements of renal function were therefore performed by the Spearman rank method.

**Results**

Comparison of Blood Rheology in Diabetic Nephropathy, Chronic Renal Failure, and Healthy Subjects

Rheological indices were similar in patients with diabetic nephropathy to those with chronic renal failure (Table 2). Plasma viscosity and standardized whole blood viscosity were higher in both groups of patients than in healthy control subjects. However, both groups of patients were anaemic and had low haematocrit values. Uncorrected whole blood viscosity was consequently reduced in patients compared with healthy control subjects (Table 2).

No differences were seen in erythrocyte deformability in the three groups (Table 3).

**Correlation of Blood Rheology with Creatinine Clearance**

Because of the good correlation between calculated creatinine clearance (Hull formula) and ⁵¹Cr EDTA GFR, the former are used to quantify renal function. Creatinine clearance showed a statistically significant correlation with haematocrit (rₛ = 0.68, p < 0.001) in the diabetic patients and hence with uncorrected whole blood viscosity measured at low (rₛ = 0.60, p < 0.001) and high shear rate (rₛ = 0.57, p = 0.001). No other significant correlations were found.

**Correlation of Blood Rheology with Proteinuria**

Both plasma viscosity and plasma fibrinogen showed a significant correlation with proteinuria (rₛ = 0.45, p = 0.012, and 0.40, p = 0.027, respectively).

**Correlation of blood rheology with rate of progression of renal failure**

The following laboratory measurements showed a significant correlation with rate of progression of renal failure: plasma viscosity (rₛ = 0.50, p = 0.005) (Figure 1); standardized whole blood viscosity at 23 s⁻¹ (rₛ = 0.41, p = 0.021); haematocrit (rₛ = -0.56, p = 0.001); fibrinogen (rₛ = 0.46, p = 0.010); C reactive protein (rₛ = 0.40, p = 0.023).

**Correlation of Blood Rheology with HbA₁c**

No rheological measure showed a significant correlation with HbA₁c.

**Other Analyses**

Rate of progression of renal failure was significantly correlated with proteinuria (rₛ = 0.52, p = 0.003) (Figure 2) and creatinine clearance (rₛ = 0.45, p = 0.012). No significant correlation was seen between either duration of diabetes or HbA₁c levels and proteinuria, rate of progression of renal failure, or creatinine clearance.
Table 2. Blood rheology in diabetic nephropathy, chronic renal failure, and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic nephropathy</th>
<th>Chronic renal failure</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Plasma viscosity (mPa s)</td>
<td>1.65</td>
<td>1.65</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>(1.59-1.71)</td>
<td>(1.61-1.69)</td>
<td>(1.49-1.61)</td>
</tr>
<tr>
<td>Uncorrected blood viscosity 23 s(^{-1}) (mPa s)</td>
<td>5.82</td>
<td>5.81</td>
<td>6.76</td>
</tr>
<tr>
<td></td>
<td>(5.36-6.28)</td>
<td>(5.27-6.35)</td>
<td>(6.36-7.16)</td>
</tr>
<tr>
<td>Uncorrected blood viscosity 230 s(^{-1}) (mPa s)</td>
<td>3.88</td>
<td>4.00</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>(3.67-4.09)</td>
<td>(3.75-4.25)</td>
<td>(4.18-4.64)</td>
</tr>
<tr>
<td>Corrected blood viscosity 23 s(^{-1}) (mPa s)</td>
<td>8.50(^b)</td>
<td>7.81</td>
<td>7.03</td>
</tr>
<tr>
<td></td>
<td>(7.87-9.13)</td>
<td>(7.56-8.06)</td>
<td>(6.63-7.43)</td>
</tr>
<tr>
<td>Corrected blood viscosity 230 s(^{-1}) (mPa s)</td>
<td>4.84(^a)</td>
<td>4.79</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>(4.71-4.97)</td>
<td>(4.69-4.89)</td>
<td>(4.28-4.78)</td>
</tr>
<tr>
<td>Haematocrit (l l(^{-1}))</td>
<td>0.348(^c)</td>
<td>0.364</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>(0.329-0.367)</td>
<td>(0.342-0.386)</td>
<td>(0.421-0.449)</td>
</tr>
<tr>
<td>Fibrinogen (g l(^{-1}))</td>
<td>3.43(^b)</td>
<td>3.77</td>
<td>2.57</td>
</tr>
<tr>
<td></td>
<td>(3.03-3.83)</td>
<td>(3.25-4.29)</td>
<td>(2.26-2.88)</td>
</tr>
<tr>
<td>C reactive protein (mg l(^{-1}))</td>
<td>1.56(^b)</td>
<td>1.48</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>(0.92-2.20)</td>
<td>(0.71-2.25)</td>
<td>(0.49-0.73)</td>
</tr>
</tbody>
</table>

Mean (95% confidence interval).  
\(^{a}\)p < 0.05, \(^{b}\)p < 0.01, \(^{c}\)p < 0.001 vs control group; \(^{d}\)p < 0.05 vs chronic renal failure group.

Table 3. Erythrocyte indices in diabetic nephropathy, chronic renal failure, and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>Diabetic nephropathy</th>
<th>Chronic renal failure</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Erythrocyte transit time</td>
<td>11.9</td>
<td>11.9</td>
<td>11.7</td>
</tr>
<tr>
<td>(5 (\mu)m) (arbitrary units)</td>
<td>(11.2-12.6)</td>
<td>(11.5-12.3)</td>
<td>(11.2-12.2)</td>
</tr>
<tr>
<td>Clogging rate (5 (\mu)m)</td>
<td>1.36</td>
<td>1.04</td>
<td>1.08</td>
</tr>
<tr>
<td>(arbitrary units)</td>
<td>(1.07-1.65)</td>
<td>(0.92-1.16)</td>
<td>(0.85-1.31)</td>
</tr>
<tr>
<td>Erythrocyte transit time</td>
<td>114</td>
<td>119</td>
<td>116</td>
</tr>
<tr>
<td>(3 (\mu)m) (arbitrary units)</td>
<td>(110-118)</td>
<td>(112-126)</td>
<td>(107-125)</td>
</tr>
<tr>
<td>Clogging rate (3 (\mu)m)</td>
<td>1.83</td>
<td>1.76</td>
<td>2.24</td>
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<tr>
<td>(arbitrary units)</td>
<td>(1.66-2.00)</td>
<td>(1.55-1.97)</td>
<td>(1.82-2.66)</td>
</tr>
<tr>
<td>MCV (fl)</td>
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<tr>
<td></td>
<td>(86-90)</td>
<td>(88-92)</td>
<td>(90-94)</td>
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<tr>
<td>MCHC (g dl(^{-1}))</td>
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<td>(33.1-33.7)</td>
<td>(33.0-33.8)</td>
<td>(32.9-33.7)</td>
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</table>

Mean (95% confidence interval).  
Measured erythrocyte indices are erythrocyte transit time, filter clogging rate, mean erythrocyte volume (MCV) and mean erythrocyte haemoglobin concentration (MCHC).

Discussion

The results of the present study suggest increased plasma viscosity and standardized whole blood viscosity in patients with diabetic nephropathy, when compared with matched healthy control subjects. This pattern of rheological abnormality was similar in nature and degree to that in non-diabetic patients with a similar degree of renal insufficiency. Thus the superimposition of diabetes on chronic renal failure did not appear to cause any additional rheological derangement.

Erythrocyte deformability was normal in our diabetic group, in contrast to some previous studies.\(^{13,14}\) Erythrocyte filtration has generally been assessed in whole
Figure 1. Correlation between rate of progression of renal failure (measured as rate of decline in creatinine clearance) and plasma viscosity in patients with diabetic nephropathy ($n = 30$); $r_s 0.50, p = 0.005$

Figure 2. Correlation between rate of progression of renal failure (measured as rate of decline in creatinine clearance) and proteinuria in patients with diabetic nephropathy ($n = 30$); $r, 0.52, p = 0.003$
blood, or in patients' plasma, but Stuart and colleagues showed that this measurement is influenced by the presence of plasma proteins and leucocytes, and that filtration results are normal in diabetes if the effects of these extrinsic variables are removed. Our findings of normal filtrability using washed, leucocyte-depleted suspensions of erythrocytes in buffer, were consistent with this.

Plasma hyperviscosity in diabetic nephropathy may be explained by changes in levels of plasma proteins, in particular of those which are raised as a consequence of an acute phase response. Acute phase proteins have high intrinsic viscosity due to their molecular shape and size, and elevated levels of these proteins contribute to the rise in serum viscosity which is seen in diabetes. Consistent with this, we found that plasma concentrations of fibrinogen and C reactive protein, both acute phase proteins, were significantly elevated in our patients. Other proteins, such as α2-macroglobulin and immunoglobulins, may also contribute, but have not been measured in the present study.

Both plasma viscosity, and standardized whole blood viscosity, were significantly correlated with the rate of progression of diabetic nephropathy. Since plasma levels of both fibrinogen and C reactive protein also correlated with progression, it is likely that the relationship between viscosity and the rate of decline of renal function reflected an acute phase response in patients with a more aggressive disease. More severe proteinuria may also have contributed to the link between plasma viscosity and progression, since hepatic production of proteins such as fibrinogen is stimulated in response to protein loss. Consistent with this, we found a positive relationship between proteinuria and both plasma fibrinogen and viscosity in the diabetic group, as previously in patients with non-diabetic renal failure. The question remains, however, whether the hyperviscosity is a cause or an effect of the more active progression.

Is there evidence that increased viscosity may cause or accelerate renal failure? Studies in experimental animals have shown that blood rheology may certainly influence glomerular haodynamics. Elevation of either plasma or whole blood viscosity results in renal vaso-dilation in order to maintain renal blood flow, and a direct relationship exists between haematocrit, a major determinant of blood viscosity, and both glomerular hydraulic pressure and filtration fraction. Since increased intraglomerular hydraulic pressure is thought to promote proteinuria and initiate endothelial injury and glomerular sclerosis, a mechanism exists by which rheological disturbance may promote glomerular injury. These considerations led Garcia and colleagues to postulate that the anaemia of renal failure is a haemodynamically favourable adaptation in that, by reducing blood viscosity, it limits glomerular hypertension and glomerular injury. In our study, uncorrected blood viscosity was lower than normal in the diabetic patients, due to anaemia. Although this might be expected to protect against glomerular hypertension, we found a negative correlation between haematocrit and the rate of progression of renal failure, a result inconsistent with a causative relationship between haematocrit and glomerular injury.

Observations of chronic renal failure patients receiving recombinant human erythropoietin have also failed to confirm a harmful role for rising haematocrit. Furthermore, Ditzel found no correlation between glomerular filtration rate and viscosity in Type 1 diabetic patients with hyperfiltration (but without overt nephropathy) and interpreted this as evidence against a viscosity-induced increase in intraglomerular pressure.

In view of the discrepancy which exists between the haematocrit (and consequently the viscosity) of blood perfusing the microcirculation and that of blood flowing in the larger vessels, it may be misleading to use viscosity values measured in venous blood to predict events occurring in the capillaries, such as those of the glomerulus. Simpson has proposed that rheological disturbance in diabetes leads to an exaggerated rise in efferent arteriolar blood viscosity following loss of filtrate along the glomerular capillary, and that hyperviscosity thus produced may promote glomerular capillary hypertension. The correlation of plasma viscosity with the rate of progression of renal failure which we have found, while consistent with Simpson's hypothesis, does not constitute proof of it.

In summary, the present study suggests that the rate of progression of renal failure in diabetic nephropathy correlates with the plasma viscosity and with proteinuria. The increased viscosity is probably a reflection of the increased acute phase protein response also found. Although these rheological changes may be expected to worsen glomerular injury, the question remains as to whether the changes are principally a cause or an effect. We believe that it is more likely that they are predominantly a consequence of the glomerular injury and proteinuria, but even so would act in a vicious cycle to worsen glomerular injury. This question should be resolved by long-term studies, if therapeutic measures to slow the progression of renal failure and reduce proteinuria, for example careful control of blood pressure with an angiotensin-converting enzyme inhibitor, result also in an improvement in the rheological abnormalities.

Acknowledgements

We thank the St Peter's Trust and the Sir Halley Stewart Trust for supporting this project, and A.B. Kurtz, D.J. Betteridge, S. McHardy-Young, and G.A. Nelstrop for allowing us to study patients under their care.

References

Protein changes.

between erythrocyte deformability and platelet aggregation.

of diabetic glomerulosclerosis: analogies to atherosclerosis.


ANTI-PLATELET THERAPY IN DIABETIC AND NON-DIABETIC PROGRESSIVE RENAL FAILURE

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Acknowledgements

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ABSTRACT

A role for the activation of platelets and coagulation in the glomerular microcirculation has been proposed in the pathogenesis of progressive glomerulosclerosis. In a group of 14 patients with non-diabetic and non-immunologically mediated renal failure and eight with diabetic nephropathy, we have examined the effect of dipyridamole (300mg/day), aspirin (75mg/day) and fish oil (10mls/day), added sequentially at 3 monthly intervals, on the rate of progression of renal failure, on proteinuria, on systemic mean arterial pressure and on indices of blood rheology, platelets and coagulation. When compared with values obtained during a run-in period, the anti-platelet therapy slowed the rate of progression in 7 out of 14 non-diabetic patients and in 5 out of 8 with diabetic nephropathy. Response to treatment tended to occur in those patients whose initial rate of progression was faster.

During the twelve month period of anti-platelet therapy the mean reduction in rate of progression in the non-diabetic group was 0.12 mls/min/month (95% confidence intervals -0.11 to 0.35) (p = 0.345), and in the diabetic nephropathy group was 0.14 mls/min/month (95% confidence intervals -0.03 to 0.31) (p = 0.142). Mean proteinuria was reduced from 2.51 to 2.06 g/24 hours in non-diabetics (NS), and from 2.19 to 1.02 g/24 hours in diabetics (p<0.01). Mean arterial pressure did not change significantly, but there was a reduction in plasma D dimer (p<0.05), serum thromboxane (p<0.001) and whole blood viscosity (p<0.05). Treatment with dipyridamole and aspirin was well tolerated, but fish oil less so. Thus anti-platelet therapy slowed the rate of progression of renal failure in half the patients studied and reduced proteinuria. Although not statistically significant, the results of this small study are sufficiently encouraging to warrant further trials of anti-platelet therapy in progressive chronic renal failure.
INTRODUCTION

Progression of chronic renal failure (CRF) appears to be inevitable once renal function has fallen to a critical level in both non-diabetic renal disease and diabetic nephropathy. It is characterized by hypertension and proteinuria, as well as an inexorable decline in glomerular filtration rate. Histologically, the renal lesion is one of focal glomerulosclerosis (FGS) (1) superimposed on any underlying glomerular disease.

Sub-total nephrectomy in rats is an important experimental model to study progressive CRF and the pathogenesis and treatment of FGS (reviewed by El Nahas (2)). Endothelial injury and platelet-fibrin microthrombi occur in affected glomeruli (3,4). Histopathologically, FGS shares many characteristics with atherosclerosis (5) and a number of observations have suggested that the haemostatic system may participate in its pathogenesis. Therapy for the retardation of CRF in man is limited. Control of hypertension slows the decline in function (6,7) and restriction of dietary protein and phosphorus may also be of benefit, although this is controversial (8). Experimental progressive CRF may be ameliorated by treatment with anti-coagulant and anti-platelet agents such as heparin (9), coumadin (10), thromboxane synthetase inhibitors (11) and ticlopidine (12). There is haematological evidence of haemostatic activation in patients with progressive CRF (13,14).

We now report the use of anti-platelet therapy in both non-diabetic and diabetic patients with progressive CRF.
PATIENTS AND METHODS

65 patients with progressive CRF and proteinuria were studied. After recruitment they were observed during a run-in period of a minimum of 9 months before anti-platelet therapy was started.

CRF Patients without diabetes

44 non-diabetic patients with chronic renal failure, not receiving any renal replacement therapy, were recruited. Patients with systemic disease, or "immune complex" glomerulonephritis were excluded. Only 14 patients were able to complete the study.

30 patients did not complete the study. Of these, 10 patients progressed to end-stage renal failure requiring dialysis during the run-in phase of the study, and a further 3 during the first six months of the treatment phase. 11 patients were excluded because their rate of progression of CRF during the run-in period was too slow to be measurable. 6 other patients were lost from the study: 2 due to intercurrent illness (myocardial infarction in one, and infectious mononucleosis leading to acute renal failure in the other), 2 due to non-compliance, and 2 because they went abroad. The results presented are therefore based on the 14 patients who completed the study. This group included 11 males and 3 females, with a mean age of 31.4 years (range 19 - 52). CRF was due to surgically corrected obstructive uropathy in 9 patients (caused by post urethral valves (4), stones (2), solitary kidney with obstruction of contralateral ureter (2) and bladder extrophy (1)), reflux nephropathy in 2, familial glomerulonephritis in 2 and polycystic kidneys in 1 patient.

13 patients were being treated for hypertension by the time that the treatment phase of the study commenced; the agents used were enalapril (8), atenolol (5), nifedipine (2) and frusemide (2). Our aim was to maintain
resting, supine blood pressure at a level of 130/85 or less. Anti-hypertensive therapy remained unchanged once the treatment phase of the study had started.

Other drug therapy was sodium bicarbonate (2 patients), 1-α vitamin D (2 patients), alucaps (1 patient), allopurinol (1 patient) and cephalexin (1 patient).

Diabetic nephropathy patients

21 insulin-dependent (type I) diabetics were recruited into the study. Diabetic nephropathy was diagnosed by the presence of persistent proteinuria of 0.5 g/24 hours or greater. All patients had had diabetes for at least 5 years and there was no other likely cause of nephropathy.

Thirteen patients did not complete the study. Of these, 4 required dialysis while still in the run-in phase, 5 were excluded because their rate of progression was too slow to measure, and 4 were lost to follow-up for other reasons. The remaining 8 patients completed the study.

In this group there were 6 males and 2 females; mean age was 43.1 years (range 33 - 59). Duration of diabetes ranged from 5 - 28 years with a mean of 22.6 years. All 8 patients were white, and had both retinopathy and neuropathy. All were being treated for hypertension; drugs included enalapril (8), frusemide (8), atenolol (3) and prazosin (1). Other drugs (apart from insulin) were ranitidine (1 patient) and thyroxine (1 patient).

Study protocol

Patients were formally assessed at 3 monthly intervals during the study, which consisted of a run-in period, a treatment period and a wash-out period.

a) Run-in period: patients were interviewed by a dietician at the start of the run-in period of the study, and their dietary protein intake adjusted so that it did not exceed one gram /kg body weight /day. During the run-in
period of the study, which lasted at least 9 months (mean 16.7 months), patients' dietary protein intake and blood pressure were carefully controlled. At each attendance, blood samples were taken for the measurement of plasma creatinine, urea and other standard biochemical indices. Proteinuria and haemostatic indices were measured at 3 monthly intervals during the last 6 months of the run-in period.

b) Treatment period: treatment consisted of dipyridamole (300 mg/day) for 3 months, followed by the addition of aspirin (75 mg/day) and, after a further 3 months, MaxEPA (10 ml/day). The drugs were introduced in this stepwise manner so that the effect of each on proteinuria and haemostasis could be measured. Patients then continued on these 3 anti-platelet drugs for a further 6 months, making a treatment period of 12 months in all. Anti-platelet therapy was then stopped. Other drug therapy remained constant throughout this period, and proteinuria and haemostatic indices were measured 3 months after the addition of each anti-platelet agent.

c) Wash-out period: patients were assessed 3 months after stopping anti-platelet therapy, and measurements were made of proteinuria and haemostasis. Plasma creatinine measurements taken during a period of at least 6 months (mean 14.9 months) after stopping anti-platelet therapy were used to assess the rate of progression of CRF during this wash-out period.

In order to assess the risk of bleeding caused by anti-platelet therapy, skin capillary bleeding time was measured in 12 patients (3 diabetics and 9 non-diabetics) at 3 points in the study: (1) during the run-in period, (2) during the period in which patients were receiving a combination of dipyridamole and aspirin and (3) during the period in which patients were receiving a combination of dipyridamole, aspirin and MaxEPA.

Measurements of renal function

Plasma creatinine was measured by the Jaffe technique using an autoanalyser
(Chemlab Instruments, Hornchurch UK). For assessment of urinary protein excretion, urine was collected for 24 hours and preserved with thymol. Urinary protein was measured by turbidometric assay following precipitation with trichloroacetic acid.

Values of creatinine clearance were derived from the plasma creatinine concentration by a mathematical formula described by Hull et al (15). The formula used to derive creatinine clearance in ml/min/70Kg was as follows:

In men: \[
88 \times (145 - \text{age in years}) - 3
\]

plasma creatinine (umol/l)

In women: values obtained by the above formula were multiplied by 0.85.

These formulae are inaccurate in the presence of liver disease but all patients studied had normal liver function. Correlation of these calculated creatinine clearance values with glomerular filtration rate, measured by a single injection method using \(^{51}\)Cr EDTA (16), gave a coefficient of correlation of 0.91 (n = 79).

Each patient had their isotopic GFR measured between two and four times but rate of progression of renal failure was assessed as the slope of the linear regression line relating mathematically-derived creatinine clearance to time. Progression could thus be expressed in terms of ml/min/month. For each of the study periods, at least 5 derived creatinine clearance values, measured over a period of at least 12 months, were used to calculate this value, except in the case of one patient whose run-in value was assessed over only 9 months, and 5 patients whose wash-out values were assessed over only 6 months.

**Haemostatic measurements**

Blood was taken after overnight fasting using a 19 gauge needle with
minimal stasis, and processed at 4°C. Plasma concentrations of platelet factor 4 (PF4), D dimer and von Willebrand factor antigen (vWFAg), and serum concentrations of C reactive protein (CRP), were measured by radioimmunoassay and enzyme immunoassay, as previously described (17,18). Immunoreactive thromboxane (TxB₂) was measured by radioimmunoassay in serum obtained after allowing blood to clot at 37°C for 60 minutes, as previously described (17). Bleeding time was measured using a Simplate II device (Organon Teknika, Turnhout, Belgium). Blood viscosity was measured at shear rates of 23 s⁻¹ and 230 s⁻¹ using a Wells Brookfield cone and plate viscometer, as previously described (19).

Other measurements

HbAlc was measured by agar gel electrophoresis.

Comparisons between patients who responded and did not respond to therapy.
Mean values, obtained during the run-in period, of mean arterial pressure, creatinine clearance, proteinuria, rate of progression and haemostatic indices were compared between patients whose rate of progression was retarded during anti-platelet therapy (responders) and those whose rate of progression was unchanged or accelerated (non-responders). Mean arterial pressures during the treatment period were also compared between the two groups.

Statistics
Statistical comparisons of matched and unmatched data were performed by the Wilcoxon test or the Mann Whitney U test as appropriate. Run-in values of blood pressure, proteinuria and haemostatic indices were examined for trends; if no trend was seen, results obtained during treatment were compared in each patient with the mean value obtained from that patient during the run-in period. If there was a trend, then treatment values were compared with measurements obtained at the end of the run-in period.
(1) Blood pressure

Mean blood pressure fell during the run-in period from 101 (2.1) to 94 (2.3) (mean and SEM) (p <0.003) in non-diabetic, and from 115 (2.6) to 103 (5.3) (p <0.05) in diabetics. There was, however, no further significant change in blood pressure during either the treatment or wash-out periods (fig 1).

(2) Proteinuria

Proteinuria fell during the run-in period, and continued to fall during the treatment period. Compared with values at the end of the run-in period, the reduction during treatment was significant only in diabetic patients receiving a combination of dipyridamole and aspirin, or of dipyridamole, aspirin and Maxepa. When the anti-platelet therapy was withdrawn, proteinuria increased again towards pre-treatment levels (fig. 2).

(3) Rate of progression of renal failure

a) Non-diabetic CRF:

7 out of the 14 patients studied showed a slowing of the rate of progression of renal failure during the treatment period, 3 patients showed no change, and 4 patients showed an acceleration of their renal failure. In two of these latter patients, an acute impairment of renal function associated with Maxepa therapy contributed to the acceleration of renal failure. The mean fall in the rate of progression over the whole group was 0.12 ml/min/month (95% confidence intervals -0.11 to 0.35 ml/min/month). This was not statistically significant (p = 0.345).

During the wash-out period, the rate of progression was slower than during the run-in period in 8 out of 14 patients, unchanged in four and faster in two. When compared with run-in values, there was a mean fall in the rate of progression of 0.03 ml/min/month (95% confidence intervals -0.21 to 0.27
b) Diabetic nephropathy:

Progression of renal failure was slower during the treatment period than during the run-in period in 5 out of the 8 patients studied; in 2 patients the rate of progression was unchanged, and in one it was accelerated. The mean fall in the rate of progression was 0.14 ml/min/month (95% confidence intervals -0.03 to 0.31 ml/min/month). This was not statistically significant (p = 0.142).

During the wash-out period, the rate of progression was slower than during the run-in period in 4 patients, unchanged in two and faster in one. One patient suffered an episode of acute on chronic renal failure during this period and his rate of progression could not, therefore, be assessed. Compared with the run-in period, there was a mean fall in the rate of progression of 0.31 ml/min/month (95% confidence intervals -0.09 to 0.71 ml/min/month) (p = 0.181) (Table 1) (Fig. 4).

(4) Haemostatic indices

In both diabetic and non-diabetic patients, there were no significant alterations over the course of the study in circulating levels of PF4, vWFAg or of CRP (data not shown). Blood viscosity measured at both 23 s⁻¹ (data not shown) and 230 s⁻¹ (Fig. 5) was significantly reduced in both diabetics and non-diabetics during the period in which patients were receiving MaxEPA. In non-diabetics, blood viscosity was also reduced during treatment with dipyridamole alone (Fig. 5). Plasma D dimer fell in both patient groups during both the treatment and wash-out periods, although this reduction was significant only during the treatment period when patients were receiving dipyridamole alone (Fig. 6). Serum TxB₂ showed a marked reduction during the period of the study during which patients were receiving aspirin (Fig. 7).
Bleeding time was 5.08 (0.38) (mean and SEM) during the run-in period. This increased to 10.00 (1.08) during treatment with a combination of dipridamole and aspirin (p <0.005), and further to 11.78 (1.70) when patients were treated with dipyridamole, aspirin and MaxEPA (p <0.002).

(5) Other Indices

There was no significant change over the course of the study in patients' weight, nor, in the diabetic group, in levels of HbA1c.

(6) Comparisons between responders and non-responders.

In the non-diabetic group there were 7 patients in each category (Table 2). There were no significant differences, although there was a tendency for rate of progression, proteinuria and D dimer to be higher in responders. Blood pressure was similar between the two groups during both the run-in period (responders 99 ± 4.3; non-responders 98 ± 2.2 mmHg), and the treatment period (responders 98 ± 1.8 mmHg, non-responders 96 ± 2.5 mmHg).

In diabetic nephropathy (5 responders and 3 non-responders)(Table 3), creatinine clearance was significantly lower, and rate of progression significantly higher in responders. Mean arterial pressure was significantly higher during the run-in period in non-responders than in responders (112 ± 3.7 v 103 ± 0.6 mmHg, p <0.05), but during the treatment period values were not significantly different (103 ± 3.5 v 101 ± 2.5 mmHg).

(7) Side effects of treatment

Three patients could not tolerate the full dose of dipyridamole; this was due to gastrointestinal symptoms (one patient), and headache and muscle pains (two patients). All three patients were, however, able to continue on a reduced dose of dipyridamole, two on 150 mg/day and one on 200 mg/day.

Treatment with Maxepa had to be reduced or stopped in 7 patients, due to unpalatability (3), exacerbation of anginal pain (1), eyesight disturbance (1), and acute reduction in renal function (2). Three of these patients were able
to continue on a reduced dose of Maxepa (4 - 6 capsules per day). One patient suffered vaginal bleeding after 9 months of anti-platelet therapy, and treatment was stopped at this time. In this patient the bleeding time had risen from 5.54 minutes during the run-in period, to 12.6 minutes when bleeding occurred. No other patient suffered significant haemorrhagic complications.

DISCUSSION

There is good evidence for the participation of the haemostatic system in immunologically-mediated glomerular injury (20) and antithrombotic therapy has been used in forms of chronic renal disease which appear to have an immune basis such as membranoproliferative glomerulonephritis (21,22) and IgA nephropathy (23). In contrast, the present study was designed to investigate the role of platelets in chronic non-immune glomerular injury. We found that the haemostatic changes and clinical outcome which followed anti-platelet therapy were similar in both non-diabetic CRF and diabetic nephropathy.

In both groups, half the patients showed a slowing of the rate of progression of renal failure with treatment. In each group however, the overall mean retardation was not statistically significant. This was due not only to the variation in response, but also to the small number of recruited patients who completed the study. The reasons for this unavoidable drop-out rate are detailed above.

Analysis of clinical outcome in each group as a whole might overlook the existence of sub-groups of patients who are more likely than others to respond to anti-platelet therapy. Comparison of responders with non-
responders suggested that retardation of CRF was more likely in those patients with a faster rate of progression during the run-in phase of the study. Rather than delineating a sub-group of CRF patients who respond to anti-platelet therapy, this is more likely to reflect the fact that, over the relatively short duration of the study, it was easier to show improvement in rapidly progressive than in slowly progressive disease. This simple fact is important when analysing treatment results. In a controlled study of 38 patients with IgA nephropathy lasting a mean of 33.2 months, Chan and coworkers concluded that anti-platelet therapy did not favourably modify the course of the disease (23). In their control group, however, mean creatinine clearance only fell from 73 to 72 ml/min over the study period. Renal function was, therefore, declining at such a slow rate that it would have been impossible to show any modification in the study period. It should be noted that in our study all the diabetic patients and 60% of non-diabetics were receiving an angiotensin converting enzyme inhibitor, a drug which, in our experience, slows the decline of renal function and reduces proteinuria.

In the present study, comparison between responders and non-responders in the diabetic group showed significantly higher mean arterial pressures during the run-in period in non-responders. This might suggest that, unless blood pressure is adequately controlled in these patients, other forms of treatment have little chance of success. It is, however, difficult to draw conclusions from such small numbers, especially as the difference in blood pressure between the two groups was not significant during the treatment period.

Anti-platelet agents might reduce proteinuria, either by preventing the release of vasoactive and cationic mediators at sites of glomerular injury (24), or by inducing changes in glomerular haemodynamics (25). Our findings of a reduction in proteinuria in diabetic nephropathy agree with previous
reports in which patients with this condition have been treated with either a combination of aspirin and dipyridamole (26), or with dipyridamole alone (27). In our study, proteinuria was reduced to a greater extent with a combination of aspirin and dipyridamole than with the latter drug alone, but Maxepa appeared to confer no further benefit and was poorly tolerated. Dipyridamole has also been shown to reduce proteinuria in non-diabetic renal disease (28), but the fall in proteinuria we observed in non-diabetic CRF was not statistically significant.

In nephrotic children with primary FGS, treatment with heparin and dipyridamole preserved renal function and corrected the previously shortened half-life of both platelets and fibrinogen (29). In the present study, anti-platelet therapy reduced serum thromboxane levels although, in spite of this, there were no significant alterations in levels of PF4 (a marker of intravascular platelet activation), vWFAg (an endothelium-derived protein which might reflect vascular injury) or of CRP (a marker of the acute phase response). There was, however, a reduction in plasma D dimer, a breakdown product of crosslinked fibrin. In patients with progressive renal failure, but without nephrotic syndrome, circulating markers of haemostatic activation show only a moderate elevation, which would be consistent with the microscopic scale of the FGS lesion (14). This might explain the failure to detect significant haemostatic changes during the treatment period of this study.

In conclusion, anti-platelet therapy had a favorable influence on the course of progressive CRF in 50% of the patients studied, and in diabetics there was a significant fall in urinary protein excretion. Although suggestive of benefit, the results were not statistically significant, and further long-term studies with larger numbers of patients will be required to determine conclusively the value of anti-platelet therapy in progressive CRF. The
difficulties of performing such studies are highlighted both by the recent re-evaluation of one such project (30) and in the work reported here, where only a small proportion of recruited patients finally completed the study.

REFERENCES


Table 1. Rate of progression of renal failure (mls/min/month) during the three periods of the study.

### a) Non-diabetic CRF

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Mean: 0.58 0.46 0.55
SEM: 0.08 0.09 0.15

### b) Diabetic Nephropathy

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<td>3</td>
<td>0.61</td>
<td>0.50</td>
<td>0.39</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>-0.30</td>
<td>0.13</td>
</tr>
<tr>
<td>5</td>
<td>0.13</td>
<td>0.37</td>
<td>0.23</td>
</tr>
<tr>
<td>6</td>
<td>0.44</td>
<td>0.22</td>
<td>-0.03</td>
</tr>
<tr>
<td>7</td>
<td>0.14</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0.46</td>
<td>0.47</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Mean: 0.38 0.24 0.11
SEM: 0.09 0.10 0.13

Values of rate of progression of renal failure are in mls/min/month. A negative value indicates an improvement in renal function. In both non-diabetic CRF and diabetic nephropathy, statistical comparison of the groups, using the Wilcoxon test for matched pairs, showed no significant differences.
Table 2. Comparison of renal and haemostatic indices between responders and non-responders in non-diabetic CRF patients.

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>99 (4.3)</td>
<td>98 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>30.6 (7.6)</td>
<td>30.9 (7.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Proteinuria (g/24 hours)</td>
<td>3.72 (0.76)</td>
<td>1.84 (0.46)</td>
<td>NS</td>
</tr>
<tr>
<td>Rate of progression (ml/min/month)</td>
<td>0.75 (0.24)</td>
<td>0.41 (0.09)</td>
<td>NS</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>239 (52.2)</td>
<td>108 (24.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet factor 4 (ng/ml)</td>
<td>13.3 (3.1)</td>
<td>12.5 (2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>von Willebrand factor antigen (U/ml)</td>
<td>1.79 (0.19)</td>
<td>1.38 (0.16)</td>
<td>NS</td>
</tr>
<tr>
<td>C reactive protein (mg/l)</td>
<td>1.13 (0.46)</td>
<td>1.43 (0.46)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum thromboxane (ng/10^9 platelets)</td>
<td>170 (49.2)</td>
<td>225 (67.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figures are mean (SEM) of values obtained during the run-in period of the study. Statistical comparisons were performed using the Mann-Whitney U test.
### Table 3. Comparison of renal and haemostatic indices between responders and non-responders in diabetic nephropathy.

<table>
<thead>
<tr>
<th></th>
<th>Responders</th>
<th>Non-responders</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Mean arterial pressure</strong></td>
<td>103 (0.6)</td>
<td>112 (3.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(mm Hg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Creatinine clearance</strong></td>
<td>18.5 (1.9)</td>
<td>48.3 (4.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(ml/min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Proteinuria</strong></td>
<td>2.4 (0.66)</td>
<td>2.9 (0.47)</td>
<td>NS</td>
</tr>
<tr>
<td>(g/24 hours)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rate of progression</strong></td>
<td>0.46 (0.13)</td>
<td>0.26 (0.10)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(ml/min/month)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>D dimer</strong></td>
<td>291 (114.7)</td>
<td>158 (12.9)</td>
<td>NS</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Platelet factor 4</strong></td>
<td>21.4 (7.9)</td>
<td>20.0 (7.0)</td>
<td>NS</td>
</tr>
<tr>
<td>(ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>von Willebrand factor antigen</strong></td>
<td>1.55 (0.21)</td>
<td>1.42 (0.27)</td>
<td>NS</td>
</tr>
<tr>
<td>(U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C reactive protein</strong></td>
<td>0.86 (0.26)</td>
<td>2.02 (1.03)</td>
<td>NS</td>
</tr>
<tr>
<td>(mg/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serum thromboxane</strong></td>
<td>258 (55.7)</td>
<td>214 (80.2)</td>
<td>NS</td>
</tr>
<tr>
<td>(ng/10^9 platelets)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures are mean (SEM) of values obtained during the run-in period of the study. Statistical comparisons were performed using the Mann-Whitney U test.
Legends to Figures

Figure 1. Mean arterial pressure (mean and SEM) in diabetic (closed circles) and non-diabetic patients (open circles). Dp, ASA and Max indicate the points at which treatment was started with dipyridamole, aspirin and maxEPA respectively.

Figure 2. Proteinuria (mean and SEM) in diabetic (closed circles) and non-diabetic patients (open circles). Dp, ASA and Max indicate the points at which treatment was started with dipyridamole, aspirin and maxEPA respectively.

Figure 3. Decline in creatinine clearance in the 14 patients with non-diabetic CRF during the three periods of the study.

Figure 4. Decline in creatinine clearance in the 8 patients with diabetic nephropathy during the three periods of the study.

Figure 5. Blood viscosity (mean and SEM) measured at 230 s⁻¹ in diabetic (closed circles) and non-diabetic patients (open circles). Dp, ASA and Max indicate the points at which treatment was started with dipyridamole, aspirin and maxEPA respectively.

Figure 6. Plasma D dimer (mean and SEM) in diabetic (closed circles) and non-diabetic patients (open circles). Dp, ASA and Max indicate the points at which treatment was started with dipyridamole, aspirin and maxEPA respectively.
Figure 7. Serum thromboxane (mean and SEM) in diabetic (closed circles) and non-diabetic patients (open circles). Dp, ASA and Max indicate the points at which treatment was started with dipyridamole, aspirin and maxEPA respectively.
Fig 1

Mean arterial pressure (mmHg)

-6 -3 0 3 6 9 15 Months

Dp ASA Max Wash out

Fig 2

Proteinuria (g/24 hours)

-6 -3 0 3 6 9 15 Months

Dp ASA Max Wash out

p<0.01 p<0.01
HAEMOSTATIC ACTIVATION AND PROTEINURIA AS FACTORS IN THE PROGRESSION OF CHRONIC RENAL FAILURE.

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Fax 071 831 0784
SUMMARY

Haemostatic activation was measured in both patients with non-diabetic chronic renal failure (CRF) and diabetic nephropathy. We have investigated the relationship between these haemostatic markers and the rate of progression of renal failure.

When compared with age- and sex-matched healthy controls, both patient groups showed significantly elevated plasma concentrations of D dimer, von Willebrand factor antigen (vWFAg) and C reactive protein (CRP) (all p<0.001), as well as an increase in spontaneous platelet aggregation (p<0.01). Plasma concentration of platelet factor 4 was slightly, but not significantly, increased. Serum thromboxane was subnormal (p<0.01).

Multiple regression analysis showed that, in non-diabetic CRF, proteinuria and serum TxB₂ were independently related to the rate of progression of renal failure; in diabetic nephropathy proteinuria and vWFAg were independently related to the rate of progression. In both groups, the relationship was stronger with proteinuria (standardized regression coefficients 0.56 and 0.45 respectively) than with serum TxB₂ (0.29) or with vWFAg (0.37).

We have found haemostatic activation in both non-diabetic and diabetic progressive renal failure. Proteinuria, and also in this study serum TxB₂ and vWFAg, appear to be determining factors in the progression of renal failure, and their measurement may have prognostic value.

Key Words

Chronic renal failure; glomerulosclerosis; haemostasis; proteinuria.

Running title.

Haemostasis in progressive chronic renal failure.
A number of observations suggest that the haemostatic system may participate in the progression of CRF. In both non-diabetic CRF and diabetic nephropathy, the lesion characteristic of progressive renal failure is focal glomerulosclerosis (FGS). FGS shows histopathological similarities to atherosclerosis [1], and is associated with endothelial injury and microthrombosis within the glomerular capillaries. In the sub-total nephrectomy model of progressive CRF, early glomerular lesions are endothelial injury with platelet aggregation at these sites. Experimental animals given heparin were protected against the development of hypertension, FGS and progressive CRF, although heparin modified to have no anti-coagulant activity was also effective [2]. Anticoagulation with coumadin was also beneficial, though to a lesser degree than with heparin [3]. Further studies have demonstrated that both the thromboxane synthetase inhibitor OKY 1581 [3], and ticlopidine [4], at doses which inhibited ex-vivo platelet aggregation, ameliorated glomerular injury and progressive CRF in this model. These findings strongly supported a causal role for thrombosis in the pathogenesis of the FGS, although heparin may also act in other ways, in particular to inhibit mesangial cell proliferation [5].

In order to investigate further the role of haemostasis in human progressive CRF, we have measured markers of haemostatic activation in both non-diabetic patients with CRF, and in patients with diabetic nephropathy, and studied their relationship to the rate of progression of renal failure. Since haemostasis may be altered in patients with a nephrotic syndrome [6], as well as in those with immunologically mediated glomerular injury [5], we have excluded such patients from our study.

PATIENTS AND METHODS

Patients
(a) Non-diabetic CRF
39 patients were studied (26 males, 13 females; median age 31, range 17 - 70). Causes of CRF were obstructive uropathy (20), reflux nephropathy (17), hypertension (1) and polycystic kidneys (1). Of the 39 patients, 28 were on
antihypertensive therapy; drugs used were enalapril, atenolol, nifedipine and frusemide.

(b) Diabetic nephropathy
30 patients were studied (19 males, 11 females; median age 50 years, range 31 - 70). Diabetic nephropathy was diagnosed by the presence of persistent proteinuria of 0.5 g/24 hours or greater. Renal biopsies were performed if the cause of the disease was not certain to be diabetes. 20 patients had type I and 10 patients had type II diabetes mellitus (DM). HbA1c levels ranged from 6.3 - 16.3% (mean 10.0), and duration of DM from 5 - 34 years (mean 17.5). 23 patients were on antihypertensive therapy; drugs used were enalapril, atenolol, nifedipine, hydrallazine and frusemide.

In both groups our aim was to maintain the resting, recumbent blood pressure at <130/85, or mean arterial pressure <105 mmHg. No patient in either group was undergoing dialysis treatment, nor was any suffering from a nephrotic syndrome. Comparison of haemostatic indices in patients on antihypertensives with values in those who were not on such therapy showed no statistical differences.

Methods
Venesection
Blood was taken after overnight fasting, using a 19 gauge needle and minimal stasis. For measurement of D dimer, plasma was obtained from blood anticoagulated with EDTA (4 mmol/l final concentration); for von Willebrand factor antigen (vWFAG) and platelet aggregation blood was anticoagulated with a 1/10 volume of 3.13% (w/v) trisodium citrate, and for platelet factor 4 (PF4) blood was anticoagulated with a mixture of EDTA, theophylline and PGE1. C reactive protein (CRP) and immunoreactive thromboxane (TxB2) were measured in serum from blood which had been allowed to clot for 60 minutes at 37°C. Plasma and serum samples were obtained by centrifugation at 4°C for 30 minutes at 1500g, and stored at -40°C until assayed. Platelet rich plasma (PRP) for platelet aggregation studies was obtained by centrifugation for 10 minutes at 150g and room temperature. Citrate anticoagulant concentration in blood used for aggregation studies was corrected for the effects of differences in hematocrit, as previously described [7].
Haemostatic markers

The following measurements were made in order to reflect different aspects of haemostatic activation;

(1) D dimer was used as a measure of the formation and subsequent breakdown of crosslinked fibrin. We have previously shown that plasma levels of D dimer are not influenced by loss of renal excretory function, making it particularly suitable as a haemostatic marker in patients with renal failure [8].

(2) PF4 was used as a measure of intravascular platelet activation since, like D dimer, it is a reliable haemostatic marker in renal failure [9]. Platelet reactivity was further characterized by measurements of spontaneous aggregation (the degree of aggregation occurring in platelet rich plasma stirred for 15 minutes at 37°C) and of serum TxB2 levels.

(3) Plasma levels of the endothelium-derived protein vWFAg were measured as an indicator of vascular injury [10].

(4) CRP was used as an indicator of the acute phase response [11].

Assay techniques

D dimer, vWFAg and CRP were measured by enzyme immunoassay, and PF4 and serum TxB2 by radioimmunoassay, as previously described [7,8]. Spontaneous platelet aggregation was measured using a turbidometric aggregometer, as previously described [7] and expressed in terms of percent aggregation, where 100% was the difference in light transmission between platelet rich- and platelet-poor plasma.

Renal function

Plasma creatinine was measured by the Jaffe technique using an autoanalyser (Chemlab Instruments, Hornchurch, UK), and urinary protein by turbidometric assay following precipitation with trichloroacetic acid. For assessment of urinary protein excretion, urine was collected for 24 hours and preserved with thymol. Glomerular filtration rate was measured by a single injection method using 51Cr EDTA. Creatinine clearance was calculated from the plasma creatinine concentration by a mathematical formula described by Hull et al [12]. The formula used to derive creatinine clearance in mls/min/70 Kg was as follows:

In males: \[ \frac{88(145 - \text{age in years}) - 3}{\text{plasma creatinine (umol/l)}} \]
In females, results obtained using the above formula were multiplied by 0.85. There was a good correlation between these calculated creatinine clearance values and isotopic GFR measurements ($r = 0.91; n = 79$).

Rate of progression of renal failure (measured as rate of decline in creatinine clearance) was calculated for each patient by performing regression analysis of derived creatinine clearance values (in mls/min) against time (in months). The slope obtained was taken as the rate of progression (in mls/min/month). At least 5 creatinine clearance values (median 7, range 5-18), measured over a period of at least 9 months (median 14, range 9-24), was used to calculate the rate of progression.

Analyses

Each patient was studied on 2 to 3 occasions during the period over which the rate of progression of renal failure was assessed, and the mean result of each haemostatic measurement used in the analysis of data.

(1) In order to detect haemostatic abnormality in non-diabetic CRF and diabetic nephropathy, haemostatic indices were compared in 21 patients selected from each of the two patient groups to match one another in terms of age, sex and degree of renal impairment, and in 21 healthy controls matched for age and sex. Details of these groups are given in table I. Statistical comparisons were performed by the Mann Whitney test for non-parametric data.

(2) Since the majority of non-diabetic patients were suffering from either obstructive uropathy or reflux nephropathy, renal and haemostatic indices were compared between these two groups, in order to determine whether there were any significant differences.

(3) The relationship between haemostatic indices and the severity of renal disease were investigated by Spearman rank correlation of these indices with creatinine clearance, proteinuria and rate of progression of renal failure. Data from all 39 non-diabetic patients, and all 30 diabetic patients were included in this analysis.

(4) In order to determine which factors had a significant independent effect on the rate progression of renal failure, stepwise multiple regression analysis was performed using the statistical package for the social sciences (SPSS) on an IBM personal computer. Rate of progression was the dependent variable, with proteinuria, creatinine clearance, and the different haemostatic measurements
as the indicator variables. Values of proteinuria and creatinine clearance were log. transformed prior to analysis. The non-diabetic CRF and diabetic nephropathy groups were analysed separately, using data from all patients studied in each group.

RESULTS

(1) Comparison of haemostatic indices in CRF, diabetic nephropathy and healthy controls.
Spontaneous platelet aggregation, and plasma concentrations of D dimer, vWF Ag and CRP were all elevated to a similar degree in both CRF and diabetic nephropathy (table II). In contrast, serum TxB2 was reduced. PF4 was higher than controls in both patient groups, but this was not statistically significant.

(2) Comparison of renal and haemostatic indices in patients with obstructive uropathy and reflux nephropathy.
Creatinine clearance was significantly lower in the 20 patients with obstructive uropathy (median 16.5 mls/min, range 6.6-48.4) than in the 17 patients with reflux nephropathy (median 29.4 mls/min, range 7.5-79.1) (p = 0.016). There were, however, no other significant differences between the two patient groups in terms of proteinuria, rate of progression of renal failure or haemostatic indices.

(3) Correlation of haemostatic indices with indicators of the severity of renal disease.
Correlations performed by the Spearman rank technique showed a number of significant relationships.
(a) Correlations with creatinine clearance.
In non-diabetic CRF, creatinine clearance showed significant inverse correlations with vWF Ag (rs = -0.37, p = 0.02) and spontaneous platelet aggregation (rs = -0.35, p = 0.029). There were no significant correlations in diabetic patients between creatinine clearance and haemostatic parameters.
Correlations with the rate of progression of renal failure.
In non-diabetic CRF, the rate of progression of renal failure correlated positively with proteinuria ($rs = 0.50, p = 0.001$) (fig. 1), vWFAg ($rs = 0.37, p = 0.021$) and serum TxB$_2$ ($rs = 0.46, p = 0.003$) (fig. 2). The correlation between rate of progression and proteinuria was stronger in those patients with reflux nephropathy ($rs = 0.70, p = 0.002$) than in those with obstructive uropathy ($rs = 0.31, p = 0.183$); other correlations were similar between the two groups.

In diabetic nephropathy, rate of progression showed a negative correlation with creatinine clearance ($rs = -0.45, p = 0.012$) and a positive correlation with proteinuria ($rs = 0.52, p = 0.003$), vWFAg ($rs = 0.38, p = 0.042$) (fig. 3) and CRP ($rs = 0.40, p = 0.023$).

It should be noted that all these correlations, though significant, showed wide scatter (Figs 1-3).

Multiple regression analysis
Regression of all indicator variables together against rate of progression yielded $R^2$ values of 0.57 ($p = 0.008$) for the non-diabetic group, and 0.48 ($p = 0.014$) for the diabetic group. The factors which showed a significant independent effect on the rate of progression of renal failure were proteinuria and serum TxB$_2$ in non-diabetic CRF, and proteinuria and vWFAg in diabetic nephropathy (table III).

DISCUSSION
We have demonstrated haemostatic activation in both non-diabetic CRF and diabetic nephropathy. This is consistent with published evidence of intraglomerular deposition of platelets and fibrin in human chronic renal disease [5], and supports the hypothesis that glomerular vascular injury and microthrombosis participate in the development of the FGS lesion. We found $R^2$ values of 0.57 and 0.48 respectively in multiple regression analysis of our data from non-diabetic CRF and diabetic nephropathy, suggesting that approximately half the variation in rate of progression could be explained by the indicator variables included in the analysis.
Previous haematological evidence of thrombin generation in CRF includes elevation of plasma fibrinopeptide A (FpA) [13], but FpA may be raised in CRF due to decreased elimination, as well as by haemostatic activation [14]. Our demonstration of raised plasma concentrations of D dimer, which is a reliable haemostatic marker in renal failure [8], suggests a true increase in fibrin turnover in CRF. This is consistent with reports of a shortening of the half-life of fibrinogen in primary FGS [15], and might reflect glomerular fibrin deposition. The levels of D dimer we observed (200 to 300 ng/ml) are not greatly elevated above the normal range, but would be consistent with the scale of the FGS lesion. It is important to note, however, that there was no correlation between the rate of progression of renal failure and plasma D dimer, and it is therefore possible that we have detected non-renal thrombosis, especially since the incidence of atheroma-related cardiovascular disease is increased in these patients. Blood pressure is also known to have an important effect on the progression of renal disease, as well as being a risk factor for atherosclerosis. We have not examined separately the effect of blood pressure on haemostatic changes or rate of progression.

Plasma concentrations of vWF Ag were raised in our study. This has been reported by others, and interpreted as a reflection of vascular injury, either due to glomerular damage [16] or to atherosclerosis [17]. Caution is required, however, when interpreting vWF Ag levels in renal failure, due to the influence of reduced clearance [18], and of the acute phase response [19]. In the present study, there was a small, but significant, elevation of CRP, and an acute phase response might therefore explain the increase in vWF Ag. Nevertheless, vWF Ag, but not CRP, correlated independently with the rate of progression of renal failure in the diabetic nephropathy group; plasma vWF Ag may therefore be a true reflection of glomerular vascular injury in these patients.

Platelet activation promotes renal injury in glomerulonephritis [5], but in our patients plasma PF4 showed only a small elevation which was not statistically significant. PF4 may lack some sensitivity as a marker of intravascular platelet activation [20], and is cleared from the circulation very rapidly [21]; a chronic, microthrombotic lesion such as FGS might therefore be too subtle to be detected by measurement of this marker in peripheral blood. Platelets in both progressive CRF and diabetic nephropathy showed a greater tendency to
aggregate spontaneously than in healthy controls, and this in spite of reduced TxB2 generation. Recent evidence has shown a close relationship between spontaneous platelet aggregation and coronary mortality in survivors of myocardial infarction, suggesting that spontaneous aggregation may be a useful biological marker of atherosclerotic vascular damage [22]. Reduction in serum TxB2 is a manifestation of the uraemic platelet defect, which we have previously shown to be present even in the early stages of progressive CRF [7]. Despite being lower than in healthy controls, serum TxB2 showed a weak, but significant independent relationship to the rate of progression of renal failure in non-diabetics. This finding is of interest since thromboxane synthetase inhibitors protect the kidney in experimental models of progressive CRF [3], and inhibition of platelet aggregation and intraglomerular thrombosis might be an important mechanism by which this benefit was achieved. Our findings suggest that, at least in non-diabetics, this platelet defect associated with renal failure may be beneficial.

We have found an independent correlation between proteinuria and the rate of progression of renal failure in both diabetic nephropathy and in non-diabetic CRF. These results confirm those of Williams and colleagues, who found significant correlations between proteinuria and the rate of progression in CRF due to glomerulonephritis and pyelonephritis, and a suggestive, though not statistically significant, correlation in diabetic nephropathy [23]. We would support their conclusion that proteinuria may be a useful prognostic index in established renal failure.

In conclusion, we have found haemostatic changes which may reflect the microthrombotic component of FGS. We are currently conducting a trial of antithrombotic therapy in progressive CRF which may indicate more clearly the role of haemostasis in this disorder.

ACKNOWLEDGEMENTS

We thank the St. Peter's Trust and the Sir Halley Stewart Trust for supporting this project, and Drs. A.B.Kurtz, D.J.Betteridge, S.McHardy-Young and G.A.Nelstrop for allowing us to study patients under their care.
REFERENCES


Table I. Groups for comparison of haemostatic markers in diabetic nephropathy (DN), CRF and healthy controls.

<table>
<thead>
<tr>
<th></th>
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<th>CRF*</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>M:F</td>
<td>13:8</td>
<td>13:8</td>
<td>13:8</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean (range)</td>
<td>48.4</td>
<td>47.6</td>
<td>41.2</td>
</tr>
<tr>
<td></td>
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<td>(30 - 70)</td>
<td>(31 - 56)</td>
</tr>
<tr>
<td>Cr.Cl.(ml/min)</td>
<td>28.8</td>
<td>21.9</td>
<td>Not tested</td>
</tr>
<tr>
<td>mean (range)</td>
<td>(8 - 58)</td>
<td>(7 - 78)</td>
<td></td>
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* Causes of renal failure: Reflux nephropathy (7), obstructive uropathy (12), hypertensive nephropathy (1), polycystic kidneys (1).
Table II Haemostatic markers in non-diabetic progressive CRF and diabetic nephropathy.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRF</th>
<th>Diabetic Nephropathy</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>D dimer (ng/ml)</td>
<td>52</td>
<td>220&lt;sup&gt;c&lt;/sup&gt;</td>
<td>280&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(27 - 260)</td>
<td>(42 - 1020)</td>
<td>(34 - 740)</td>
<td></td>
</tr>
<tr>
<td>vWF Ag (U/ml)</td>
<td>1.00</td>
<td>1.58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>(0.67 - 1.60)</td>
<td>(0.91 - 2.69)</td>
<td>(0.91 - 2.77)</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.58</td>
<td>0.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(0.13 - 1.45)</td>
<td>(0.18 - 7.50)</td>
<td>(0.28 - 5.00)</td>
<td></td>
</tr>
<tr>
<td>PF4 (ng/ml)</td>
<td>10</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>(2 - 26)</td>
<td>(6 - 26)</td>
<td>(2 - 97)</td>
<td></td>
</tr>
<tr>
<td>Serum TxB&lt;sub&gt;2&lt;/sub&gt; (ng/10&lt;sup&gt;9&lt;/sup&gt; plats)</td>
<td>288</td>
<td>140&lt;sup&gt;c&lt;/sup&gt;</td>
<td>173&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(41 - 1043)</td>
<td>(17 - 524)</td>
<td>(35 - 1169)</td>
<td></td>
</tr>
<tr>
<td>Spont. Aggn. (%)</td>
<td>3</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(1 - 11)</td>
<td>(2 - 24)</td>
<td>(3 - 38)</td>
<td></td>
</tr>
</tbody>
</table>

D dimer, von Willebrand factor antigen (vWF Ag), C reactive protein (CRP), platelet factor 4 (PF4), serum thromboxane (TxB<sub>2</sub>) and spontaneous platelet aggregation (spont. aggn.) in non-diabetic progressive renal failure (CRF), diabetic nephropathy, and matched healthy controls. Figures are median (range). a p<0.05; b p<0.01; c p<0.001.
Table III Results of multiple regression analysis.

<table>
<thead>
<tr>
<th>All Variables</th>
<th>Multiple R</th>
<th>$R^2$</th>
<th>P</th>
<th>Multiple R</th>
<th>$R^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRF (n = 39)</td>
<td>0.75</td>
<td>0.57</td>
<td>0.008</td>
<td>0.70</td>
<td>0.48</td>
<td>0.014</td>
</tr>
<tr>
<td>Diabetic nephropathy (n = 30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Independent Variables</td>
<td>Beta</td>
<td>P</td>
<td>Beta</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinuria</td>
<td>0.56</td>
<td>0.001</td>
<td>0.45</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum TxB$_2$</td>
<td>0.29</td>
<td>0.032</td>
<td>0.11</td>
<td>0.533</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWFAg</td>
<td>0.18</td>
<td>0.195</td>
<td>0.37</td>
<td>0.046</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple correlation coefficients ($R$), and standardized regression coefficients (beta) of proteinuria, serum thromboxane (TxB$_2$) and von Willebrand factor antigen (vWFAg) against rate of progression of renal failure (as the dependent variable) in non-diabetic progressive chronic renal failure (CRF) and diabetic nephropathy.
Legends to Figures

Figure 1.
Correlation between 24 hour protein excretion and rate of progression of renal failure (measured as rate of decline in creatinine clearance) in 39 non-diabetic patients with chronic renal failure.

Figure 2.
Correlation between serum immunoreactive thromboxane (TxB$_2$) and rate of progression of renal failure (measured as rate of decline in creatinine clearance) in 39 non-diabetic patients with chronic renal failure.

Figure 3.
Correlation between plasma von Willebrand factor antigen and rate of progression of renal failure (measured as rate of decline in creatinine clearance) in 30 patients with diabetic nephropathy.
AN ASSESSMENT OF WHOLE BLOOD IMPEDANCE AGGREGOMETRY USING BLOOD FROM NORMAL SUBJECTS AND HEMODIALYSIS PATIENTS

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ABSTRACT

Impedance aggregometry allows the measurement of platelet responses in whole blood as well as in PRP. The variability of haematocrit values encountered when applying this technique to hemodialysis patients prompted an investigation of the effects of red cells on platelet aggregation in whole blood. Collagen induced aggregation was measured in both PRP and whole blood from hemodialysis patients and healthy controls. Platelets from hemodialysis patients were less aggregable than those from the controls when tested in PRP, but more aggregable when tested in whole blood. Blood samples with a range of haematocrit values were prepared by mixing PRP and autologous red cells, and used to study the effect of haematocrit on platelet aggregation. In blood from control subjects aggregation rate was reduced by rising haematocrit but no reduction of maximum aggregation occurred until haematocrit exceeded 40%. In contrast uraemic platelets showed increased responses in the presence of red cells. In a limited cross over study no significant difference was found in the effect on platelet aggregation of washed erythrocytes from uraemic and non-uraemic donors. It is concluded that red cell presence influences platelet aggregation by complex mechanisms during impedance aggregometry and that this effect must be considered when interpreting results.

INTRODUCTION

Simulation of in-vivo conditions would seem to be desirable when investigating platelet function and the recent development of the electronic impedance aggregometer allows the measurement of platelet aggregation

Key words: Platelet aggregation, uraemia, erythrocytes.
responses in whole blood as well as in platelet-rich plasma (PRP) (1). Anticoagulated whole blood samples can be tested immediately after venesection, and the technique thus offers potential advantages over turbidometric aggregometry where centrifugation of the sample is required for the production of PRP, involving delay and the possible removal of certain populations of platelets, and where the influence of red cells on platelet reactions is excluded.

Uraemic patients on chronic haemodialysis suffer from both an increased incidence of atheroma-associated cardiovascular mortality and also from a bleeding tendency which is due in part to platelet dysfunction (2). Aetiology of the platelet defect is complex and may involve toxins (2,3), defective factor VIII Von Willebrand factor (4), excessive PGI₂ production by vascular endothelium (5), high levels of parathyroid hormone (6) and anaemia (7). Haemodialysis has been shown either to correct (8,9), partially correct (10) or fail to correct (11) platelet function measurements. The variety of methods used in these studies may in part account for the differences in results obtained, and variation in patient population and dialysis technique may also contribute. The observed bleeding and thrombotic tendencies in haemodialysis patients may be a function, not only of intrinsic platelet reactivity, but also of platelet count, haematocrit (7) and perhaps intrinsic red cell properties.

We have used the impedance aggregometer to compare aggregation responses in whole blood and PRP from haemodialysis patients and healthy controls in order to assess whether aggregation responses in these two groups are altered by the presence of red cells. The influence of haematocrit on platelet aggregation in uraemic and non-uraemic whole blood was then studied using blood samples with a range of artificially prepared haematocrit values. Lastly, a cross over study was performed in which washed erythrocytes from haemodialysis patients and controls were interchanged prior to the induction of platelet aggregation to compare the effects of uraemic and non-uraemic red cells.

MATERIALS AND METHODS

Use of the impedance aggregometer. Blood samples were collected into a 0.1 volume of 3.13% w/v trisodium citrate and used for whole blood aggregometry without further treatment. Haematocrit values were measured in EDTA blood samples using a microhaematocrit centrifuge (Gelman Hawksley Limited). PRP was prepared by centrifugation at 150 g for 15 minutes followed by correction of platelet count to match that obtained in whole blood. Counts were adjusted using platelet-poor plasma (PPP), obtained by centrifugation at 1500 g for 15 minutes. Platelets were maintained at room temperature throughout and platelet counts performed using an electronic counter (Coulter Electronics, Luton, Beds).

Aggregation responses in both whole blood and PRP were carried out using the impedance aggregometer (prototype model developed by Wellcome Research Laboratories). This technique employs the detection of changes in electrical resistance between two electrodes immersed in the test sample. 1 ml samples were mixed using a magnetic stirrer and allowed to equilibrate at 37°C for two minutes before the addition of aggregating agent. Electron microscope studies have shown that during this period a platelet monolayer forms on the electrodes (1). Rapid accretion of platelets onto this monolayer occurs following aggregation stimulus, with consequent impedance of
electrical conduction between the electrodes. The measured increase in resistance is proportional to the magnitude of the aggregation response.

Calibration of the instrument was performed using a 5 ohm resistor connected across the electrodes. The deflection given by this resistor with the electrodes immersed in the test sample was used as a standard. Test results were compared with this and expressed in ohms (Ω).

Comparison of platelet responses in whole blood and PRP. Blood was taken prior to dialysis from six uremic patients on chronic haemodialysis (haematocrit mean: 22%, range: 18-26%. Platelet count mean: 119.5 × 10⁹/l, range: 127-250 × 10⁹/l) and also from six healthy laboratory personnel (haematocrit mean: 41%, range: 38-44%. Platelet count mean: 250.3 × 10⁹/l, range: 184-308 × 10⁹/l). No drugs likely to affect platelet function had been taken in the previous two weeks. Whole blood and PRP were prepared as described above and platelet aggregation was induced in parallel on different channels of the instrument using collagen (Hormon Chemie) at final concentrations of 0.5, 1.0 and 1.5 μg/ml. Aggregation was carried out within one hour of blood collection and results of initial aggregation rate and maximum extent of aggregation obtained in whole blood and PRP were compared using a paired t test.

Effect of haematocrit on platelet aggregometry. PRP and PPP from healthy volunteers and from haemodialysis patients were prepared as described above and packed red cells obtained from the tube which had been centrifuged to produce PPP. Packed red cells were added to autologous PRP to give a range of artificial haematocrit values from 10% to 50% (e.g. 0.3 ml packed red cells added to 0.7 ml PRP gave a haematocrit of 30%). PRP was also mixed with PPP in the same proportions to give control samples with similar platelet counts. Aggregation was then induced in both test blood samples and control PRP samples using 1 μg/ml collagen, and compared in order to elucidate the effects of increasing haematocrit independent of its dilutional effect on the platelet count. The experiments were performed using both normal blood and blood from haemodialysis patients.

Effects on platelet aggregation of erythrocytes from haemodialysis patients and healthy subjects. In order to assess the respective influence of red cells from haemodialysis patients and healthy subjects on platelet aggregation, red cells from the two groups were interchanged prior to the induction of an aggregation response. PRP and packed red cells were obtained as described above from six haemodialysis patients and six healthy volunteers. Following removal of the buffy layer, red cells were washed twice in 0.9% saline and then added to PRP to give a haematocrit value of 30%. Both autologous red cells and also red cells from one of the other group (with identical ABO blood type) were used. Platelet aggregation was then induced using 1 μg/ml collagen and the effects of the two sets of red cells compared by means of a paired t test.

RESULTS

Platelet aggregation studies in whole blood and PRP. Comparison of results obtained in whole blood with those obtained in PRP shows that platelets from haemodialysis patients gave similar values for aggregation rate when tested in whole blood and PRP but values of aggregation maximum were significantly higher in whole blood than in PRP (Fig 1). In contrast, when using platelets from healthy subjects, values of aggregation rate in whole blood were significantly lower than in PRP, whereas maximum values were unchanged (Fig 2).
Comparison of platelet aggregation in whole blood and PRP in haemodialysis patients

Comparison of platelet aggregation in whole blood and PRP in healthy subjects

**FIG 1**
Aggregation rate (A) and maximum (B) in PRP (open circles) and whole blood (closed circles) from haemodialysis patients. NS: Not Significant, *p<0.01

**FIG 2**
Aggregation rate (A) and maximum (B) in PRP (open circles) and whole blood (closed circles) from healthy subjects. NS: Not Significant, *p<0.005
Comparison of platelet aggregation in PRP from haemodialysis patients and healthy subjects

**FIG 3**
Aggregation rate (A) and maximum (B) in PRP from healthy subjects (open circles) and haemodialysis patients (closed circles). NS: Not Significant, *p = < 0.05, **p = < 0.02

Comparison of platelet aggregation in whole blood from haemodialysis patients and healthy subjects

**FIG 4**
Aggregation rate (A) and maximum (B) in whole blood from healthy subjects (open circles) and haemodialysis patients (closed circles). NS: Not Significant, *p = < 0.05, **p = < 0.02, ***p = < 0.005
If the same data is plotted to compare responses of platelets from haemodialysis patients with those from healthy subjects it is seen that responses of platelets from haemodialysis patients were reduced in comparison with normals in PRP (Fig 3), although this in part might be accounted for by the lower platelet count in haemodialysis patients. However, in whole blood this trend is reversed and platelets from haemodialysis patients were more aggregable than normal platelets despite lower platelet counts (Fig 4).

Effective concentration of citrate anticoagulant will be lower in blood from haemodialysis patients due to anaemia. This may enhance platelet aggregation in this group but cannot explain the reversal in trends seen when comparing aggregation responses between groups in whole blood and PRP.

A similar pattern of results was obtained in identical experiments using ADP (Sigma) at concentrations of 2, 5 and 10 μmolar (data not shown).

Effect of haematocrit on whole blood aggregometry. Following the addition to PRP of autologous erythrocytes to give increasing haematocrit values, the presence of erythrocytes was shown to have differing effects on platelet aggregation in blood from normal subjects and from haemodialysis patients.

Comparison of platelet aggregation in PRP from healthy subjects to which has been added either autologous red cells or PPP (as a control) to show the effect of haematocrit.

![Comparison of platelet aggregation in PRP from healthy subjects to which has been added either autologous red cells or PPP (as a control) to show the effect of haematocrit.](image)

FIG 5

Aggregation rate (A) and maximum (B) in PRP (open circles) and whole blood (closed circles) from healthy subjects. NS: Not Significant, *p= < 0.05, **p= < 0.01, ***p= < 0.002.
In normal blood (Fig 5) red cells caused retardation of the aggregation response and aggregation rate was lower in whole blood than in PRP samples with compatible platelet count. Values of maximum aggregation were unchanged in whole blood until haematocrit values exceeded 40%, after which significant reduction was shown. (Statistical comparison was performed using a paired t test).

When using blood from haemodialysis patients (Fig 6) values of aggregation rate and maximum were higher in whole blood than in PRP at all haematocrit values, this increase reaching statistical significance at haematocrits of 20% and 30%.

Comparison of platelet aggregation in PRP from haemodialysis subjects to which has been added either autologous red cells or PPP (as a control) to show the effect of haematocrit

![Graph showing effects of haematocrit on platelet aggregation rate and maximum](image)

**FIG 6**
Aggregation rate (A) and maximum (B) in PRP (open circles) and whole blood (closed circles) from haemodialysis patients. NS: Not Significant, \(*p < 0.05\), \(**p < 0.002\), \(**p < 0.01\)

Effects on platelet aggregation of erythrocytes from haemodialysis patients and healthy subjects. In the cross over study in which erythrocytes from healthy subjects and haemodialysis patients were interchanged, platelets from both groups showed a trend towards greater responses in the presence of red cells from haemodialysis patients, but this was not statistically significant (TABLE 1). We have not therefore been able to confirm that these red cells have a greater capacity to activate platelets than do normal red cells.
### TABLE 1

**Platelet Aggregation in the Presence of Normal and Uraemic Red Cells (PCV = 30%)**

<table>
<thead>
<tr>
<th>Platelets</th>
<th>Red Cells</th>
<th>Rate (μ/min)</th>
<th>Paired Maximum (μ)</th>
<th>Paired t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>5.0 ± 0.29</td>
<td>NS</td>
<td>11.2 ± 0.85</td>
</tr>
<tr>
<td>Normal</td>
<td>Uraemic</td>
<td>5.5 ± 0.43</td>
<td>NS</td>
<td>12.0 ± 0.74</td>
</tr>
<tr>
<td>Uraemic</td>
<td>Normal</td>
<td>2.4 ± 0.78</td>
<td>NS</td>
<td>7.4 ± 1.93</td>
</tr>
<tr>
<td>Uraemic</td>
<td>Uraemic</td>
<td>2.5 ± 0.76</td>
<td>NS</td>
<td>8.6 ± 2.13</td>
</tr>
</tbody>
</table>

### DISCUSSION

This study has shown that platelet aggregation measurements obtained using the impedance aggregometer may be influenced by the presence of red cells. This influence is different in blood from haemodialysis patients and healthy subjects and varies with haematocrit value.

The most prominent effects of increasing haematocrit were a reduction of aggregation rate in normal blood with no change in maximum extent and an increase in maximum extent in blood from haemodialysis patients with no change in aggregation rate. Thus haemodialysis patients' platelet responses exceeded those of normals when measured in whole blood even though they were lower than normals in PRP.

The explanation of these findings is unclear. Both chemical and physical mechanisms of red cell influence on platelet function have been postulated by various workers. Chemical activation of platelets by red cell-derived ADP has been suggested (12), and experimental support for this has been given by evidence of reduced platelet function in whole blood following red cell membrane stabilisation by chlorpromazine at concentrations lower than those necessary to inhibit platelets directly (13,14). Our findings of platelet enhancement in blood from haemodialysis patients might be explained by increased chemical activation by uraemic red cells. Indeed we have demonstrated using the impedance aggregometer that prevention of ADP loss from red cells by the use of chlorpromazine causes reduction of platelet aggregation in whole blood from uraemic subjects as well as from normal subjects (unpublished observations). However this effect was not more prominent in uraemics than in normals. In the cross over study reported in this paper we found no significant difference between platelet aggregation in the presence of red cells from healthy subjects and haemodialysis patients. Thus there was no definite indication that uraemic red cells themselves contribute to increasing platelet aggregation.

In flowing blood red cells are thought to enhance the radial movements of platelets towards the blood vessel wall, and in experimental...
systems employing conditions of linear flow, red cells have been shown to increase platelet deposition onto exposed subendothelium, this increase being correlated with haematocrit (15,16). Thus a physical mechanism for red cell influence on platelet function exists in flowing blood. Since linear flow effects are absent in the impedance aggregometer this form of interaction is excluded, but other physical mechanisms of red cell/platelet interaction may still occur in this test system. On one hand the contribution of erythrocytes to the size of the aggregate mass will cause an increase in electrical resistance between the detector electrodes and thus serve to increase aggregation responses in whole blood. In contrast the presence of red cells may cause physical obstruction to platelet collisions and interactions and thus inhibit platelet aggregation. The relative importance of these two opposing effects may depend upon several factors such as the metabolic state of the platelets, plasma constituents and viscosity, and haematocrit value. A possible explanation for the results obtained in this study may therefore be that conditions in normal blood favour inhibition by red cells whereas conditions in blood from haemodialysis patients favour enhancement. Further work is required to clarify this, especially since platelet responses in haemodialysis patients are subject to varied and complex influences. If these interactions have a physiological counterpart then they may help in our understanding of uraemic haemostasis.

In considering the value of impedance aggregometry in the investigation of platelet function, it is important to recognise that the information given in whole blood will not necessarily parallel that given in PRP, by this or any other technique. Impedance aggregometry may therefore be used in conjunction with turbidometric methods, but not as an alternative to them. The effect of haematocrit value must be considered when comparing data obtained from different patient groups. Haematocrit can affect impedance aggregometry results directly, as we have shown here, and also indirectly via alterations in citrate anticoagulant concentration (17). We have found that in healthy subjects values of maximum aggregation are affected less than values of aggregation rate by haematocrit variations. Aggregation maximum may therefore be the most useful parameter to measure when using impedance aggregometry in non-uraemic populations. Indeed a recent study has shown that this parameter gives the best correlation with measurements of aggregate mass (18).

Despite its limitations impedance aggregometry in whole blood offers advantages for ex-vivo platelet function studies in that it involves minimal delay between withdrawal of blood and measurement of platelet aggregation. It gives reproducible results in an environment that simulates in-vivo conditions more closely than does PRP, and it might be particularly suitable for monitoring within patient changes of platelet function where other variables as previously discussed remain constant. It is therefore a useful addition to the established methods of platelet function testing.

ACKNOWLEDGEMENTS

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REFERENCES


