THE ROLE OF THE COAGULATION SYSTEM IN THE PATHOGENESIS OF LIVER INJURY

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STATEMENT OF ORIGINALITY

I confirm that the work described in this thesis is my own.

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ABSTRACT

There is a growing body of evidence that suggests a role for the coagulation cascade in promoting liver injury, in particular fibrosis. With the exception of thrombin, the expression and role of individual coagulation proteins in liver injury is poorly understood. The aim of this body of work was to further our understanding of the role of specific coagulation proteins in liver injury and consider viable targets as anti-fibrotic therapies. We examined these concepts using immunohistochemical, cell line, and animal based studies as well as translational studies in human subjects.

Using immunohistochemical techniques, upregulation of coagulation proteins in both acute and chronic liver injury in mice and humans was demonstrated and correlated with severity of injury. Data from cell line studies demonstrated stellate cells, the principal cell involved in hepatic fibrosis, were sensitive to both thrombin and Factor Xa. Furthermore the direct inhibition of both thrombin and Factor Xa using novel anticoagulants in cell line studies and chemical induced murine models of liver fibrosis exhibited anti-fibrotic effects. Two unique translational studies were undertaken to determine the applicability of these findings in humans. A pilot study demonstrated that warfarin anticoagulation resulted in a significant reduction in liver stiffness measurements, a surrogate marker of liver fibrosis, in patients with pre-existing hepatitis C related liver fibrosis, and the interim results of a larger study, performed in the setting of hepatitis C post liver transplantation, has shown a reduction in fibrosis scores.

The studies presented in this thesis add to the growing body of evidence suggesting a role for coagulation proteins in the pathogenesis of liver injury and are the first to show the potential anti-fibrotic benefits of both novel anticoagulants in murine models of liver fibrosis and warfarin anticoagulation in patients with hepatitis C related liver fibrosis pre and post transplantation.

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ABBREVIATIONS

APTT	Activated partial thromboplastin time
ALF	Acute liver failure
ALT	Alanine aminotransferase
αSMA	Alpha smooth muscle actin
PIIINP	Aminoterminal peptide of procollagenase 3
ATIII	Antithrombin III
AST	Aspartate aminotransferases
APRI	AST platelet ratio index
BSA	Bovine serum albumin
C1 INH	C1-inhibitor
CB	Cannabinoid
CCL4	Carbon tetrachloride
CCL4	Carbon Tetrachloride
CCL21	Chemokine ligand 21
CCR	Chemokine receptor
CD 31	Cluster of Differentiation 31
CD 68	Cluster of Differentiation 68
CTGF	Connective tissue growth factor
DAB	3,3'-Diaminobenzidine
DER	Derriford Hospital
DDR-2	Discoidin domain receptor-2
DMEM	Dulbecco's minimal essential medium
EDIN	Edinburgh Royal Infirmary.
ET-1	Endothelin-1
EGF	Epidermal growth factor
ECM	Extracellular matrix
ELF	Enhanced Liver Fibrosis test
FVL	Factor V Leiden
FXa	Factor Xa
FPA, FPB	Fibrinopeptides
FBS	Foetal bovine serum
GGT	Gamma-glutamyl transpeptidase
GVHD	Graft versus host disease
H&E	Haemtoxylin & Eosin
HSC	Hepatic stellate cell
HTT	Hepatic transit time
HBV	Hepatitis B virus
HCV	Hepatitis C

HCC	Hepatocellular carcinoma
HKa	High-molecular-weight kininogen
HIV	Human immunodeficiency virus
HA	Hyaluronic acid
HIF-1a	Hypoxia induced factor-1- alpha
ICSS	Imperial College Scoring system
IGF-1	Insulin growth factor-1
INR	International Normalised Ratio
KCH	Kings College Hospital
LSM	Liver stiffness measurements
LMWH	Low molecular weight heparins
MMP-2	Matrixmetalloproteinase-2
MCP-1	Monocyte chemotactic protein-1
NAPQI	N-acetyl-p-benzoquinoneimine
NPV	Negative predictive value
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NO	Nitric oxide
PBS	Phosphate buffered saline
PL	Phospholipid
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PPV	Positive predictive value
PAR	Protease activated receptor
FII	Prothrombin
PT	Prothrombin time
QEH	Queen Elizabeth Hospital, Birmingham
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RFH	Royal Free Hospital
SJUH	St. James University Hospital, Leeds
SMH	St. Mary's Hospital, London
SEM	Standard error of mean
SPSS	Statistical Package for the Social Sciences
TAA	Thioacetamide
FIIa	Thrombin
TAT	Thrombin–Antithrombin complex
TM	Thrombomodulin
TSP-1	Thrombospondin-1
TF	Tissue Factor
TFPI	Tissue factor pathway inhibitor
TIMPs	Tissue inhibitors of metalloproteinases
TNF-alpha	Tissue necrosis factor- alpha
TLR4	Toll Like Receptor 4

TGF-beta-1	Transforming growth factor beta-1
TBS	Tris buffered saline
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand Factor
WAFT-C	Warfarin anticoagulation for liver fibrosis in patients transplanted for hepatits c virus infection

1. GENERAL INTRODUCTION

1.1. LIVER DISEASE

Liver disease represents a significant global health problem. Acute liver failure is a rare, but life threatening illness accounting for over 2000 deaths in the United States per year (Hoofnagle *et al.,* 1995; Ostapowicz *et al.,* 2002). In contrast, the majority of liver related morbidity and mortality is related to chronic liver disease, with cirrhosis having an estimated worldwide prevalence of 1% (Schuppan *et al.,* 2008).

1.1.1. Acute liver failure

Acute liver failure (ALF) was initially defined as a syndrome of rapidly progressing liver failure in which encephalopathy develops within eight weeks of onset of symptoms, in the absence of previous liver disease (Trey, 1970). Further modifications to this classification have evolved to incorporate both aetiology and speed of onset of encephalopathy. A more recent classification defines ALF, as the development of encephalopathy within 12 weeks of the onset of jaundice with three subgroups: hyperacute, acute and subacute (O'Grady *et al.,* 1993). In acute and subacute liver failure, the onset of encephalopathy occurs within 8-28 days and 5-12 weeks respectively after the onset of jaundice; whereas in hyperacute liver failure the encephalopathy develops within seven days of the onset of jaundice.

1.1.1.1. Aetiological factors in ALF

The aetiological factors responsible for ALF vary markedly according to geographical location. In the United Kingdom (UK) paracetamol overdose accounts for greater than 50%, whereas in France it accounts for 4% cases of ALF (O'Grady *et al.,* 2000). More recently,

the incidence of paracetamol induced ALF has declined due to the introduction of UK legislation limiting the amount of paracetamol that can be bought over the counter. This is in contrast to what is being observed in the United States, where an increase in inadvertent paracetamol poisoning is occurring. Worldwide, viral hepatitis is the most common cause of ALF. In Japan the high incidence and prevalence of viral hepatitides account for up to 55% causes of ALF. More recently, the increased use of herbal remedies and recreational drugs constitute a small but significant number of cases of severe heptatoxicity and ALF (Steadman, 2002). In both the UK and USA, the second most common 'cause' is seronegative, or ALF of unknown aetiology (O'Grady *et al.,* 2000).

1.1.1.2. Paracetamol toxicity and acute liver injury

Paracetamol toxicity is a leading cause, in humans, of acute hepatic failure (Ostapowicz *et al.,* 2002). Despite extensive research, the mechanisms involved in the process are not fully understood. Numerous factors contribute to paracetamol induced injury including microchrondrial dysfunction, reactive oxygen and nitrogen species, and cytokines (James *et al.,* 2003; Cover *et al.,* 2005; Chiu *et al.,* 2003; Gardner *et al.,* 2002; Hinson *et al.,* 2004; Jaeschke *et al.,* 2003). Animal models have demonstrated that paracetamol is bioactivated by the hepatic cytochrome P450 pathway to a highly toxic and reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) (Whitcomb *et al.,* 1994; Manyike *et al.,* 2000; Corcoran *et al.,* 1980; Bessems *et al.,* 2001; Jollow *et al.,*1973; Mitchell *et al.,* 1973). At therapeutic doses, 90 percent of paracetamol is metabolized in the liver to sulfate and glucuronide conjugates, which are then excreted in the urine (Forrest *et al.,* 1982; Mitchell *et al.,* 1973; Manyike *et al.,* 2000). However, with toxic doses of paracetamol, the sulfation and glucuronidation pathways are saturated, and more paracetamol is metabolized to NAPQI (Lee *et al.,* 1996).

When hepatic glutathione stores are depleted by approximately 70 to 80 percent, NAPQI begins to react with hepatocytes, (Prescott, 1983; Mitchell *et al.,* 1973; Gemborys *et al.,* 1980; Linden *et al.,* 1984). This process leads to oxidative injury and hepatocellular centrilobular necrosis. Although not fully characterized, lipid peroxidation and mitochondrial injury likely play a role in the progression of hepatocellular injury (Larson *et al.,* 2005, Knight et al., 2003). In addition, it appears that the release of cytokines, reactive nitrogen and oxygen species from damaged hepatocytes may also contribute to propagation of hepatic injury.

The clinical course of poisoning is often divided into four sequential stages (Singer *et al.,* 1995):

Stage I: In the first 24 hours after overdose, patients often manifest nausea, vomiting, diaphoresis, pallor, lethargy, and malaise. Some patients remain asymptomatic. Laboratory studies are typically normal.

Stage II. From 24 to 72 hours after ingestion, the clinical and laboratory evidence of hepatotoxicity and, occasionally, nephrotoxicity become evident. Initially, stage I symptoms usually resolve and patients appear to improve clinically while subclinical elevations of hepatic aminotransferases (AST, ALT) occur. Cytokine release from hepatocytes may initiate a secondary inflammatory response from Kupffer cells and other inflammatory cells, extending the zone of hepatic injury (Michael *et al.,* 1999; Blazka *et al.,*1995; Liu *et al.,* 2004; Liu *et al.,*2006; Ishida *et al.,* 2002). As stage II progresses, patients develop right upper quadrant pain, with liver enlargement and tenderness. Elevations of prothrombin time (PT),

international normalized ratio (INR) and total bilirubin can occur with oliguria and renal function abnormalities.

Stage III: Liver function abnormalities peak from 72 to 96 hours after ingestion. The systemic symptoms of stage I can reappear in conjunction with jaundice and hepatic encephalopathy, Signs of severe hepatotoxicity include plasma ALT and AST levels that often exceed 10,000 IU/L, prolongation of the PT or INR, hypoglycemia, lactic acidosis, and an elevated bilirubin concentration.

Stage IV (4 days to 2 weeks) — Patients who survive stage III enter a recovery phase that usually begins by day 4 and is complete by 7 days after overdose (Shakil *et al.,* 2000). Recovery can be slower in severely ill patients; symptoms and laboratory values may not normalize for several weeks. Histological changes in the liver vary from cytolysis to centrilobular necrosis. The centrilobular region is preferentially involved because it is the area of greatest concentration of CYP2E1 and therefore the site of maximal production of NAPQI.

1.1.1.3. Coagulopathy and acute liver injury

Haematological abnormalities occur in acute liver injury since the liver is responsible for the synthesis of coagulation factors. In paracetamol induced ALF patients have lower serum concentrations of various coagulation factors, with a reduction in circulating levels of fibrinogen, factors II, V,VII, IX and X (Kerr, 2003). This is thought to be primarily due to decreased synthesis of coagulation factors within the damaged liver. Alternatively, increased consumption via activation of the coagulation cascade could contribute to this phenomenon.

Indeed, increased peripheral consumption of coagulation factors and platelets is well documented in this type of injury. The concentration of plasma tissue factor (TF) is elevated in patients following paracetamol injury, and this is associated with an increase in plasma concentrations of the thrombin–antithrombin (TAT) complexes (Kerr, 2003). Hence the profound coagulopathy in acute liver injury is likely multi-factorial, with reduced synthesis of coagulation proteins in the acutely failing liver accompanied by consumption of coagulation proteins peripherally. The extent of consumption or activation of coagulation factors centrally within the liver during acute liver injury remains less defined. One can hypothesise that in paracetamol induced injury, hepatic sinusoidal endothelial cells are damaged, which would favour hepatic consumption of coagulation factors, via up-regulation of TF and the activation of the coagulation cascade (DeLeve *et al*., 1997, Williams *et al*., 2003). A single study has demonstrated hepatic fibrin deposition in a mouse model of paracetamol injury (Ganey *et al*., 2007). No study to date has evaluated whether activated coagulation proteins are expressed in human liver tissue following paracetamol injury. Furthermore, activation of coagulation factors in the acutely failing liver may not only contribute to coagulopathy via consumption but also have an important role in the initiation or propagation of injury. Microcirulatory dysfunction, caused by activation of the cascade and fibrin deposition could result in a potent ischaemic environment which contributes to injury further. These concepts need to be studied further to understand the role coagulation proteins in the generation of acute liver injury and whether they represent viable therapeutic targets.

1.1.2. Chronic liver disease

Chronic Liver disease is now the fifth most common cause of mortality in the UK, but unlike the first four causes (ischaemic heart disease, cerebrovascular disease, respiratory disease and cancer), liver disease mortality is increasing (Office for National Statistics: http://www.statistics.gov.uk/). The majority of liver disease results from chronic inflammation driving tissue fibrosis leading to cirrhosis, diminished hepatic function and hepatocellular carcinoma (HCC). This results in increased mortality and morbidity with reduced quality of life and life expectancy (Younossi *et al*., 2001). Indeed, hepatocellular carcinoma now has the fastest rising cancer incidence of any cancer in the western world (Friedman, 2010).

1.1.2.1. Liver fibrosis

Liver fibrosis represents the uniform response of the liver to toxic, infectious, and metabolic agents and is characterised by an increased synthesis and altered deposition of extracellular matrix components (Friedman, 2008). It is a complex dynamic process, involving the recruitment and activation of platelets, inflammatory cells, hepatic stellate cells and other extracellular matrix producing cells, including portal fibroblasts and bone marrow derived cells (Calvaruso *et al*., 2008; Beaussier *et al*., 2007; Forbes *et al*., 2004). For the most part, it is a process which evolves over many years, however it can occasionally be rapid, in the case of drug induced liver injury, Hepatitis C virus (HCV) co-infection with human immunodeficiency virus (HIV) or HCV infection post transplantation (Schluger *et al.,* 1996; Bonnard *et al.,* 2007; Friedman, 2008).

In viral hepatitis, alcoholic steatohepatitis and non alcoholic steatohepatitis, the commonest causes of liver fibrosis, fibrogenesis is invariably initiating by inflammation. This is in contrast

to haemachromatosis, where there is characteristically a lack of inflammatory cell infiltrate, but still fibrosis, meaning other mediators in addition to cellular inflammation can initiate fibrosis (Friedman 2008). Possibilities include: reactive oxygen species generated by lipid peroxidation, via induction of cyochrome P450 (Castillo *et al.,* 1993; Chitturi, 2001); apoptosis, which can result from liver injury itself (Jaeschke, 2002); and innate immunity pathways including toll like receptor 4 (TLR4) signalling (Seki *et al.,* 2007).

1.1.2.1.1. Extracellular matrix

The extracellular matrix (ECM) is a dynamic structural component of the normal liver (Wells, 2008). It is made up of macromolecules which provide the scaffolding of the liver and act as transducers of extracellular signals. In the normal liver, the basement membrane-like matrix or ECM, is made up of collagens, glycoproteins, fibronectin, laminin, tenascin, von Willebrand factor (vWF) and proteoglycans (Schuppan *et al.,* 2001). Various cellular sources of the ECM have been demonstrated, the main source being the hepatic stellate cell.

In fibrotic livers, the quantity and quality of the ECM changes, increasing up to eight fold compared to normal livers. A significant increase in collagen content and proteoglycans occurs, resulting in a higher density interstitial type matrix (Rojkind *et al.,* 1970; Gressner, 1998). With progressive fibrogenesis the ECM composition changes from one predominantly made up of type IV collagen, proteoglycans and laminin to a matrix consisting of type I and III collagen. Fibronectin released from sinusiodal endothelial cells into the ECM contributes to transforming growth factor-beta (TGF-beta) mediated stellate cell activation (Jarnagin *et al.,* 1994).

The constitutional change in the ECM has resulting consequences on surrounding cells, including hepatocytes, endothelial cell dysfunction, and perpetuation of stellate cell activation (McGuire *et al.,* 1992). Angiogenic stimuli, including platelet derived growth factor (PDGF)

and vascular growth factor (VEGF) cause capillarisation of sinusoids (Rosmorduc *et al.,* 2010). The accumulation and change in matrix composition also drives fibrosis further, by stimulating positive feedback mechanisms. Altered signalling promotes intergins, and membrane bound proteins Rho and Rac to further provoke stellate cell migration and activation (Yang *et al.,* 2003; Zhou *et al.,* 2004; Melton *et al.,* 2007; Shafiei *et al.,* 2006; Zhang et *al.,* 2006; Yee, 1998; Choi *et al.,* 2006), and activation of matrix metalloproteases, result in release of growth factors stimulating further fibrogenesis (Schuppan *et al.,*1998; Schuppan *et al.,* 2001).

1.1.2.1.2. Signalling in hepatic fibrosis

Fibrogenesis is a complex mechanism involving an array of cellular and extra-cellular signalling. Cytokines, chemokine, adipokine, neuroendocrine, angiogenic and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) signalling have all been found to play an important function (Freidman 2010). The release of cytokines in the context of fibrogenesis, is controlled in part by activation of metalloproteinases. TGF-beta-1, PDGF, connective tissue growth factor (CTGF), tissue necrosis factor-alpha (TNF-alpha), and vascular endothelial growth factor (VEGF) are all involved in key mechanisms of fibrosis (Schuppan *et al.,* 2001; Pinzani *et al.,* 1994; Marra *et al.,* 2001). TGF-beta-1 is of particular importance, mediating stellate cell activation and stellate cell collagen production (Inagaki *et al.,* 2007; Breitkopf *et al*., 2006). VEGF and PDGF mediate angiogenesis, which contributes to ECM production and portal hypertension, and regeneration post injury (Fernandez *et al.,* 2009).

Chemokines such as CCRI and CCR5 receptors are profibrotic mediators (Seki *et al.,* 2009; Seki *et al.,* 2009). Adipokines, such as leptin and adiponection, are synthesised by hepatic stellate cells, and involved in fibrogenesis (Ding *et al.,* 2005; Ikejima *et al.,* 2007).

Neuroendocrine signalling including the cannabinoids and their respective receptors have fibrogenic properties. Cannaboid 1 receptor (CB1) is a profibrotic mediator (Teixeira-Clerc *et al.,* 2006). CB2 conversely has anti-fibrotic but proinflammatory properties (Freidman, 2010).

Neutrophil and monocyte chemoattractants are also released leading to increased local inflammatory infiltration (Marra *et al.,* 1998). Oxidative stress is long recognised to be a important inducer of liver fibrosis. Recently the NADPH oxidase enzyme complex has been found to be vital in this process (De Minicis *et al.,* 2007). Indeed inhibition of NADPH, using losartan has demonstrated anti-fibrotic potential in mice.

1.1.2.1.3. Platelet activation

Platelets are the first cells recruited to a site of injury. They have a vital role by limiting blood loss via the formation of aggregates, in damaged blood vessels, and converting fibrinogen into fibrin. Furthermore their alpha-granules are rich in growth factors such as PDGF, TGF-beta and VEGF. These factors are released upon activation and are potent stimulators of fibrogenic cells, including hepatic stellate cells (Henderson *et al*., 2007).

1.1.2.1.4. Extracellular matrix producing cell populations

Hepatic stellate cells are the primary source of the ECM in both normal and fibrotic livers (Friedman 2003). However it is increasingly evident that fibrogenic cells not only arise from hepatic stellate cells, but other cell lines, including portal fibroblasts, hepatocytes, cholangiocytes, circulating fibrocytes and bone marrow recruitment of mesenchymal cells (Wells 2008; Forbes *et al*., 2004; Jhandier *et al,* 2005; Beaussier *et al*., 2007; Kisseleva *et al*., 2006). The degree of contribution from these different cellular sources likely differs with

aetiology and stage of liver injury. Portal fibroblasts may be of importance in cholestatic liver disease (Beaussier *et al*., 2007) The primary fibrogenic cellular source therefore needs to be considered when developing novel anti-fibrotic therapies for specific liver diseases (Friedman, 2008).

1.1.2.1.5. Hepatic stellate cells

A considerable body of research implicates the hepatic stellate cell (HSC) as the principal cell type involved in hepatic fibrogenesis. (Friedman, 2008). Its activation is the common pathway leading to hepatic fibrosis. HSCs normally lie in a quiescent state in the sub-endothelial space of Disse and comprise 15% of liver cells (Wells, 2008). In the normal liver they are the principal storage site for retinoids and are thought to be derived from neural crest tissue, since they express neural crest markers, including glial fibrillary acidic protein and nestin (Niki *et al*., 1999). They are activated by a number of stimuli, including pro-inflammatory cytokines and protease activated receptor-1 (PAR1) ligation, which results in a myofibroblastic phenotype (Friedman, 2008). This myofibroblast is proliferative, fibrogenic and contractile. Activation consists of two major phases, an initiation phase followed by a phase of perpetuation (Freidman, 2000).

1.1.2.1.5.1. Hepatic stellate cells activation: Initiation

Initiation refers to the early changes that occur in gene expression and phenotype initiated by a range of mediators, that render the previously quiescent hepatic stellate cell responsive to other stimuli (Friedman, 2000). The characteristic feature of the initiation phase is the production of a fibrogenic, contractile, phenotype with induction of PDGF receptors. Initiation is invariably stimulated by a variety of neighbouring cells including apoptotic hepatocytes, injured cholangiocytes, Kupffer cells, injured sinusoidal endothelial cells, other

stellate cells or other inflammatory cells (Freidman 2008): Hepatocyte apoptosis generates oxidative stress since hepatocytes are a rich source of lipid peroxides and stellate cell activation is mediated by TLR9 and Fas, a protein involved in apoptosis (Canbay *et al.,* 2002; Watanabe *et al.,* 2007); Kupffer cells via TGF-beta-1 and production of reactive oxygen species stimulate stellate cell activation (Bilzer *et al.,* 2006). Endothelial cells, when injured, via fibronectin activate stellate cells (Jarnagin *et al.,* 1994). Platelets via PDGF, TGF-beta-1 and epidermal growth factor (EGF) (Bachem *et al.,* 1989) are another source of activation.

1.1.2.1.5.2. Hepatic stellate cells activation: Perpetuation

Initiation activates the stellate cells, while perpetuation is the response to stimuli which maintains the activated stellate cell in the myofibroblastic state to allow fibrotic scar formation (Freidman, 2008). Perpetuation consists of several discrete changes in stellate cell function aimed to contribute to excess abnormal matrix production. These changes include stellate cell proliferation, chemotaxis, fibrogenesis, increased contractility, altered matrix degeneration, and cytokine signalling, (Friedman, 2000). These actions work in synergy to propagate fibrogenesis. For example proliferation and chemotaxis ensure increased numbers of collagen producing cells are present at the site of injury to amplify fibrogenesis.

1.1.2.1.5.2.1. Proliferation

The release of a variety of mitogenic factors causes proliferation of stellate cells. PDGF is a potent stellate cell mitogen (Borkham-Kamphorst *et al.,* 2007), and has been shown to be up-regulated in liver injury (Pinzani *et al.,* 1994). Indeed induction of PDGF receptors on stellate cells occurs early in the initiation phase (Wong *et al.,* 1994). Other factors include VEGF (Yoshiji *et al.,* 2003) thrombin, (Marra *et al.,* 1995; Marra *et al.,* 1998) EGF, TGF alpha, kerinocyte growth factor, and fibroblast growth factor (Steiling *et al.,* 2003, Yu *et al.,* 2004).

Matrixmetalloproteinase-2 (MMP-2), act as stellate cell mitogens, via activation by discoidin domain receptor-2 (DDR-2) binding to fibillar collagen (Olaso *et al.,* 2001; Olaso *et al.,* 2002).

1.1.2.1.5.2.2. Chemotaxis

Activated stellate cells migrate to towards areas of hepatic injury. A number of growth factors have been identified within the liver, which have chemoattractant properties. These include PDGF, insulin growth factor-I (IGF-I), endothelin-I (ET-I), monocyte chemoattractant protein I (MCP-I) and the chemokine receptor CXCR3 (Bonacchi *et al.,* 2001; Gentilini *et al.,* 2000; Marra *et al.,* 1998; Tangkijvanich *et al.,* 2001). Adenosine in contrast to these mediators, inhibits further chemotaxis, allowing cells to fix at the site of injury (Hashmi *et al.,* 2007).

1.1.2.1.5.2.3. Fibrogenesis

The primary event of fibrogenesis is increased matrix production and scar formation, which is predominantly mediated by stellate cells and results in hepatic fibrosis (Friedman 2003). Stellate cells regulate the expression of type I, III and IV collagen. Type I collagen, which is the major constituent of scar tissue, is up-regulated post-transcriptionally in stellate cells by the actions of TGF-beta via SMADs (Pinzani *et al.,* 2001). Other less potent mediators of type I collagen production include angiotensin II, interleukin -1b and TNF (Battellier *et al.,* 2003). Connective tissue growth factor (CTGF) also promotes fibrogenesis (Paradis *et al.,* 2002; Rachfal *et al.,* 2003; Gao *et al.,* 2004; Chen *et al.,* 2010).

1.1.2.1.5.2.4. Contractility

On activation, the hepatic stellate cell contracts, a state that is characterised by the increased expression of alpha smooth muscle actin (αSMA), a contractile filament protein (Rockey *et al*., 1992). This results in impedance of sinusoidal blood flow increasing portal resistance, and eventually increasing portal pressure once advanced fibrosis has developed (Rockey 2001; Pinzani *et al.,* 1992; Melton *et al.,* 2006). Progressive development of intrahepatic shunting occurs in conjunction with the constriction of hepatic sinusoids. The process is mediated by ET-1 as well as angiotensinogen II, and atrial natriuretic peptide (Rockey 2003; Reynaert *et al.,* 2002; Racine-Samson *et al.,* 1997). Nitric oxide (NO) has an opposing effect, resulting in sinusoidal relaxation (Rockey, 2001).

1.1.2.1.5.2.5. Matrix degradation

The concept of matrix degradation evolved following evidence that resolution of fibrosis can occur and subsequently that cirrhosis is reversible. This has given rise to the notion that fibrosis is a dynamic balance between matrix production and degradation. Initial matrix degradation may be termed 'pathologic', and corresponds to the disruption of the normal low density matrix of the subendothelial basement membrane, which occurs in the early stages of fibrosis. This allows for normal matrix to be replaced by a higher density pathological matrix, containing fibrils. Matrix degrading collagenases, including the metalloproteinases (MMP) break down collagen type IV and are central to this (Arthur 1995; Benyon et al., 2001; Iredale 2001). Stellate cells and Kupffer cells are the primary sources of MMP-2 and MMP-9 respectively (Benyon *et al.,* 2001; Arthur *et al.,* 1992). Conversely MMP-1 degrades type I collagen and is involved in the 'restorative' degradation of the pathological matrix (Friedman, 2003), and upregulation of this enzyme would favour resolution of scar tissue. Tissue inhibitors of metalloproteinases (TIMPs), inhibit the actions of collagenases,

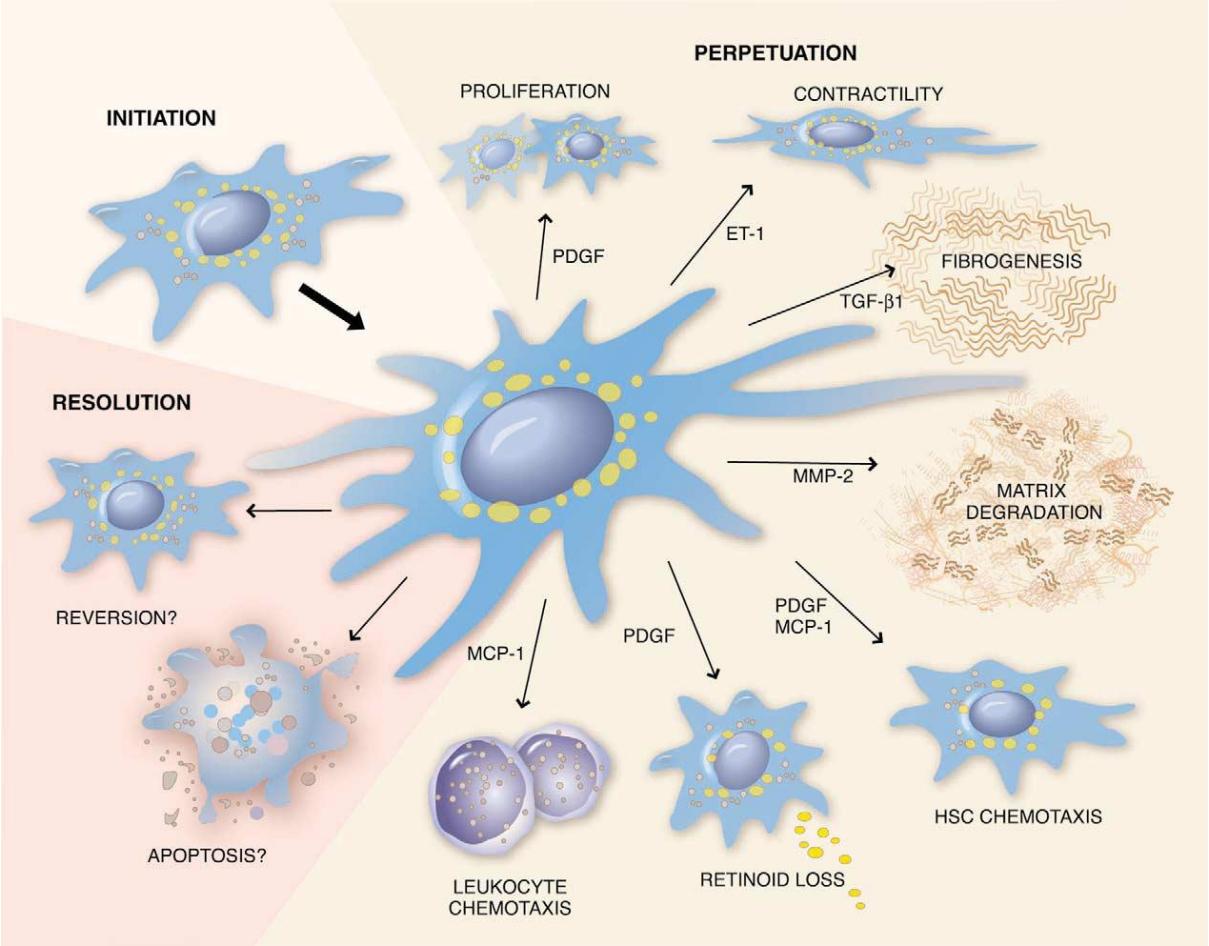
resulting in reduced matrix degradation, and inhibit stellate cell apoptosis, which inhibits resolution of fibrosis and favours matrix accumulation (Iredale, 2007). TIMP-1 and 2 are up-regulated in progressive fibrosis, (Murawaki *et al.,* 1997; Iredale *et al.,* 1998; Herbst *et al.,* 1997) and their sustained release from stellate cells (Iredale, 2001) is potentially a key determinant of progressive fibrosis. The emergence of these concepts further highlight the need to develop viable antifibrotics, since inhibiting fibrosis and matrix accumulation would not only prevention further fibrosis but could potentially cause regression, by tipping the balance away from matrix accumulation towards degradation.

1.1.2.1.5.2.6. Hepatic stellate cell mediated inflammatory signalling

The concept that stellate cells are purely effectors of fibrosis no longer exists. Evidence suggests stellate cells also have a central role amplifying inflammatory signalling, via toll like receptors, and cytokines including MCP-1, CCL21, CCR5 and RANTES. They demonstrate antigen presenting capabilities, and also produce neutrophil chemoattractants (Paik *et al.,* 2003; Marra *et al.,* 2002; Bonacchi *et al.,* 2003; Schwabe *et al.,* 2003).

Figure 1-1: Pathways of stellate cell activation and resolution during liver injury

The activated stellate cell or myofibroblast is proliferative, fibrogenic and contractile. Activation consists of two major phases, an initiation phase followed by a phase of perpetuation (From Freidman 2003).



1.2. EXPERIMENTAL MODELS OF LIVER INJURY

1.2.1. In vitro hepatic stellate cell lines

The development of techniques for isolating and cultivating HSCs represented a major advance in exploring the roles of these cells in the complex mechanisms involved in fibrogenesis (Friedman, 2008). Rodent stellate cell lines have been characterised (Greenwel *et al.,* 1993; Vogel *et al.,* 2003) but now have been been superseded by human cell lines. The most commonly used human HSC cell lines include the LX1 and LX2 cell lines. The LX-2 cell line has been generated by isolating stellate cells from normal human livers and immortalizing them by culturing in low serum conditions. When activated these cells bear close similarities to in vivo human activated HSCs (Xu *et al.,* 2005). The LX2 cell line has been extensively validated and already used in a number of studies exploring the effects of mediators on stellate cell activity (Freidman, 2008).

Utilising these cell lines therefore allows us the unique opportunity to study the factors that both inhibit and activate HSCs. Culturing HSC cells on plastic results in cellular activation (Bachem *et al.,* 1992; Friedman *et al.,* 1989; Geerts *et al.,* 1989) whereas growing on non adherent surfaces, maintains a quiescent state (Friedman *et al.,* 1994). Stellate cells additionally grow well on a variety of extracellular matrices including ones consisting of type I collagen matrix. This has given rise to the use of contractility assays which permit a quantitative measure of HSC activation and contractility (Sohail *et al.,* 2009).

1.2.2. Murine Models of Liver Injury

The use of murine models is an invaluable tool in providing insight into the pathogenesis of liver injury. Ideally an animal model should reproduce the identical pathological process seen in humans, while reproducibly demonstrating similar pathophysiological complications, patterns of morphology, disease progression, reversibility and mortality (Tsukamoto *et al.,* 1990). As yet no animal model reproduces human fibrosis exactly but rodent models still offer us the opportunity to study the pathogenesis of liver injury, and in particular the interactions of different cell types or systems while controlling environmental and genetically conditions offering, in intact organisms, whose physiological and pathological responses closely mimic those of humans (Anstee *et al.,* 2006). Various different models have been described, each having modelled different types of liver injury. The majority of studies investigating liver fibrosis employ mouse models utilising chemically induced fibrosis. Acute liver injury can be studied using animal models of paracetamol injury, which allow us a unique opportunity to examine the pathogenesis of acute liver injury in an analogous manner to that of human injury (Birge *et a*l., 1990).

1.2.2.1. Chemically induced liver fibrosis

A variety of agents, often carcinogenic can be employed to chemically induce fibrosis. The most commonly used are carbon tetrachloride and thioacteamide. Fibrosis generated in this manner has both a degree of reproducibility and robustness (Weiler-Norman *et al.,* 2007), which are criteria vital to any method of disease modelling. These models also have the added advantages that they can be used in conjunction with either transgenic or wild type mice to explore the underlying molecular mechanisms involved in liver fibrosis or can be

used to evaluate the potential of novel antifibrotic agents, using strictly controlled environmental and genetic conditions.

1.2.2.1.1. Carbon Tetrachloride (CCL4)

Carbon tetrachloride (CCL4) is the oldest and commonest method of inducing liver fibrosis in rodent models (Cameron *et al.,* 1936; Marrione *et al.,* 1949). It is a halothane, which results in hepatic injury when given at a repetitive low dose. It can be administered through a variety of routes, but is most commonly administered by intraperitoneal injection up to three times per week. CCl4 is bioactivated by oxidases. This leads to a combination of effects including CCL3 radical formation in the liver, which causes hepatocyte damage via lipid peroxidation, hepatic stellate cell activation, Kuppfer cell activation and TGF-Beta 1 upregulation, and increased oxidative stress (Knittel *et al.,* 1999; Hillebrant *et al.,* 2002; Kim *et al.,* 2006; Yu *et al.,* 2002). Histological changes result in fatty change with necrosis and intense necroinflammation in centrilobular areas. This progresses onto both septal and non-septal fibrosis resulting in extensive fibrosis and cirrhosis. The fibrous septae are classically thin, and can regress when CCL4 is withdrawn (Weiler-Norman *et al.,* 2007). CCL4 provides us a model of fibrosis originating from hepatocellular necrosis, hence can be used as an indirect model of HCV related liver fibrosis, since no direct murine model of this infection exists. The reproducibility and ease of induction of fibrosis are its main advantages. Disadvantages include heterogeneity in amounts of fibrosis produced between animals (Weiler-Norman *et al.,* 2007).

1.2.2.1.2. Thioacetamide

Thioacetamide (TAA) was first described over sixty years ago as method of inducing liver fibrosis (Fitzhugh *et al.,* 1948). A selective hepatoxin, chronic administration not only induces liver fibrosis but can result in tumors, including cholangiocarcinoma and HCC (Yeh *et al.,* 2004). TAA is usually given in drinking water over a period of 8 to 18 weeks to induce liver fibrosis (Muller *et al.,* 1988; Dashti *et al.,* 1989). Acute administration of TAA can result in both necrosis and nephrotoxic damage (Constantinou *et al.,* 2006).

TAA is an organic sulphuric compound that is metabolised to acetamide and TAA S-oxide, by a microsomal monooxygenase requiring NADPH and cytochrome P450 (Chieli *et al.,* 1984; Lee *et al.,* 2003). TAA S-oxide is then metabolised to TAA-S-dioxide, an unstable reactive metabolite that is thought to initiate necrosis and oxidative stress by covalently binding to liver macromolecules (Hunter *et al.,* 1977; Porter *et al.,* 1978; Low *et al.,* 2004; Chilakapati *et al.,* 2005; Kornek *et al.,* 2006; Dashti *et al.,* 1989; Wang *et al.,* 2000; Ramaiah *et al.,* 2001).

Histologically, mild to moderate amounts of fibrosis develop by 8 weeks. By 12 weeks of exposure, parenchymal damage occurs, with hepatocyte swelling, necrosis and proliferation (Zimmerman *et al*., 1987). This results in fibrous enlargement of the portal tracts, with portal-portal and portal-central septae developing resulting in cirrhosis (Zimmerman *et al*., 1987; Li *et al.,* 2006; Muller *et al.,* 1988). Inflammation and fatty infiltration is less extensive than in CCL4 induced injury (Kreft *et al.,* 1998). Histological similarities with human viral hepatitis, have led to its use as an indirect model of both fibrosis secondary to Hepatitis B virus (HBV) and HCV infection. Withdrawal of thioacetamide results in resolution of fibrosis over an 8 week period (Zimmerman *et al.,* 1987). A longer period of resolution, when compared to CCL4 models, makes it a more suitable model, when evaluating potential antifibrotic therapies, and the occurrence of regenerative nodules make it more comparable

to human cirrhosis (Zimmerman *et al.,* 1987; Dashti *et al.,* 1989). When given orally it has ease of use, cost, and animal safety benefits with less mortality compared to CCL4 based regimens (Li *et al.,* 2002). A degree of heterogencity in fibrosis levels between study animals can be observed, but this is less than in CCL4 models (Li *et al.,* 2006). TAA does require a longer period of administration to develop significant levels of fibrosis, which has cost implications regarding animal husbandry.

1.2.2.2 Other rodent models of liver fibrosis

Alternative models to chemical induced liver fibrosis have been developed. Rats opposed to mice can be utilized to induce cholestatic fibrosis using a bile duct ligation model (Weiler-Norman *et al.,* 2007). In this model ligation of the bile duct surgically results in a cholestatic injury leading to fibrosis within 2 weeks and cirrhosis within one month (Weiler-Norman *et al.,* 2007). The advantage of this model is the study of mechanisms underlying cholestatic liver injury and trialling anti-fibrotics in an animal setting that may be of benefit in biliary cirrhosis.

1.3. THE COAGULATION CASCADE AND CHRONIC LIVER INJURY

1.3.1. Coagulation cascade

The coagulation cascade traditionally is divided into an extrinsic pathway, which is the principal pathway, and an intrinsic pathway. Both pathways converge to form a final common pathway resulting in thrombin (Factor IIa) production (See Figure 1-1). Recently in vivo evidence suggests the division between the two pathways is increasingly indistinct, with both being initiated by similar factors with multiple interactions existing downstream between the two pathways (Mann et al., 2009). Vascular or endothelial injury, including inflammation, initiates the extrinsic pathway by the binding of plasma factor VII/VIIa to tissue factor (TF). TF acts with activated factor VIIa and phospholipid (PL) to convert factor IX to IXa and factor X to Xa. The intrinsic pathway includes contact XIa and activation of factor XI by the XIIa–activated high-molecular-weight kininogen (HKa) complex. Factor X1a converts factor IX to IXa, and factor IXa, in turn, converts factor X to Xa, in concert with factor VIIIa and PL (the "tenase" complex). Factor Xa is the active catalyst of the "prothrombinase" complex, which includes factor Va and PL and converts prothrombin to thrombin. Thrombin cleaves fibrinopeptides (FPA, FPB) from fibrinogen, allowing the resultant fibrin monomers to polymerise, and converts factor XIII to XIIIa, which cross-links the fibrin clot. Among the procoagulant drivers, factor VIII plays a key role, being responsible together with factor IX and the negatively charged phospholipids of activated platelets to boost thrombin generation (Mann *et al.,* 2009). Thrombin accelerates this process by its potential to activate factors V and VIII, however continued proteolytic action dampens the process by activating protein C, which in complex with its endothelial receptor thrombomodulin, degrades factor Va and VIIIa (Dahlback *et al.,* 2004). Natural plasma inhibitors also provide negative feedback mechanisms: C1-inhibitor (C1 INH) neutralises

factor XIIa, tissue factor pathway inhibitor (TFPI) blocks factor VIIa/TF, and antithrombin III (ATIII) blocks factors IXa, Xa and thrombin. The balance of coagulation in normal conditions is therefore ensured by the tight control of thrombin generation resulting from a fine equilibrium between procoagulants and endogenous anticoagulants.

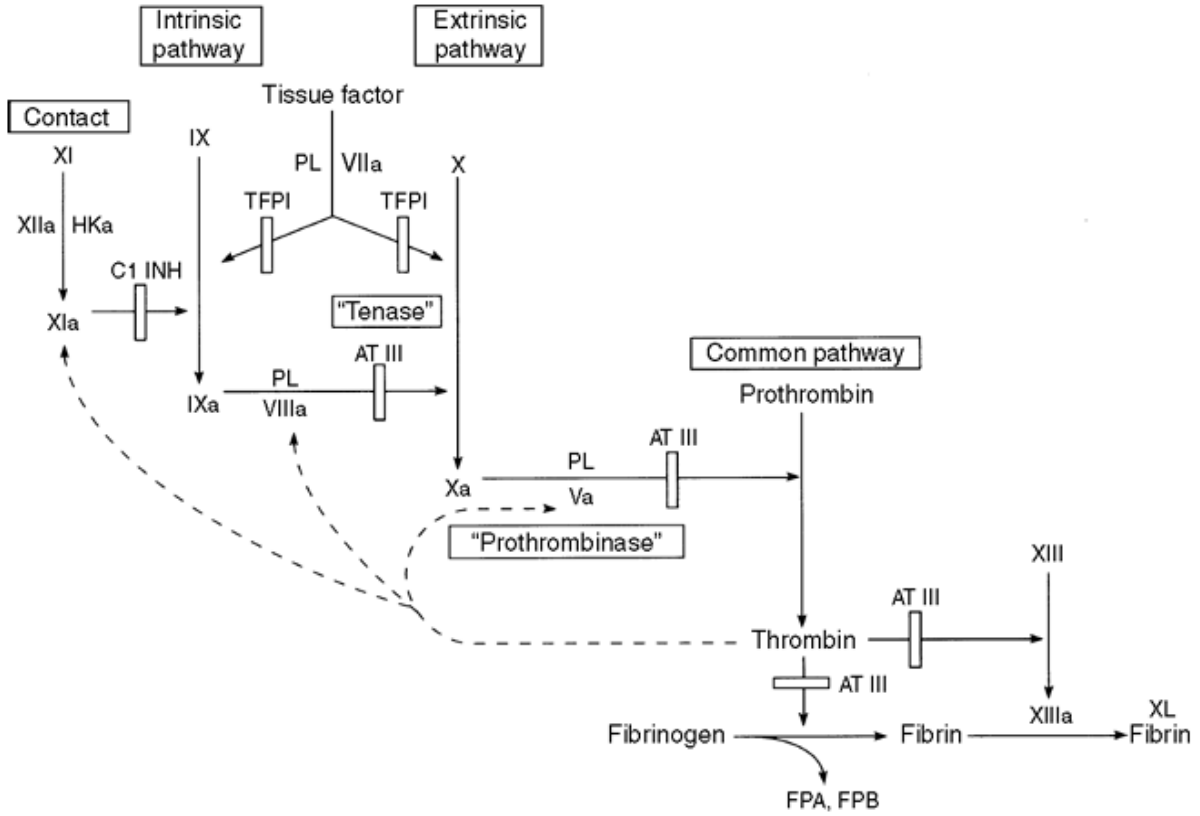


Figure 1-2: The coagulation cascade.

The Coagulation cascade: Mechanism of Thrombin and production: The extrinsic and intrinsic pathway unite to form a final common pathway resulting in the production of thrombin (Factor IIa) which results in fibrin deposition at the site of injury. Arrows, active enzymes; filled rectangles, site of inhibition; dashed line, feedback reactions. From Chapter 1.Haemostasis and Thrombosis, Edition 5, LWW.

1.3.2. Mechanisms of interaction between the coagulation system and chronic liver injury

The interaction between the coagulation cascade and liver injury is multifaceted (Borensztajn *et al*., 2010). On the one hand, coagulopathy is a well documented sequelae of chronic liver failure. On the other hand, evidence is emerging which suggests pro-fibrotic states are prothrombotic and activation of the coagulation cascade and individual coagulation proteins results in the generation of chronic liver injury.

1.3.2.1. Coagulopathy in liver injury

1.3.2.1.1. Platelet dysfunction in chronic liver disease

Cirrhotic patients have elevated levels of vWF, which can contribute to induction of primary haemostasis (Lisman *et al.,* 2006), and compensate for defects in platelet function and numbers that are demonstrated in cirrhosis. Indeed platelets in cirrhotics still have the qualitative capability to support thrombin production, but reduction in platelet numbers may be a key limiting factor in these patients (Tripodi *et al.,* 2006).

1.3.2.1.2. The balance of procoagulant versus anticoagulant factors in liver injury

The effects of liver injury on the coagulation system have been described as a paradox (Borensztajn *et al*., 2010). Advanced fibrosis is associated with impaired synthesis of all clotting factors, except Factor VIII and vWF (Tripodi *et al.,* 2005; Tripodi *et al*., 2006). This defect is demonstrated by prolongation of the PT and the activated partial thromboplastin time (APTT), which are tests that represent the status of procoagulant proteins synthesised by the liver. The use however of these conventional tests of haemostasis on peripheral

blood correlate poorly with bleeding in chronic liver disease (Tripodi *et al.,* 2010), since this reflects the inability of these tests to take into account an imbalance in endogenous anticoagulants and procoagulants. Patients with advanced fibrosis have significantly lower levels of protein C and antithrombin (Tripodi *et al*., 2005). This partial deficiency of anticoagulant proteins counteracts any reduction in thrombin generation in advanced fibrosis, resulting in a normal net effect in plasma coagulation or even an imbalance favoring a procoagulant state (Tripodi *et al*., 2005). Indeed this in part can explain the paradox of why in chronic liver disease an increased relative risk of venous thromboembolic disease is observed in a case control population based study (Søgaard *et al.,* 2009). In this study of 99,444 patients with thromboembolic disease, cirrhosis had a 1.7 fold increase in relative risk of venous thrombosis compared to the general population.

Advanced fibrosis therefore represents a fragile balance of pro and anticoagulant factors. Imbalances caused by portal hypertension, bacterial infections or renal failure can promote an anticoagulant state resulting in bleeding. Conversely vascular dysfunction secondary to endothelial damage or chronic inflammation can promote a prothrombotic state resulting thrombosis. No studies have examined the extent of activation of the coagulation cascade in early fibrotic states in the liver. Since various other organs, including skin and lungs, demonstrate local activation of the coagulation cascade in the setting of ongoing fibrosis, (Borensztajn *et al*,. 2008; Borensztajn *et al*,. 2009) it is conceivable that in early fibrotic states, a balance towards a local hypercoagulable state exists, which in part drives further fibrosis, by the mechanisms that will be discussed below.

1.3.2.2. Coagulation system and the pathogenesis of liver Injury

Two mechanisms have been postulated by which activation of the coagulation system contributes to liver injury and specifically the generation of hepatic fibrosis. The first describes a hypothesis by which activation of the coagulation cascade results in fibrin deposition and microthrombi formation within the liver parenchyma, resulting in hypoxia and fibrosis, and has been termed parenchymal extinction (Wanless *et al*., 1995; Wanless *et al*., 2005). More recently an alternative pathway, promoting the profibrotic effects of coagulation proteins independent of their pro-coagulant action, in part via PAR receptor ligation, has been suggested. Furthermore epidemiological evidence demonstrates a relationship between prothrombotic tendencies and rate of hepatic fibrosis, which indirectly supports these concepts. The evidence for these hypotheses is discussed in further detail below.

1.3.2.2.1. Microemboli and Parenchymal extinction: a role in liver injury

1.3.2.2.1.1. Microthrombi and hepatic damage in animal models

Mice with acute murine hepatitis virus infection demonstrated some of the initial evidence for a role for coagulation in the pathogenesis of liver disease. Microthrombi within the hepatic microvasculature were associated with areas of tissue necrosis (MacPhee *et al*., 1985; MacPhee *et al*., 1988; Levy *et al*., 1983) and disease severity. This effect may have been mediated by Fgl2/Fibroleukin, a prothrombinase whose expression on the surface of hepatic endothelial cells is induced by viral infections including HBV (Marsden *et al.,* 2003).

1.3.2.2.1.2. Parenchymal extinction in human liver disease

Wanless et al (1995) formulated a hypothesis for liver tissue remodelling and fibrosis, in which microthrombi deposition is central. They demonstrated microthrombi within branches of the hepatic vein and portal vein in cirrhotic livers from a range of pathologies including HCV, HBV, primary biliary cirrhosis, alcoholic liver disease, and cardiac cirrhosis. Based on these observations they proposed that hepatic necroinflammation causes local intimal injury and occlusive thrombi generation within branches of the hepatic and portal vein, which leads to sinusoidal congestion and tissue ischaemia. This in turn leads to hepatocyte death, collapsing the region between the central veins and their adjacent portal tracts. The collapsed tissue would be replaced by fibrous tissue, and the process was termed "parenchymal extinction" (see Figure 1-2). They postulated that cirrhosis results from multiple areas of parenchymal extinction becoming confluent (Wanless *et al.,* 1995). These findings were validated by the same group, using animal models of chronic liver disease including a rabbit model of fibrosing steatohepatitis induced by a combination of diethylstilbestrol injections and a high-cholesterol diet (Wanless *et al.,* 1996; Wanless *et al*., 2004). At the time of reporting, the above concept was not entirely unique. In Budd-Chiari syndrome, the underlying occlusion of the hepatic vein by thrombus is well recognised to result in hepatic congestion and subsequent hepatic fibrosis (Tanaka *et al*., 1998).

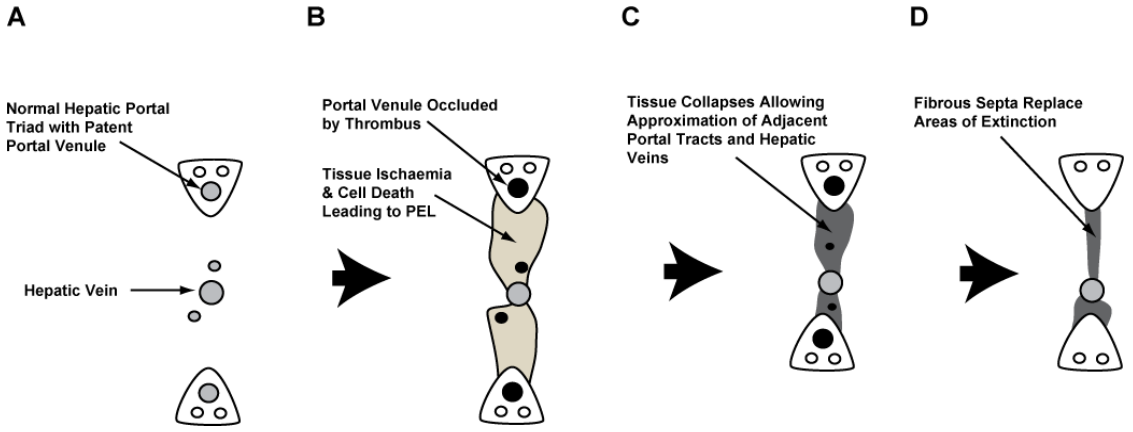


Figure 1-3: Natural history of parenchymal extinction and fibrous septa formation.

A Normal liver with patent portal and hepatic veins. **B** Inflammatory injury causes venous thrombosis (black). The consequent hepatocyte ischaemia and death leads to parenchymal extinction. **C** Extinction allows tissue to collapse so that adjacent portal tracts and hepatic veins are approximated. **D** Regions of extinction are replaced by fibrous septa. The obliterated venules are no longer evident. (Reproduced from Anstee *et al.,* 2009).

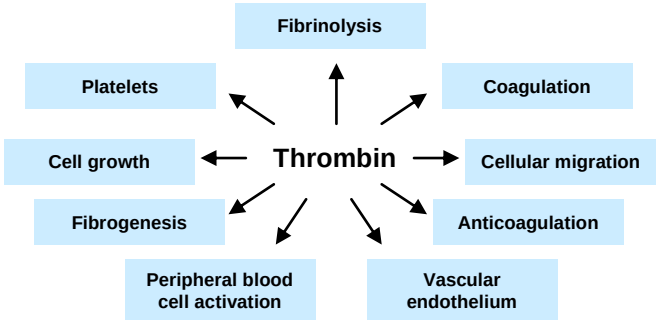
1.3.2.2.2. Profibrotic effects of coagulation proteins independent of their procoagulant activity

1.3.2.2.2.1. Thrombin: an overview

Activation of the coagulation system generates thrombin (FIIa) from its precursor protein, prothrombin (FII), which is synthesised primarily in the liver. Thrombin is best known for the proteolytic cleavage of fibrinogen to form fibrin (Coughlin 2000) but it has a multitude of other cellular actions (See Figure 1-2). It promotes platelet aggregation; is a potent activator of endothelial cells, smooth muscle cells, and macrophages; acts as a chemoattractant for

inflammatory cells, and acts as both a mitogen and chemoattractant for fibroblast and vascular smooth muscle cells (Henderson *et al*., 2007). Recent studies have demonstrated the role of thrombin in hepatic stellate cell activation.

Figure 1-4: Functions of thrombin.



1.3.2.2.2.2 Thrombin and PAR receptors: A Role in Direct Stellate cell activation

The cellular actions of thrombin are in part mediated by protease activated receptor (PAR) signalling (Rullier *et al*., 2007). PAR receptors are a family of widely expressed G-protein-coupled receptors, activated by cleavage of the amino-terminal sequence of the extracellular exodomain, producing a new N-terminus tethered ligand that transduces transmembrane signalling (Coughlin., 2000). Four PARs have been identified. PAR1, PAR3, and PAR 4 are thrombin receptors, with PAR 1 and 3 both preferentially activated by thrombin. PAR4 has reduced affinity to thrombin and is activated also by trypsin (Di, 2003). PAR2, which is selectively activated by trypsin and mast cells tryptase, and is resistant to thrombin activation. PAR1 is expressed on human platelets, macrophages and endothelial cells. Hepatic stellate cells have been shown to express PAR1. A substantial body of evidence from in vivo and in vitro studies is now accumulating to suggest PAR-1 activation leads to

stellate cell activation and hepatic fibrogenesis (Fiorucci *et al*., 2004; Marra *et al*., 1998; Gaca *et al*., 2002).

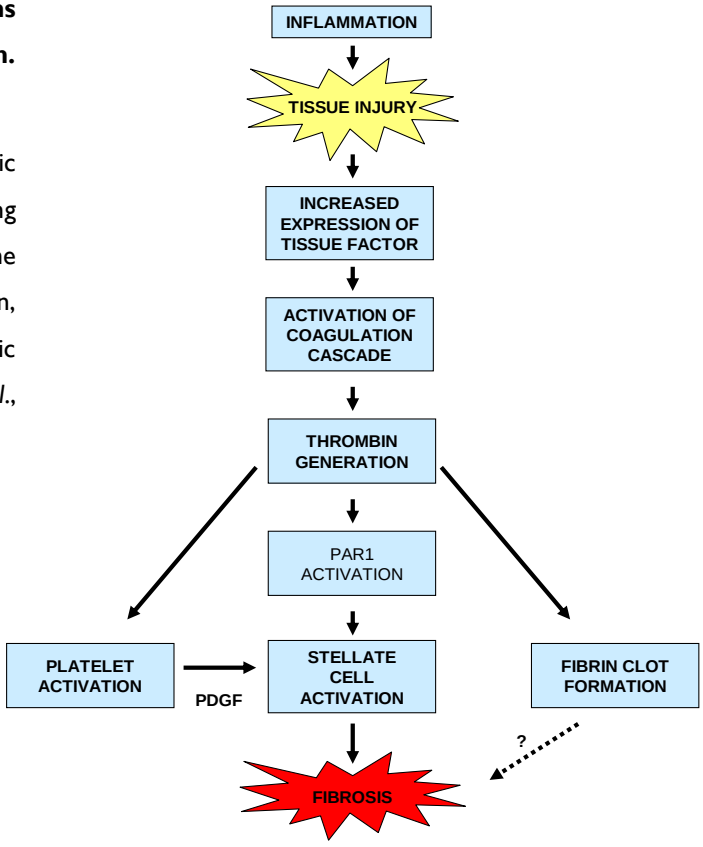
Selective PAR 1 agonists in vitro have demonstrated increased stellate cell proliferation and contractility. Conversely PAR 1 antagonists inhibited these actions in a rodent bile duct ligation model of fibrosis, with reduced collagen type I collagen expression (Fiorucci *et al*., 2004). Equally invalidation of PAR 1 in transgenic knockout mice induces a significant protection against liver fibrosis (Rullier *et al.,* 2007). PAR-1 also facilitates the posttranslational activation of TGF-beta, a key mediator of fibrogenesis (Jenkins *et al*., 2006). PAR 1 expression is up-regulated during both acute and chronic liver disease and with both severity and duration of liver disease. (Marra *et al.,*1998, Fouricci *et al.,* 2004, Rullier *et al.,* 2005). Finally a recent study has demonstrated that carriage of the D-1426C/T transition mutation in the 5' regulatory region of the PAR-1 gene in HCV patients is associated with increased rates of hepatic fibrosis (Martinelli *et al.,* 2008). These studies heavily promote the role of PAR1 and thrombin via PAR activation in hepatic fibrosis (see Figure 1-4).

1.3.2.2.2.3. Thrombin: Other mechanisms of Hepatic Stellate cell activation

In addition to thrombin activating stellate cells via PAR 1, indirect activation via other PAR receptors has been demonstrated. PAR mediated platelet degranulation results in the release of PDGF and PAR mediates the activation of TGF-beta. Both PDGF and TGF-beta stimulate HSC activation (Friedman, 2003).

Figure 1-5: PAR-1–mediated actions of thrombin in stellate cell activation.

Inflammation within the hepatic parenchyma leads to tissue injury causing tissue factor induced initiation of the coagulation cascade, thrombin production, PAR-1 activation and activation of hepatic stellate cells. (Adapted from Anstee *et al*., 2009).



1.3.2.2.3. The role of other coagulation factors in chronic liver injury

1.3.2.2.3.1. Tissue factor

Tissue factor (TF) is a 47 kDA transmembrane glycoprotein that is expressed physiologically in vascular adventitial cells, a number of epithelial cell types, including mucous membranes, macrophages and blood monocytes (Nakasaki *et al.,* 2002). TF is classed as a type I integral membrane protein with the amino-terminus extracellular and a carboxy terminus is located within the cell. The extracellular domain is vital for procoagulant activity and the cytoplasmic domain is involved in cell signalisation. TF provides a haemostatic barrier to limit bleeding

after injury but does not come in contact with the circulation in normal circumstances. During injury it is locally expressed and binds to factor VII, forming the TF:VIIa complex thereby activating the extrinsic coagulation system.. In inflammatory lesions, TF is expressed by endothelial cells, monocytes and macrophages (Shoji *et al,* 1998). Rullier et al has reported a predominantly hepatocytic expression of tissue factor in normal and cirrhotic livers, which would be unexpected based on the above principles (Rullier *et al.,* 2005). However samples classed as normal liver tissue in this study were actually taken from the periphery of livers with metastasis or benign tumours. This may have confounded the distribution of TF demonstrated in liver tissue classed as normal in this study since malignancy has been shown to upregulate TF expression (Rullier *et al.,* 2005). It is vital that the role of TF in chronic liver injury is studied further. In particular its expression in fibrosis and whether it initiates activation of coagulation in this setting would have important implications to planning therapeutic interventions.

1.3.2.2.3.2. Fibrin and Fibrinogen

Fibrinogen is a plasma glycoprotein consisting of three disulfide bond-linked pairs of alpha, beta, and gamma chains coded by separate genes. It is involved in the final step of the coagulation cascade. Thrombin-induced cleavage of alpha and beta chains results in a conversion of the soluble protein into fibrin monomers. These fibrin monomers polymerise to form a fibrin clot. Fibrin clot dissolution or fibrinolysis is primarily maintained by plasmin and inhibited by plasminogen activator inhibitor -1 (PAI-1). Fibrin production in liver injury is in keeping with the theories of microemboli deposition and parenchymal extinction. Neubauer et al investigated fibrin deposition in animal models with short and long term liver damage. In short-term liver damage, large amounts of fibrinogen/fibrin deposits were

detected in the areas of necrosis, with positivity along the hepatic sinusoids. In long-term liver damage, fibrinogen/fibrin was localised within the fibrous septa. (Neubauer *et al.,* 1996) No studies have as yet attempted to demonstrate these findings in liver fibrosis or acute liver injury in humans. This is in part is due to the advent of studies demonstrating the role of thrombin in PAR-1 activation, which has questioned the significance of microemboli and fibrin deposition in liver injury (Rullier *et al.,* 2007, Fiorucci *et al.,* 2004). Further studies are still required to clarify this as it is likely neither pathway is mutually exclusive (Anstee *et al.,* 2009).

1.3.2.2.3.3. Factor Xa

Factor Xa (FXa) is a coagulation factor generated at the point of convergence of the intrinsic and extrinsic coagulation pathways. It is an essential component of the prothrombinase complex, which is responsible for the conversion of prothrombin to thrombin by proteolytic activity. Evidence is now emerging that it may have profibrotic properties across a range of fibrotic diseases.

Chambers et al have demonstrated it to be a potent promoter of connective tissue growth factor, a cytokine involved in fibrogenesis in foetal lung fibroblasts. It also acts as a potent mitogen for lung fibroblasts (Chambers *et al.,* 2000) and is produced in the lungs of both mice and humans with pulmonary fibrosis, a paradigm for liver fibrosis (Scrotton *et al.,* 2010). Recent evidence suggests that FXa activates PAR 1, in a similar fashion to thrombin, as well as PAR 2 (Ruf *et al.,* 2003; Borensztajn *et al.,* 2008). PAR1 activation has been demonstrated to be the dominant effector in FXa related lung fibroblast activation (Scrotton *et al.,* 2009). PAR 2 has been studied in less detail, and fewer studies have explored the role of PAR 2 in liver fibrosis, since its role is less well defined compared to PAR 1,3 and 4 (Borensztajn *et al.,*

2010). PAR2 expression has been demonstrated on both hepatic stellate cells and hepatocytes (Borensztajn *et al.,* 2010; Fiorucci *et al.,* 2004; Gaca *et al.,* 2002), and shown to be up-regulated in fibrotic livers (Borensztajn *et al.,* 2010). PAR2 agonists demonstrate hepatic stellate cell proliferation and collagen synthesis in rats (Fiorucci *et al.,* 2004; Gaca *et al.,* 2002), and PAR2 activation results in the release of the profibrotic cytokines, PAI-1 and TGF-beta from fibroblasts (Borensztajn *et al.,* 2008). Recently PAR2 deficiency, using PAR2 knockout mice, has been shown to reduce CCL4 induced liver fibrosis (Knight *et al.,* 2011). Additionally FXa activation of PAR2, has been demonstrated to result in the release of pro-inflammatory cytokines in a number of cell lines, including endothelial, mesangial, epithelial and fibroblastic cells (Pan *et al.,* 2008; Tanaka *et al.,* 2008; Wilson *et al.,* 2009).

These studies suggest that FXa in a similar fashion to thrombin, and independent of its pro-coagulant function, may promote fibrosis via PAR activation. Although many of these studies have been in the setting of pulmonary fibrosis, this is a well established paradigm for liver fibrosis. The role of FXa and related therapeutics therefore need to be characterised further in the setting of hepatic fibrogenesis.

1.3.2.2.3.4. Other coagulation related proteins

1.3.2.2.3.4.1. Thrombospondin-1

Thrombospondin-1(TSP-1) is a member of the thrombospondins, which are extracellular glycoproteins involved in cell to cell and cell to matrix signalling (Armstrong *et al.,* 2003; Bornstein *et al.,* 2001; Lawler *et al.,* 2000). TSP-1 is a major constituent of platelet alpha-granules and is released by thrombin activated platelets and inflammatory cells (Bornstien *et al.,* 2001). TSP-1is involved in platelet aggregation, wound healing and inhibition of nitric oxide, allowing platelet aggregation to occur faster (Chung *et al.,* 1997; Isenberg *et al.,* 2008).

TSP-1 release can contribute to liver injury. In rodent models TSP-1 has been demonstrated to be upregulated in fibrogenesis, via activation of TGF-beta. No studies have defined the expression of TSP-1 in acute liver injury.

1.3.2.2.3.4.2. Thrombomodulin

Thrombomodulin (TM) is an endothelial cell membrane glycoprotein that inactivates thrombin and activates protein C (Esmon *et al.,* 2004; Dittman *et al.,* 1990). Protein C in complex with TM degrades factor Va and VIIIa, resulting in decreased generation of thrombin and thus an anticoagulant state. TM is expressed in a variety of cells, including endothelial cells (Raife *et al.,* 1996), and has been demonstrated in large blood vessels in normal human liver tissue (Laszik *et al.,* 1997).

To date, only one study demonstrating TM expression in a model of human chronic liver injury has been reported (Zeniya *et al.,* 1995). Therefore, further studies are required to determine the role of TM expression in chronic liver disease.

1.3.3. Epidemiological evidence: the coagulation system in liver fibrosis

1.3.3.1. HCV and prothrombotic factors

A number of coagulation factor polymorphisms have been shown to have functional effects on the clotting cascade leading to an increased risk of thrombosis or thrombophilia. Several studies have confirmed that presence of thrombophilia increases the risk of severe fibrosis in chronic viral hepatitis. Papatheodoridis et al demonstrated that hepatitis B and C patients with advanced fibrosis (Ishak stage 4–6) were significantly more likely to have thrombophilia related to deficiencies of protein C, plasminogen and anti-thrombin III compared to patients with milder fibrosis (Papatheodoridis *et al.,* 2003). Factor V Leiden (FVL) a well characterised mutation in the coagulation system, which causes activated protein C resistance and amplification of the coagulation cascade, conferred a near fourfold increase in the risk of rapid fibrosis in a caucasian HCV population (Wright *et al.,* 2003 A). These findings have also been validated in other human studies (Poujol-Robert *et al.,* 2004) and confirmed by animal studies using C57BL/6 mice carrying the FVL mutation treating with carbon tetrachloride to induce hepatic fibrosis, demonstrating significantly more fibrosis than wild type mice (Anstee *et al.,* 2008). Furthermore, protein C deficiency, increased expression of factor VIII, and hyperhomocysteinemia have also been related to accelerated fibrosis in HCV patients (Poujol-Robert *et al.,* 2004). A single study has questioned this effect but a predominantly female study group with a low overall fibrosis stage was examined, which may have confounded the results (Goulding *et al*., 2007).

1.3.3.2. Haemophilia and liver fibrosis in HCV infection

Haemophilia in contrast to thrombophilia results in an anti-thrombotic state. Two small case series have studied the natural history of HCV in patients with haemophilia (Yee *et al.,* 2000; Assy *et al*., 2007). Yee et al, demonstrated a slow rate of disease progression in a cohort of 185 patients singly infected with HCV, 25 years post exposure (Yee *et al.,* 2000). Assy et al, demonstrated chronic HCV infection in patients with haemophilia caused less severe liver disease than in patients without haemophilia, and the extent of histological abnormalities correlated with the severity of the underlying coagulopathy (Assy *et al*., 2007). Both these studies suggest hypocoagulation states may slow rates of hepatic fibrosis. Further larger prospective studies of liver fibrosis in haemophiliacs to confirm these findings are required, though would be difficult to carry out due to inherent issues with performing liver biopsies to accurately stage fibrosis.

1.4. THERAPIES FOR LIVER FIBROSIS

1.4.1. Current antifibrotic therapies

To date there are no specific antifibrotic therapies licensed in the UK or elsewhere in the world. Several potential agents have shown antifibrotic properties in experimental animal models, but have either failed to translate to human studies or have been ineffective in human trials (Rockey *et al.*, 2008). Potential antifibrotic candidates including angiotensin II antagonists, the cannabinoid receptor I antagonist Rimonabant, and Silymarin (Milk Thistle) are currently being evaluated in ongoing trials (http://clinicaltrials.gov. Identifiers NCT 00298714, NCT00265642, NCT00680407). To date however the only clearly effective antifibrotic therapy is still clearance of the underlying disease process. A new antifibrotic agent evaluated in an adequately powered well designed clinical trial is therefore urgently required.

1.4.2. The Rationale for Anticoagulation as an antifibrotic in liver disease

As described, a convincing body of evidence exists outlining a potential role of the coagulation system in the pathogenesis of liver fibrosis. Furthermore epidemiological studies have shown that prothrombotic states promote liver fibrosis. Hence if prothrombotic states accelerate liver fibrosis, the converse should be true, in that anticoagulation should slow liver fibrosis. Several animal studies support this hypothesis.

1.4.2.1. Animal models demonstrating the benefit of anticoagulation on fibrosis.

Studies using animal models of fibrosis have been used to evaluate the impact of anticoagulation on liver fibrosis.

1.4.2.1.1. Direct thrombin antagonists

Duplantier *et al* evaluated, in a rat model of CCl4- induced chronic liver injury, the effect of thrombin inhibition using a novel synthetic thrombin antagonist SSR182289. The study demonstrated a reduction in liver fibrosis and α smooth muscle actin expression. (Duplantier *et al*., 2004). To date no human trials have been reported with this agent nor other commercially available direct thrombin antagonists.

1.4.2.1.2. Direct Factor Xa antagonists

No studies to date have evaluated the antifibrotic potential of direct FXa inhibitors in animal models of liver fibrosis. Recent evidence from studies of pulmonary fibrosis, a paradigm for liver fibrosis suggest that use of direct FXa inhibitors may offer protection from fibrosis in animal models. The direct FXa inhibitor, ZK 807834 has been shown to significantly reduce bleomycin induced lung fibrosis in mice (Scrotton *et al*., 2009). The impact of direct FXa inhibitors on hepatic fibrosis needs to be studied to assess their anti-fibrotic potential.

1.4.2.1.3. Heparin

Three studies have demonstrated an antifibrotic effect of heparin anticoagulation. Assey et al evaluated the effects of aspirin and enoxaparin on fibrosis progression in TAA treated rats. Both aspirin and enoxaparin prevented the progression of fibrosis. Aspirin additionally

stimulated liver regeneration (Assey *et al*., 2007). Adbel-Salam et al using a rat bile duct ligation model demonstrated the beneficial effects of unfractionated and low molecular weight heparins (Abdel-Salam *et al*., 2005). A reduction in the elevated bilirubin, ALT and GGT levels and prevention of hepatic necrosis and fibrosis were demonstrated. Abe et al, demonstrated heparin prevented hepatic fibrogenesis induced by chronic CCl4 administration in mice, through down regulation of TGF-beta and inhibition of HSC proliferation (Abe *et a*l., 2007).

1.4.2.1.4. Warfarin

The use of warfarin anticoagulation to reduce liver fibrosis induced by carbon tetrachloride has been tested using a transgenic mouse model of FVL (activated protein C resistance). The progression of fibrosis in FVL mice was compared to the C57BL/6 mice (controls) and C57BL/6 mice which had been anticoagulated with warfarin. The results confirmed that the thrombophilic mouse developed fibrosis at a faster rate than the control mice but warfarin anti-coagulation significantly reduced the rate of fibrosis seen in both strains. (Anstee *et a*l., 2008).

1.4.2.1.5. Other agents with anticoagulant properties

Wanless et al in a rabbit model of fibrosing steatohepatitis induced by a combination of diethylstilbestrol injections and a high-cholesterol diet reported that concomitant administration of dipyridamole, an antiplatelet and vasodilator, reduced hepatic fibrosis (Wanless *et a*l., 1996).

1.4.2.2. Human studies of anticoagulation for fibrosis

To date only one small study has suggested an efficacy of low-molecular-weight heparin as an antifibrotic in humans. Patients, with HBV infection, treated with 3 weeks of heparin, showed improved serum levels of ALT and Bilirubin, with a reduction in serum Hyaluronic acid and type IV collagen concentrations (Shi *et al*., 2003). Long term use of heparin as an antifibrotic in humans may not be practical due to side effects including osteopaenia, thrombocytopaenia and idiopathic hepatitis. Kubo et al in a clinical trial of warfarin in idiopathic pulmonary fibrosis reduced mortality at three years by 50% (Kubo *et al*., 2005). In this trial patients in the treatment group had their INR raised to between two and three with no associated morbidity which is in line with other reports of complication rates at this level of anticoagulation. Therapeutic use of anticoagulation to prevent fibrosis in humans is not therefore without precedent but needs to be evaluated in hepatic fibrosis using well designed clinical trials with relevant endpoints. Furthermore a better understanding of the role of individual coagulant proteins in fibrogenesis would inform which anticoagulant would be best suited to being trialled in this setting.

1.4.2.2.1. Safety of anticoagulation for in chronic liver disease.

Anticoagulation is widely used in medicine as a treatment for deep vein thrombosis and pulmonary embolus and prophylactically to reduce the risk of cerebrovascular accident in patients with atrial fibrillation. Despite the proven benefits of anticoagulation a number of clinicians have reservations about the use of these drugs particularly if they have witnessed a significant adverse event (Choudary *et al.,* 2006). This probably accounts for the under use of anticoagulation where clinically indicated (Rashid *et al.,* 2006). It is therefore natural for hepatologists to have safety concerns in view of the risks of bleeding from oesophageal

varices in advanced liver disease. Despite this several recent studies have demonstrated not only a beneficial effect for anticoagulation in preventing or treating thrombosis in patients with advanced fibrosis or cirrhosis, but also an acceptable safety profile. Anticoagulation in this setting has mainly been studied in the context of extrahepatic portal vein thrombosis. Benefits of anticoagulation relate to re-canalization, which is more likely to occur if thrombosis is partial or anticoagulation therapy is prolonged. Despite these prolonged periods of anticoagulation, no increased risk of bleeding has been demonstrated in several series, and anticoagulation is considered relatively safe even in the presence of advanced fibrosis (Romero-Gomez *et al.,* 2002; Amitrano *et al.,* 2010; Francoz *et al.,* 2005). Futhermore a single small study has demonstrated a role for heparin anticoagulation as a potential prophylactic agent against portal vein thrombosis in patients with Child B and C cirrhosis (Zechini *et al.,* 2010). In addition to a significant reduction in both the incidence of portal vein thrombosis and episodes of decompensation, there was no increased risk of bleeding events in these patients when heparin was given for a period of 24 months when compared to a matched control group patients treated with placebo alone (Zechini *et al.,* 2010). Not only is anticoagulation potentially safe in pretransplant patients, but Burroughs et al (*unpublished data*) have audited a group of 80 patients who received liver transplants at the Royal Free Hospital and who were anticoagulated for standard clinical indications such as Budd Chiari syndrome. In this group of patient no anticoagulation associated adverse events occurred.

Therefore prolonged anticoagulation is not without precedent in the setting of both patients with advanced fibrosis or patients who have undergone liver transplantation. No added risk of bleeding has been noted in these studies and anticoagulation can potentially be safely undertaken in patients with hepatic fibrosis whether it be in the setting of a clinical trial or treatment of thrombo-embolic disease.

1.5. METHODS OF ASSESSING LIVER FIBROSIS IN CLINICAL TRIALS

1.5.1. Liver Biopsy

Liver biopsy is currently considered the 'gold standard' for assessing hepatic fibrosis. Multiple scoring systems for the quantification of inflammation and fibrosis, when interpreting liver histology, have been developed. Knodell *et al* developed the first scoring system based on the identification and scoring of periportal necrosis, intralobular necrosis, portal inflammation, and fibrosis (Knodell *et al*., 1981). This system was modified by Ishak *et al*, to produce a scoring system that is still routinely used in clinical practice (Ishak *et al*., 1995). The Metavir scoring system was developed to specifically assess fibrosis in the setting of Chronic HCV infection. Fibrosis stage is scored on a scale of 0 to 4, with 4 representing cirrhosis (Poynard *et al.,* 1996). Scoring fibrosis via liver biopsies is not without disadvantages. It is an invasive and painful procedure with rare but potential life threatening complications. These factors limit its acceptance in asymptomatic patients and raise ethical issues when employing it to stage fibrosis in the setting of clinical trials (Cadranel *et al.,* 2000; Bravo *et al*., 2001; Foucher *et a*l., 2006). In addition, the accuracy of liver histology in assessing fibrosis has been questioned due to sampling error and inter-observer variability, which can often lead to understaging of fibrosis (Regev *et al*., 2002). There is a need therefore to develop and validate non-invasive tests that can firstly reflect the full spectrum of hepatic fibrosis and secondly act as effective longitudinal surrogates for the progression or regression of fibrosis when conducting trials of antifibrotics, since the use of liver biopsies cannot always be ethically justified in studies where there is an absence of a pre-existing clinical need for histology (Friedman *et al*., 2006).

1.5.2. Non Invasive markers of fibrosis

Non invasive markers of liver fibrosis can be classed either by the modality of the test (serum marker versus imaging) or the constituents of the test (direct versus indirect marker of fibrosis) (Guha *et al.,* 2008). A number of non-invasive markers have been developed but this section will focus on the techniques we have employed in this body of work.

1.5.2.1. The Enhanced Liver Fibrosis test

The Enhanced Liver Fibrosis (ELF) test is a serum panel of biomarkers which combines three direct markers of liver fibrosis: hyaluronic acid (HA); TIMP-1 and the aminoterminal peptide of procollagenase 3 (PIIINP). Each individual marker is measured and entered into an algorithm (the ELF algorithm) to generate a score (the ELF score). The test score was first validated in a mixed cohort of patients with chronic liver disease, and has been shown to correlate with the level of liver fibrosis assessed by liver biopsy (Rosenberg et al., 2004).

1.5.2.2. Transient Elastography

Transient elastography (FibroScan; Echosens, Paris, France) is a non-invasive technique used to measure liver stiffness. (Foucher *et al*., 2006) A shear wave at a low frequency of 50 Hz is created by a vibrating probe, which propagates through the liver. The velocity of the wave is measured by a pulse echo-ultrasound, with results represented in kPA. This measurement correlates to the stiffness or elasticity of the underlying liver. The wave propagates faster through the liver if the tissue is harder due to increased fibrosis and liver stiffness has been shown to be related to severity of fibrosis and in particular fibrosis stage in Hepatitis C patients (Sandrin *et al.,* 2003; Castera *et al.,* 2005; Ziol *et al.,* 2005). Other variables however in addition to fibrosis may contribute to liver stiffness, including inflammation, hepatic

steatosis and portal hypertension. Furthermore, measurement is heavily limited or impossible in obesity, ascites or in patients with narrow intercostal spaces (Guha *et al*., 2008). Advantages include a sampling area that is considerably greater than that of a liver biopsy.

1.5.2.3. Contrast Ultrasound and Hepatic Transit Time

Microbubble ultrasound contrast agents have been assessed in various liver diseases. After intravenous injection of the microbubble contrast, the hepatic transit time (HTT) is calculated. The HTT represents the difference between the arrival time of the contrast to the hepatic artery and hepatic vein. The HTT decreases with progressive fibrosis due to increased intrahepatic shunting and capillarization of the sinusoids (Sugimoto *et al*., 2002). Studies have shown that HTTs adequately differentiate severe and mild fibrosis in hepatitis C infected patients (Lim *et al*., 2005). This test however is not widely available.

1.5.2.4 APRI score

The AST to platelet ratio index score (APRI) uses commonly available serum tests to predict the indirect risk of underlying fibrosis, and has been validated in a variety of liver disease including HCV (Wai *et al.,* 2003). The AST to platelet ratio index (APRI) is calculated as AST level (/ ULN) X10 / Platelet count (10/l). A recent meta-analysis has demonstrated that an APRI score of 0.7 has a sensitivity and specificity of 77 and 72 percent respectively for significant fibrosis (Lin *et al.,* 2011).

1.6. GENERAL AIMS AND OBJECTIVES FOR THE OVERALL STUDY

The overall aim of the study is to further our understanding of the role of the coagulation system in the pathogenesis of liver injury and fibrosis. The work is intended to have three main objectives:

1. To demonstrate activation of the coagulation system, and investigate the role of individual coagulation proteins in the pathogenesis of acute and chronic liver injury.

2. To evaluate novel anticoagulants as potential antifibrotic therapies in vitro and murine models of liver injury.

3. To evaluate the antifibrotic potential of warfarin anticoagulation in patients with liver fibrosis.

2. THE COAGULATION SYSTEM IN LIVER INJURY: AN IMMUNOHISTOCHEMISTRY APPROACH

2.1. INTRODUCTION

Evidence is emerging to suggest that coagulation proteins have a multifaceted role in liver injury. The effects on coagulation in both acute and chronic liver injury are well documented. Recent evidence also suggests that liver injury, particularly fibrosis is in part activated by the coagulation system. The role of coagulation proteins in the pathogenesis of acute liver injury is less well defined, and warrants further study.

2.1.2. Acute Liver Injury

Paracetamol toxicity is a leading cause of hepatic failure. Despite extensive research, the mechanisms involved in the process are not fully understood. In acute liver injury, abnormalities in haemostasis are well documented. Patients with paracetamol induced liver injury have lower concentrations of coagulation factors peripherally (Kerr, 2003). Historically this has been attributed to the decreased production of these proteins in an acutely injured or failing liver. An alternative explanation would be increased consumption of factors in the damaged liver, due activation of the coagulation system. A single study has demonstrated hepatic fibrin deposition in a mouse model of paracetamol injury (Ganey *et al*., 2007). As far as we are aware, no study has evaluated whether activated coagulation proteins are expressed in human liver tissue following paracetamol injury. The consumption and activation of coagulation factors in the acutely failing liver may not only contribute to coagulopathy but hypothetically could have an important role in the generation of injury via thrombin mediated platelet aggregation, microcirculatory dysfunction and ischaemia. These

concepts can in part be evaluated further using murine models of acute liver injury induced by paracetamol, which is an analogous to the paracetamol induced human acute liver injury, (Birge *et al.,* 1990).

2.1.3 Chronic Liver Injury

The theories of parenchymal extinction and microemboli deposition as mechanisms of chronic liver injury inherently rely on intrahepatic activation of the coagulation system (Wanless *et al*., 1995). More recently thrombin, has been demonstrated to cause PAR-1 activation, leading to hepatic fibrosis (Marra *et al.,* 1998; Fiorucci *et al.,* 2004), and FXa has been demonstrated to mediate pulmonary fibrosis (Scrotton *et al.,* 2009). This pathway now seems to have superseded the earlier theories of microthrombi deposition. With the exception of thrombin, the function of individual coagulation proteins in liver injury is poorly understood. Few studies have looked directly at the expression of individual coagulation proteins in liver tissue. Fibrin has been shown to be deposited in areas of necrosis and within fibrosis septae in carbon tetrachloride treated mice (Neubauer *et al*., 1996). Tissue factor expression has been demonstrated in cirrhotic and normal human livers (Rullier *et al*., 2005). No study has examined the co-expression of fibrin, tissue factor and associated proteins in liver fibrosis, which is of importance in order to re-establish whether these factors are upregulated; the relationship between their expression and severity of injury; and whether microthrombi and thrombin mediated stellate cell activation are mutually exclusive mechanisms in the pathogenesis of hepatic fibrosis.

2.2. AIMS

1. To determine if coagulation and associated proteins, including fibrin and tissue factor, are expressed or up-regulated in the liver during acute and chronic liver injury.

2. To quantify and qualify expression of these coagulation factors in relation to the grade of severity of liver injury.

2.3. METHODOLOGY

2.3.1. Rationale

Immunohistochemistry was used to stain liver tissue sections for coagulation proteins. An indirect detection technique adapted from a previously described strepavidin-biotin labelled method was used (Golombiewski *et al*., 1997). The method allowed for amplification and increased sensitivity. It allowed a low level of binding to be detected by labelling the primary antibody with a biotinylated secondary antibody, which was then revealed by the enzyme labelled streptavidin conjugate and chromogen. Using the streptavidin-biotin staining method may however result in high levels of non-specific background staining, due to endogenous biotin in tissues, such as the liver, kidney or spleen. This was prevented by treating the tissue with avidin/biotin blocking reagents when required prior to the incubation of biotinylated antibody.

2.3.2. Materials

2.3.2.1. Tissue

Both acute and chronic liver injury tissue was studied with appropriate control tissue. For each injury, both murine and human liver tissue was studied.

2.3.2.1.1. Acute liver injury tissue

2.3.2.1.1.1. Murine acute liver injury tissue

Formalin fixed paraffin embedded blocks of liver tissue from mice, previously held under a Home Office project license (license number 70/5562), were kindly supplied by Professor

Robert Goldin (Imperial College London). Treatment group mice (n=12) had received an intraperitoneal dose of 300mg/kg of paracetamol to induce an acute liver injury analogous to that since in humans. Control mice (n=4) had received only an intraperitoneal saline injection. Animals were then culled by 24 hours post administration with a non-recoverable injection of 0.2ml sodium pentabarbitone (60ng/ml). A subset of treatment group mice was culled at 2 and 4 hours. Whole livers were extracted at the time of culling and fixed in 10% formalin immediately. The formal fixed tissue blocks were embedded in paraffin wax and sectioned at 5μm. Each section was graded according to the Imperial College Scoring system (ICSS) (see Table 2-1). This is a validated, yet to be published, scoring system, which grades mouse liver injuries secondary to paracetamol on the basis of the extent and distribution of necrosis. Briefly this system had been developed (by Robert Goldin and Helen Matthews) using 20 murine livers with paracetamol-induced damage, and validated in a further 27 murine livers, with inter-observer kappa scores between 0.85 and 0.96 (as a percentage of the maximum possible) and intra-observer scores were between 0.95 and 0.98. Sections were stained with Haemtoxylin & Eosin (H&E) and stained for fibrin, tissue factor, thrombospondin-1 (TSP-1), a marker of thrombin mediated platelet aggregation and hypoxia induced factor-1 alpha (HIF-1a), a marker of ischaemia.

Table 2-1: Imperial College Scoring System (ICSS):

A grading system for paracetamol induced liver injury in murine models	
Grade 0	no injury
Grade 1	perivenular necrosis no bridging
Grade 2	some bridging necrosis
Grade 3	extensive bridging necrosis
Grade 4	panlobular necrosis

2.3.2.1.1.2. Human acute liver injury tissue

It is not often feasible to biopsy patients when they have developed acute liver failure due to the high risk of post biopsy bleeding. A more appropriate approach was to obtain tissue sections from livers removed at time of transplantation indicated for acute liver failure from a variety of causes, including paracetamol toxicity, acute hepatitis B, autoimmune hepatitis and seronegative acute liver failure. The use of this tissue, held under a human tissue bank license, was kindly supplied by Dr Alberto Quaglia (Institute of Liver Studies, Kings College Hospital, London) and approved by the Kings College and Medical School Ethics committee (Reference number: 01/04/005). Archived transplant recipient explanted livers were extracted at the time of transplantation and fixed in 10% formalin and embedded in paraffin wax and sectioned at 5µm. Sections were stained with Haemtoxylin & Eosin (H&E) and stained for fibrin, TF, TSP-1 and HIF-1a.

2.3.2.1.2. Chronic liver injury tissue

2.3.2.1.2.1. Murine chronic liver injury tissue

Formalin fixed paraffin embedded blocks of liver tissue form mice, previously held under a Home Office project license (license number 70/5562), were kindly supplied by Dr Quentin Anstee (Imperial College London). C57BL/6J mice, aged 8 weeks old, had been treated with carbon tetrachloride (CCl_4) diluted in a corn oil vehicle and administered by intraperitoneal injection on alternate week days for a period of 4 weeks. The dose of CCl_4 administered had been increased weekly in a stepwise fashion with a maximum dose of 1 ml CCl_4 per kg body weight being reached after 3 weeks (Anstee *et al*., 2008). At completion, animals had been culled by intraperitoneal injection of 0.1ml Pentoject (Pentobarbitone Sodium Ph.Eur. 200mg/ml, Animal Care Ltd., UK) followed by cervical dislocation. Liver tissue had been

collected and preserved in 10% formalin and then embedded in paraffin wax. Sections were cut at 5µm. Sections were stained with H&E, and stained for fibrin, TF and TM.

2.3.2.1.2.2. Human chronic liver injury tissue

Archived liver biopsy specimens performed for the clinical staging of chronic HCV infection were used. Tissue sections were supplied by Professor Robert Goldin (Imperial College London) and use of tissue was approved by the Brent Research Ethics committee (Reference number: 08/H0717/34). Archived biopsies were identified via the departmental Hepatitis C database. Inclusion criteria were biopsies archived within the last 10 years with evidence of active HCV viral replication (positive HCV RNA level) at time of biopsy. Biopsies were excluded if a patient was taking anticoagulation at time of biopsy or if there was insufficient archived biopsy tissue for adequate histological re-staining and scoring. Biopsies that fulfilled the inclusion and exclusion criteria were identified (n=20), and 5µm sections were cut from the corresponding formalin fixed paraffin embedded tissue blocks. Sections were stained for fibrin, TF and TM. Some sections were utilized optimizing staining techniques.

2.3.2.1.3. Control tissue

Human formalin fixed paraffin embedded placental tissue was used as positive control tissue to establish the staining technique, due to the vascular nature of the tissue and presence of coagulation factors. Human placental tissue, with acute and chronic injury tissue from mice and humans stained with the omission of the primary antibody acted as negative controls. Sections of formalin fixed, paraffin embedded liver tissue from mice which were administered saline injections instead of paracetamol or CCL4, and then culled, acted as experimental murine controls.

2.3.2.2. Antibodies, Detection System, Blocking Agents and Reagents

2.3.2.2.1. Primary antibodies

A monoclonal murine IgG1 against human fibrin (No. 350; American Diagnostica, Greenwich, CT, USA) was used. This antibody is documented to react with the fibrin monomer of the fibrin clot, by reacting with fragments X and Y, but not D and E of human fibrinogen. A monoclonal murine IgG1 against human tissue factor (No. 4509; American Diagnostica, Greenwich, CT, USA) was used, which is directed against an eptitope, within aa 1-25, the extracellular domain of human tissue factor. Both of these primary antibodies were diluted with an assay solution made up of 0.15M Tris buffered saline (TBS), 1% Bovine serum albumin (BSA) and 0.3% of Tween 20 to a recommended dilution of 1:50. A monoclonal murine IgG1 against human TSP - 1 (sc-73158, Santa Cruz Biotechnology Inc, Santa Cruz, US.), a polyclonal rabbit IgG against human TM (sc-9162, Santa Cruz Biotechnology Inc, Santa Cruz, US.), which is directed against amino acids 22-321 of thrombomodulin, and a mononclonal murine IgG1directed against amino acids 432-528 of HIF-1α (sc-53546, Santa Cruz Biotechnology Inc, Santa Cruz, US.) were used. These primary antibodies were diluted with an assay solution made of 1.5% goat serum in phosphate buffered saline (PBS) to dilutions of 1:50-1:200, depending on tissue type.

2.3.2.2.2. Secondary antibodies

For staining using antibodies against fibrin and tissue factor, the secondary antibody used was a biotin-conjugated rabbit F(ab')2 anti-mouse IgG1 (STAR 11B; AbD Serotec, Oxford, UK). The secondary antibody was diluted with TBS. The chosen dilution of 1:50 was determined by titration to eradicate non-specific staining.

For staining using antibodies against TSP-1 and HIF-1α, a biotinylated goat anti-mouse was used (ABC staining system, Santa Cruz Biotechnology Inc, Santa Cruz, US). For staining against TM, a goat anti-rabbit secondary antibody was used (ABC staining system, Santa Cruz Biotechnology Inc, Santa Cruz, US).

2.3.2.2.3. Detection systems

For fibrin and TF staining, the detection system employed was a Streptavidin conjugated to Alkaline Phosphatase (STAR 6B; AbD Serotec, Oxford, UK). The detection system was diluted with TBS to the recommended dilution of 1:100. For TSP-1, HIF-1α and TM staining, an avidin conjugated biotinylated horseradish peroxidise was used, diluted with PBS to the recommended dilution of 1:50. (ABC staining system, Santa Cruz Biotechnology Inc, Santa Cruz, US).

2.3.2.2.4. Blocking agents

Normal rabbit serum (C12SA; AbD Serotech, Oxford, UK) was diluted with TBS to a dilution to produce 10% normal rabbit serum. Normal goat serum (Santa Cruz Biotechnology Inc, Santa Cruz, US.) was diluted with PBS to produce 1.5% normal goat serum. Avidin/Biotin blocking reagent (BUF016; AbD Serotech, Oxford, UK) was used to minimise non-specific binding from endogenous biotin when staining for fibrin and tissue factor in murine sections.

2.3.2.2.5. Chromogens and substrates

Fast red tablets with Fast Red Substrate buffer (BUF023; AbD Serotech, Oxford, UK) were used. One Fast Red Tablet added to 5mls of Fast Red substrate buffer was allowed to dissolve with gentle mixing. 3,3'-Diaminobenzidine (DAB) x 10 concentrate was diluted with peroxide buffer. (Roche Diagnostics, Mannheim, Germany).

2.3.2.2.6. Other reagents

Xylene solution (Genta Enviromental Ltd, York, UK) was kindly donated by the Department of Histopathology, St. Mary's Hospital. Ethanol solution (Biotech Solutions Ltd, York, UK) was diluted with distilled water to produce 75% and 90% Ethanol solutions. Hydroden peroxidase (H3410; Sigma, St. Louis, MO, USA) was diluted with Methanol (Fischer Scientific, Leicestershire, UK) to produce 0.3% Hydrogen Peroxidase in Methanol. Sodium Citrate buffer (HDS05; HD Supplies, Bucks, UK) was diluted with distilled water to a concentration of 0.01M. TBS 10x working solution (T5912-1L; Sigma, St. Louis, MO, USA) was diluted with distilled water to produce TBS. PBS tablets (P4417; Sigma, St Louis, MO, USA) were dissolved in distilled water to produce PBS.

2.3.3. Experimental Protocol

2.3.3.1. Deparaffinisation

Sections were immersed in 100% Xylene solution for 10 minutes twice and rehydrated by immersing in 100 % Ethanol for 5 minutes, 90% Ethanol for 5 minutes and 75% Ethanol for 5 minutes. Sections were then washed with distilled water.

2.3.3.2. Blocking of Endogenous Peroxidise Activity

Sections stained for fibrin and tissue factor were treated with 0.3% hydrogen peroxide in methanol for 30 minutes, and then washed once with TBS. Sections stained for TSP-1, HIF-1α and TM were treated with 3% hydrogen peroxide in methanol for 30 minutes, and then washed once with PBS.

2.3.3.3. Antigen Retrieval

Sections were immersed in 0.01M of sodium citrate buffer, pH 6.0 and microwaved (1500 W) two times for 5 minutes each, then allowed to cool to room temperature, and then washed once in TBS or PBS.

2.3.3.4. Blocking

To minimise non specific staining, murine sections were incubated at room temperature for 120 minutes in 10% normal rabbit serum for fibrin and TF staining, and for 60 minutes in 1.5% goat serum for TSP-1, HIF-1α and TM staining. Excess serum was tapped off. Sections were then incubated for 15 minutes with Avidin solution. Sections were washed thoroughly 3 times with TBS before incubating for 15 minutes with Biotin solution. Sections were washed 3 times with TBS or PBS.

2.3.3.5. Incubation with the Primary Antibody

Sections were incubated with the primary antibodies at the noted dilutions (1:50-1:200) overnight at 4C, and then washed three times with buffered saline**.** For tissue sections, which were to act as negative controls, the primary antibody was omitted, and sections

were incubated only in the assay solution in which the respective antibody had been diluted (0.15M TBS with 1% bovine serum albumin (BSA) and 0.3% of Tween 20; or 1.5% Goat serum diluted in PBS). All sections were washed three times in TBS or PBS.

2.3.3.6. Incubation with the Secondary Antibody

Sections stained for fibrin and TF were incubated with biotinylated secondary antibody (STAR11B) at recommended dilution (1:50) for 60 minutes at room temperature. Sections stained for TSP-1 and HIF-1α were incubated with the biotinylated goat anti-mouse secondary antibody (ABC staining system) for 60 minutes at room temperature. Sections stained for TM were incubated the biotinylated goat anti-rabbit secondary antibody (ABC staining system) for 60 minutes at room temperature. All sections were washed three times in TBS or PBS.

2.3.3.7. Application of Detection System

Sections stained for fibrin or TF were incubated with the enzyme conjugated streptavidin (STAR 6B) at recommended dilution (1:100) for 60 minutes at room temperature. Sections washed three times with TBS. Sections stained for TSP-1, HIF-1α and TM were incubated with the avidin conjugated biotinylated horseradish peroxidise (ABC staining system) at the recommended dilution (1:50) for 60 minutes at room temperature. Sections were washed three times with Phosphate buffered saline.

2.3.3.8. Chromogen and Substrates

Fibrin and TF sections were incubated with Fast red solution for 2 minutes at room temperature. Sections were then washed once in distilled water. TSP-1, HIF-1α and TM sections were incubated with DAB staining solution for 2 minutes. Sections were then washed in distilled water for 2 minutes.

2.3.3.9. Counterstaining and Mounting

Fibrin and TF sections were counterstained in haematoxylin for 1 minute and then rinsed in running water for 5 minutes**.** Sections were mounted in the aqueous mounting medium and coverslips added manually (Dako permanent aqueous mount; Dako). TSP-1, HIF-1α and TM sections were counterstained in haematoxylin for 1minute, and then dehydrated with two washes in 95% ethanol for 10 seconds each, followed by 2 washes in 100% ethanol for 10 seconds each and then 3 washes in xylene for 10 seconds each. Excess xylene was then wiped off. Sections were mounted using a non aqueous mounting medium and cover slips applied.

2.3.4. ANALYSIS

2.3.4.1. Acute Liver Injury

Each murine section was evaluated in its entirety using light microscopy by a histopathologist (Professor Robert Goldin) blinded to the injury grade of the specimen. The extent of necrosis in each section stained with H&E was reassessed using the ICSS (Table 2-1). The amount of fast red staining for fibrin or TF expression or brown stained secondary to DAB for TSP-1 expression was noted using digital image analysis and or a 4 point visual analogue

scale of 0 (none), 1 (small amount of staining), 2 (moderate staining) and 3 (heavy staining). Structural patterns and distribution of staining were noted in each section. Digital image analysis was performed to allow a semi-automated quantification of protein expression. Using an Olympus BX50 microscope at x1.5 objective under fixed lighting conditions, multiple digital images of each section stained were taken. Using the Nikon NIS Elements Software Viewing System (Nikon, Japan) the digital images acquired from each section were 'stitched' to produce a single image representative of the entire section. This image was analysed, using Nikon NIS digital image analysis software to calculate the percentage area of staining in each section. Image mapping techniques allowed for semi-automated co-localization of staining patterns between individual proteins, and or areas of necrosis.

Each human acute liver injury section was evaluated in its entirety using light microscopy by a histopathologist initially blinded to the aetiology of the cause of acute liver failure. Structural patterns and distribution of staining were noted in each section for each protein.

2.3.4.2. Chronic Liver Injury

Liver sections were evaluated using light microscopy with a histopathologist (Dr Adam Levene) blinded to the nature of specimen. Digital image analysis was performed to allow a semi-automated quantification of the coagulation proteins in each section of tissue. For archived HCV human liver, sections were graded using the Ishak Modified Histological Activity Index for fibrosis (see Table 2-2) and the Knodell Histology Activity Index for inflammation (see Table 2-3). Sections were sub-classified into one of four groups according to the reported scoring of liver fibrosis: Mild fibrosis (fibrosis score 1 and 2), moderate fibrosis (fibrosis score 3 and 4) and severe fibrosis (fibrosis score 5 and 6) (see Table 2-2).

Using an Olympus BX50 microscope under fixed lighting conditions, digital images of 8 randomly chosen microscope fields of view at x20 objective were taken from each murine sections. We evaluated the whole of each human section at x10 objective. The Nikon NIS Elements Software Viewing System (Nikon, Japan) was used to create stitched images, and the percentage area of staining for fibrin and tissue factor in each section was calculated as described above.

Table 2-2: Modified histological scoring system for quantifying liver fibrosis.

Score	Degree	Description
0	-	No fibrosis
1	Mild	Fibrous expansion (spurs) around some central veins
2		Fibrous expansion around >50% central veins
3	Moderate	Fibrous expansion around most central veins, some CV-to-CV bridging
4		Fibrous expansion around most central veins, marked (>50%) CV-to-CV bridging
5	Severe	Marked bridging fibrosis plus some nodules
6		Cirrhosis (>50% nodularity)

Table 2-3: The Knodell Histology Activity index for quantifying hepatic inflammation.

Score	Level of inflammation
0	No inflammation present
1-4	Minimal inflammation present
5-8	Mild inflammation present
9-12	Moderate inflammation present
13-18	Severe inflammation present

2.3.5. Statistics

Data was collated using Microsoft Excel (Microsoft Inc, USA). Statistical analysis was performed using SPSS version 14 (SPSS, USA). Quantitative data is expressed as mean ± standard error of mean. An independent t-test analysis was used to compare protein expression between control and experiment murine groups where applicable. A linear trend across categories was examined using regression analysis to assess expression of proteins and degree of injury. Statistical significance was accepted at $p<0.05$. Graphs were generated using Microsoft Excel (Microsoft Inc, USA) and SPSS version 14 (SPSS, USA) with any error bars representing standard error of mean.

2.4. RESULTS

2.4.1. Antibody evaluation

2.4.1.1. Fibrin and Tissue Factor

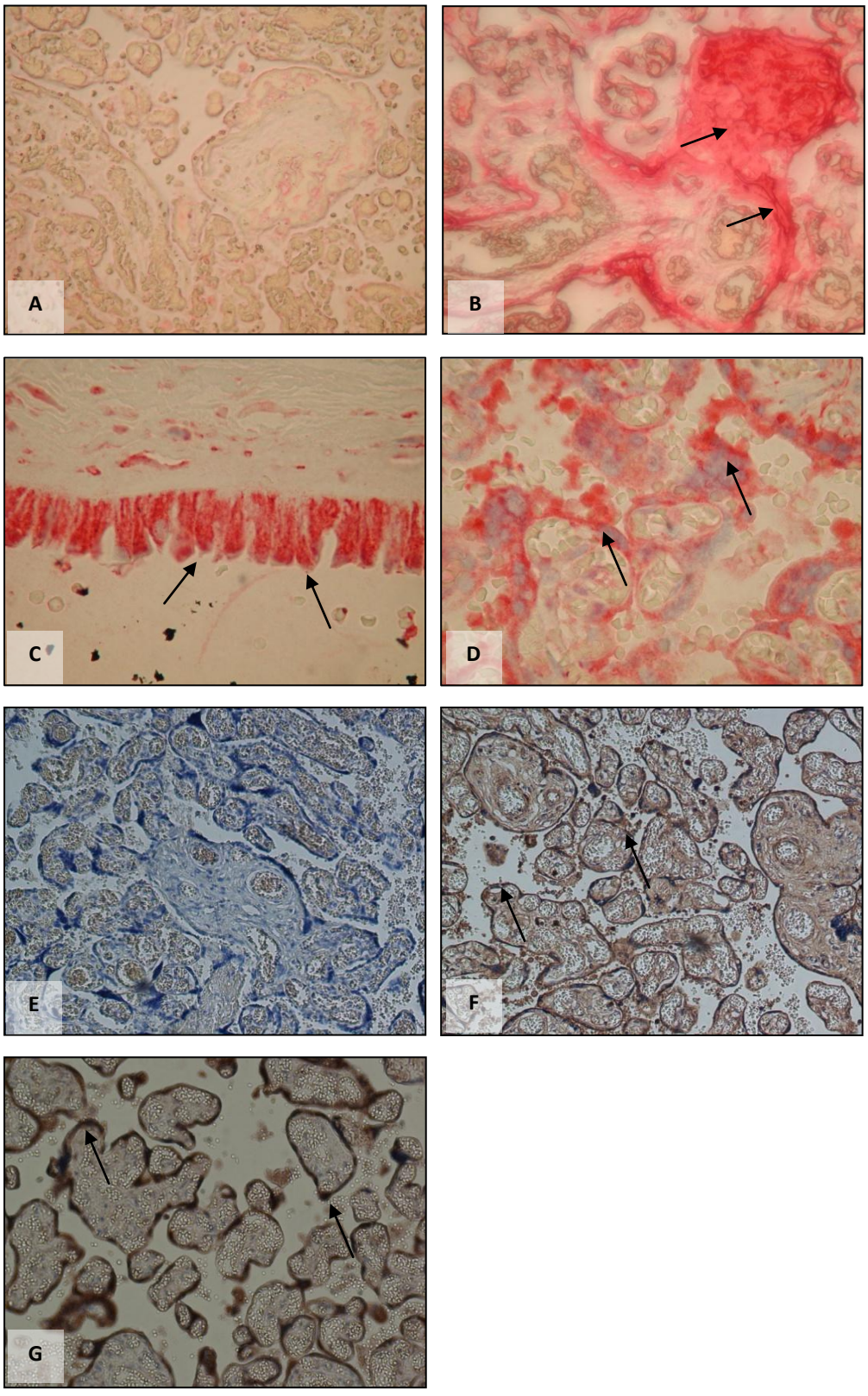
The experimental method described in section 2.3 utilised Fast Red and should produce a strong red stain at the site of the antigen. In human placenta (positive control), fibrin and tissue factor were detected in keeping with the expected pattern of distribution (see Figure 2-1.B-D). In placental tissue tested, no non specific staining occurred when the protocol was performed with the omission of the primary antibody (See Figure 2-1.A).

2.4.1.2. TSP-1, HIF-1 α and Thrombomodulin

The experimental method described in section 2.3, utilising DAB, should produce a brown stain at the site of each antigen stained with this technique. Human placenta was again used as the positive control, and TSP-1 and TM were detected in keeping with the expected pattern of distribution (see Figure 2-1 F-G). In placental tissue tested, again no non specific staining occurred when the protocol was performed with the omission of the primary antibody (See Figure 2-1.E).

Figure 2-1: Placental control tissue stained for TF, fibrin, TSP-1, TM, and HIF-1 α.

Fast red stains red, and DAB stains brown.



A: Negative control: Placenta tissue section stained with the omission of the primary (x4 objective). No fast red stain demonstrated.

B: Placental tissue section stained for fibrin (x40 objective). Findings in keeping with expected dense fibrin deposition in the placental bed.

C: Placenta tissue stained for tissue factor (x10 objective). Heavy epithelial staining around a section of the amniotic sack.

D: Placenta tissue stained for TF (x40 objective). Heavy TF staining in the placental bed in a similar pattern to fibrin.

E: Negative control: Placenta tissue stained with the omission of TSP-1, HIF-1 α or TM (x10 objective). No non specific staining demonstrated.

F: Placenta tissue stained for TM (x10 objective). Heavy staining of syncytiotrophoblast layer of placenta.

G: Placenta tissue stained for TSP-1 (x20 objective). Positive staining seen in the placental bed.

Black arrows indicate positive antigen staining.

2.4.2 Acute Liver Injury

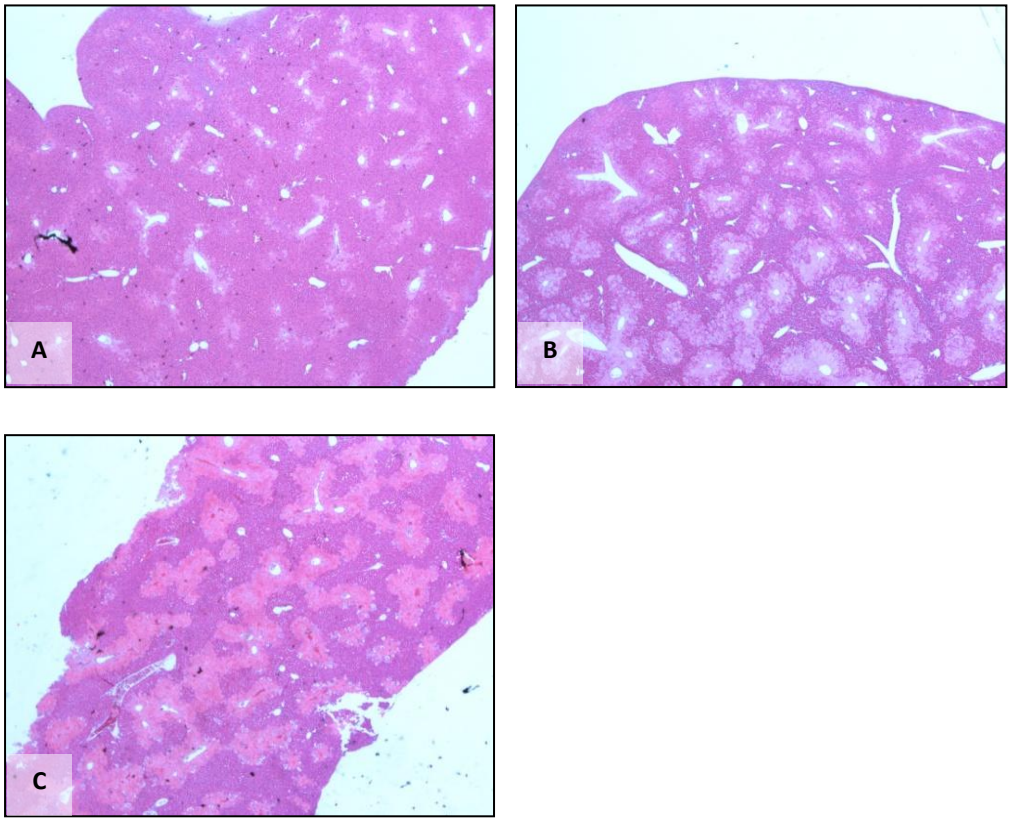
2.4.2.1. H&E Staining

2.4.2.1.1. H&E staining in murine sections

16 sections in total were stained: 12 sections from mice treated with paracetamol and 4 sections from control mice. H&E staining demonstrated varying degrees of necrosis, ranging from no evidence of necrosis in control sections to mild perivenular necrosis in Grade 1 injury sections; mild bridging necrosis in Grade 2 injury sections; extensive bridging necrosis in Grade 3 injury sections; and panlobular necrosis in Grade 4 injury sections (See Figure 2-2).

Figure 2-2: H&E staining demonstrating a selection of various degrees of necrosis in acute murine liver injury.

Necrotic areas seen as a pink discolouration



A: Grade 1 injury demonstrating perivenular necrosis with no bridging (x1.5 objective)

B: Grade 2 injury demonstrating mild bridging necrosis (x1.5 objective).

C: Grade 3: demonstrating extensive bridging necrosis (x1.5 objective).

2.4.2.2 Fibrin Staining

2.4.2.2.1. Fibrin in normal murine livers

Mice treated with saline only, represented what we would expect to demonstrate in normal livers. A small amount of intravascular fibrin, in the form of thrombus in the central veins with cut edge staining was noted. Neither hepatocellular nor centrilobular staining was noted (See Figure 2-4A). These findings confirmed the success of the staining technique as intravascular clot was correctly localised and no non-specific staining within the liver parenchyma was present.

2.4.2.2.2. Fibrin expression in murine paracetamol induced acute liver injury

Fibrin was expressed in all the sections from mice with ICSS graded acute liver injury. In grade 3 and 4 injuries there was heavy confluent staining within necrotic areas originating in the centrilobular areas. Fibrin staining was less prominent in sections from mice with milder injuries (See Figure 2-3 & 2-4). Regression analysis of the relationship between the extent of fibrin staining measured by using a visual analogue scale and the grade of liver injury revealed a very strong correlation (Spearman correlation 0.956, $p<000.1$). A comparable correlation was found when performing regression analysis between the digital quantification of fibrin staining and grade of liver injury (Spearman correlation 0.961 $p<0.0001$) (see Table 2-4 and Figure 2-3). Co-localization of fibrin staining using image mapping confirmed that fibrin staining was confined to areas of necrosis (see Figure 2-5).

Table 2-4: Fibrin staining measured by a 4 point visual analogue scale and digital quantification in murine acute liver injury

Values expressed as mean % of section stained for fibrin (SEM). (n = 16).

Grade of injury	Visual analogue score	% Fibrin stain per section
G0	0 (0)	2.4 (0.5)
G1	1.25 (0)	8.4 (2.0)
G2	2(0)	20.8(1.6)
G3	2.5 (0.3)	30.0 (4.6)
G4	3 (0)	40.5 (0.3)

Figure 2-3: Scatterplot graph showing digital quantification of fibrin staining in murine paracetamol acute liver injury.

Digital quantification of fibrin staining (% staining per section) versus Imperial College Scoring System (ICSS). Regression analysis demonstrating a strong correlation between fibrin deposition and grade of injury (r= 0.96, p<0.0001)

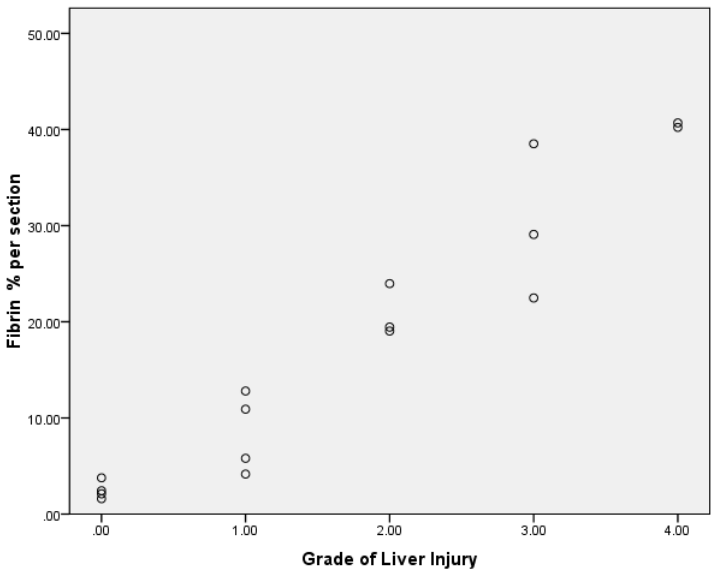
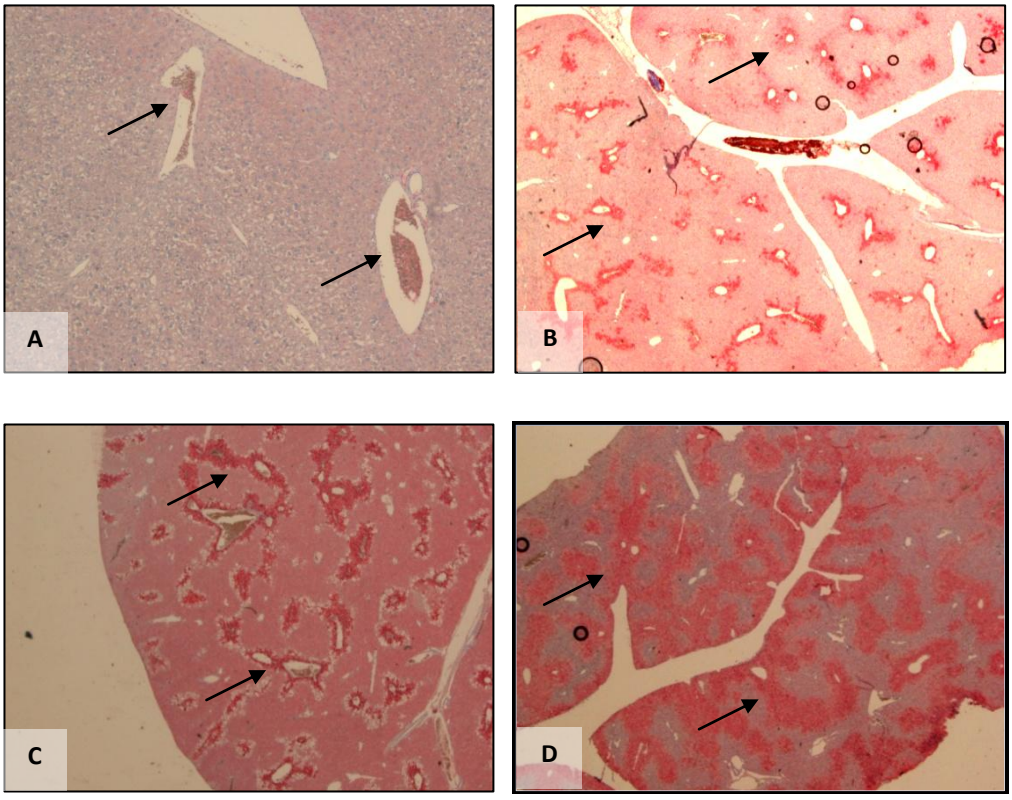


Figure 2-4: Fibrin staining with varying patterns with degree of injury.

Fibrin staining seen as red stain



A: Control tissue demonstrating only intravascular thrombus within large vessels (stained red)(x 1.5 objective).

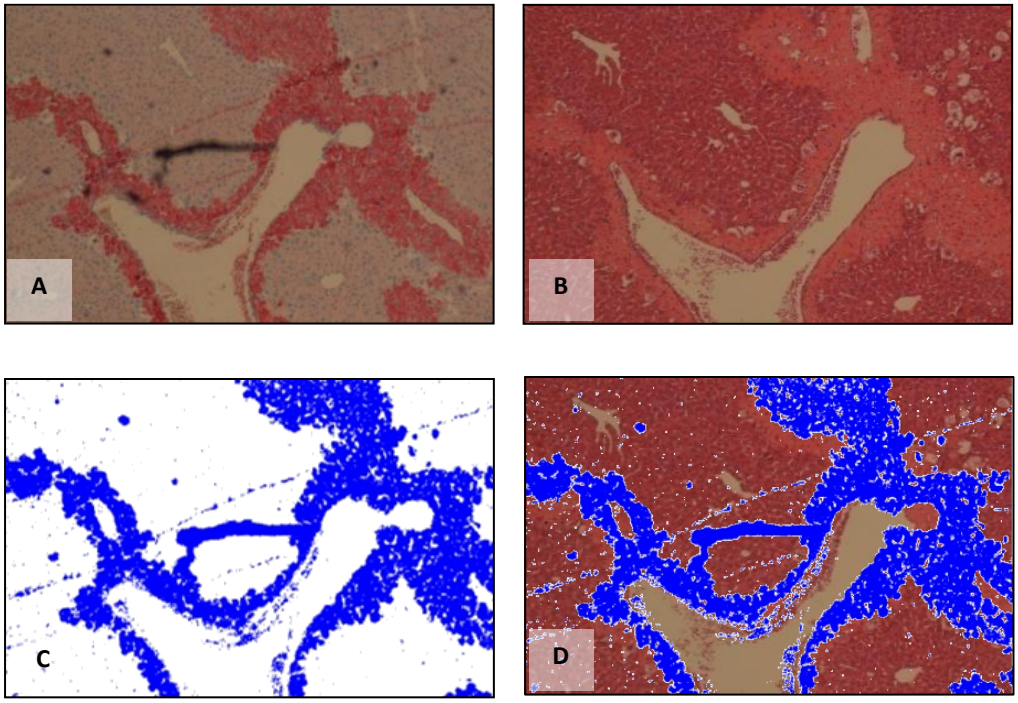
B: Grade 1 injury demonstrating a perivenular deposition of fibrin, with no bridging between veins (x 1.5 objective).

C: Grade 2 injury showing centrilobular fibrin deposition with mild bridging between the central veins (x 1.5 objective).

D: Grade 4 injury demonstrating extensive panlobular staining of fibrin (x 1.5 objective).

Black arrows indicate areas of positive staining

Figure 2-5: Digital image co-localization of fibrin with necrosis, using acute murine liver injury serial sections.



A: Grade 3 injury section stained showing extensive fibrin staining (x4 objective).

B: Grade 3 injury section H&E stain, demonstrating bridging necrosis (x4 objective).

C: Composite image produced using NIS image analysis isolating fibrin expression (blue) (x4 objective).

D: Digitally merged image of the fibrin stain (blue) superimposed over the H&E stain, hence co-localizing fibrin staining to the area of necrosis (x4 objective).

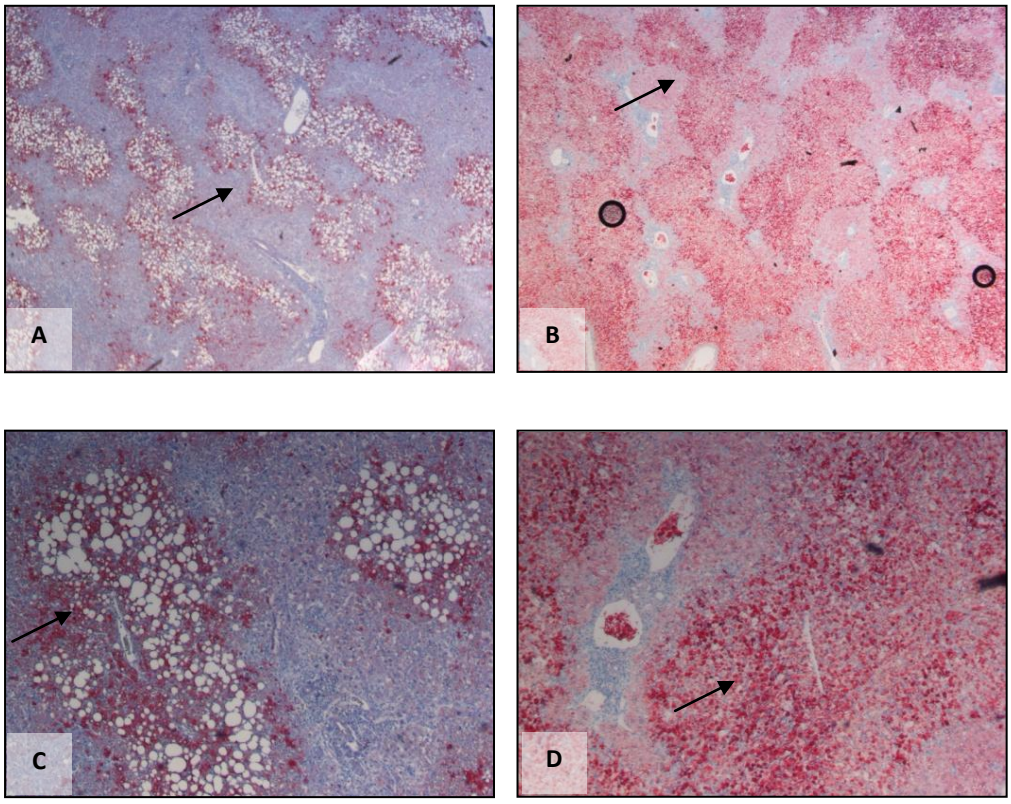
2.4.2.2.3. Fibrin expression in human acute liver failure

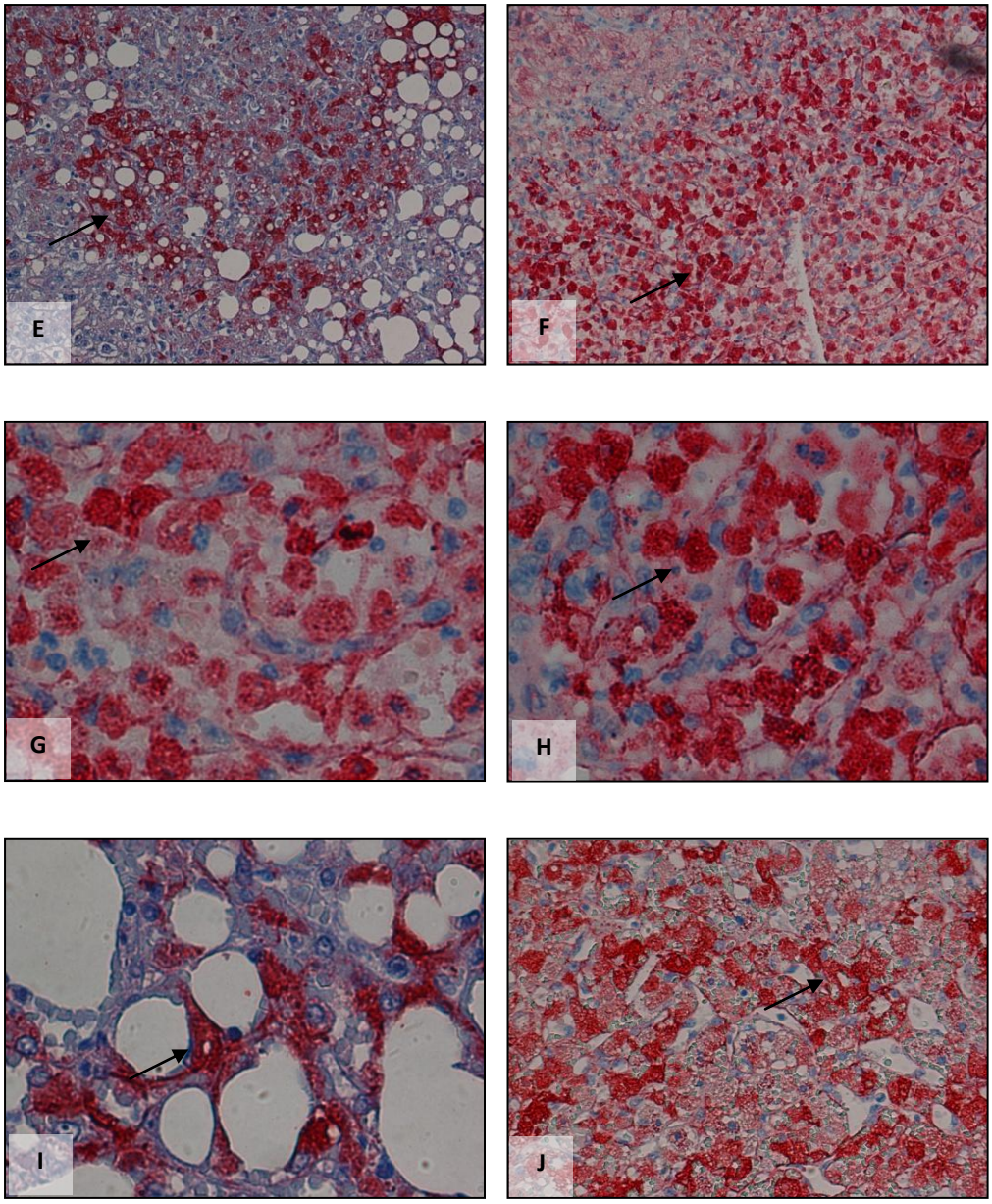
Tissue from explanted transplant recipients livers secondary to paracetamol overdose (n=3), acute HBV infection (n=1), seronegative acute liver failure (n=1) and autoimmune hepatitis (n=1) were stained for fibrin. Fibrin was expressed in all the sections but to varying degrees depending on the cause of acute liver failure (See Figures 2-6 & 2-8). Intravascular clot stained positive. All negative control tissue failed to stain as would be expected.

2.4.2.2.3.1. Fibrin expression in human acute liver failure secondary to paracetamol overdose

Fibrin was heavily expressed within the necrotic areas of all liver sections from patients, who had developed acute liver failure as a result of paracetamol toxicity. At higher objectives, staining for fibrin was demonstrated within macrophages and hepatic sinusoids (See Figure 2-6)

Figure 2-6: Fibrin staining in tissue sections from human acute liver failure secondary to paracetamol toxicity.





A,B: Paracetamol toxicity demonstrating confluent fibrin deposition at low power (x1.25 objective).

C,D: Paracetamol toxicity demonstrating fibrin deposition confined to necrotic areas (x4 objective).

E,F : Paracetamol toxicity demonstrating fibrin accumulation in macrophages (x10 objective).

G,H: Paracetamol toxicity demonstrating fibrin accumulation in macrophages at higher power (x 40 objective).

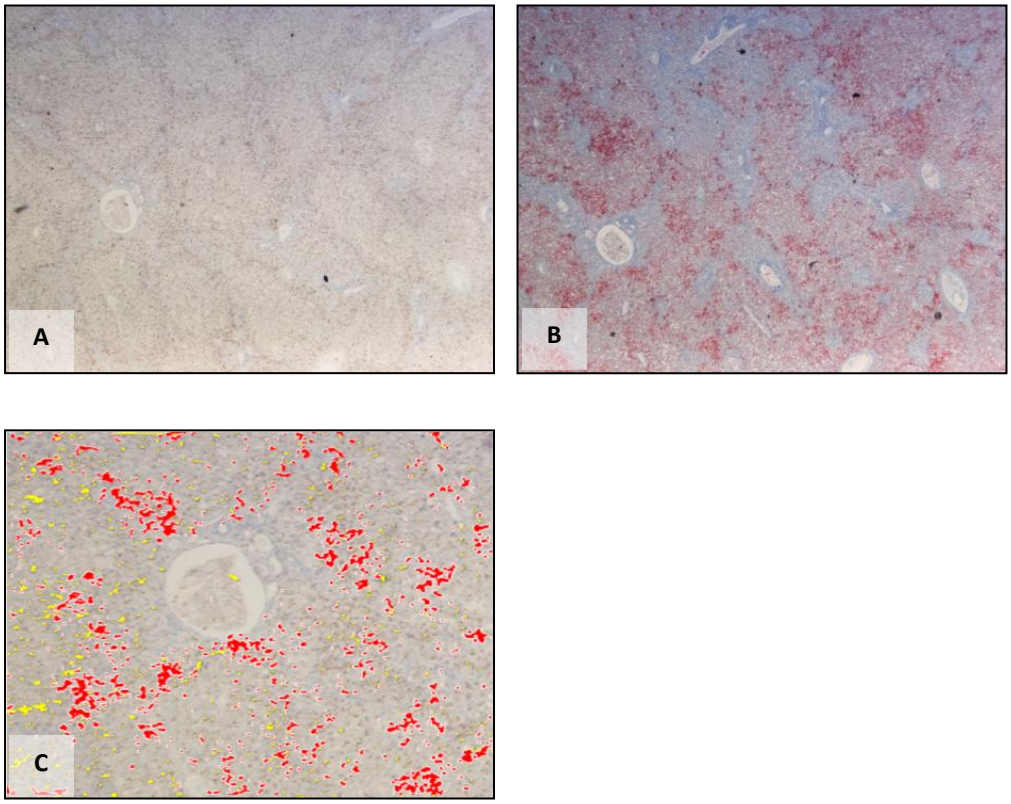
I, J: Paracetamol toxicity demonstrating sinusoidal staining (x40 objective).

Black arrows indicate areas of positive staining

CD 68, a marker for macrophages, displayed a similar pattern of distribution to fibrin, and digital image mapping demonstrated a degree of co-localization between staining of fibrin and CD68 on serial sections, confirming the localization of fibrin within macrophages (see Figure 2-7).

Figure 2-7: Fibrin and CD68 staining in tissue sections from human acute liver failure secondary to paracetamol toxicity.

Co-localization of CD68 and fibrin staining



A : Paracetamol toxicity demonstrating confluent CD68 expression at low power (x4 objective).

B : Paracetamol toxicity demonstrating confluent fibrin deposition (x4 objective).

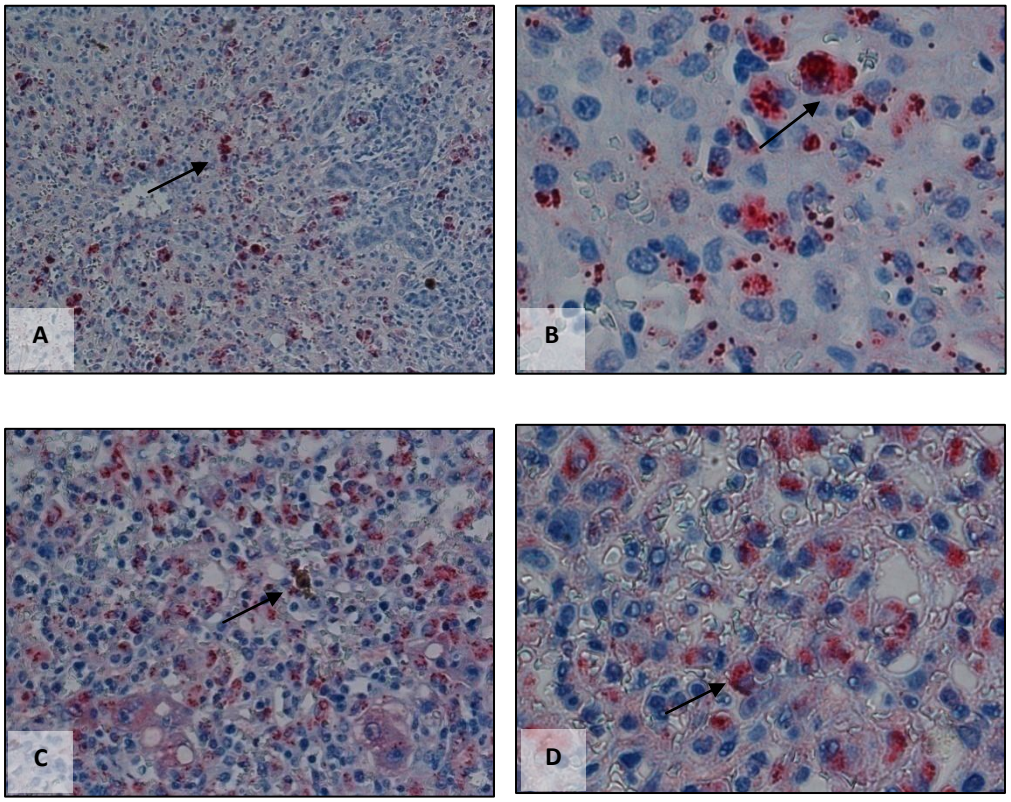
C : Digital image mapping showing colocalization of CD68 (yellow) and fibrin (red) expression (x10 objective).

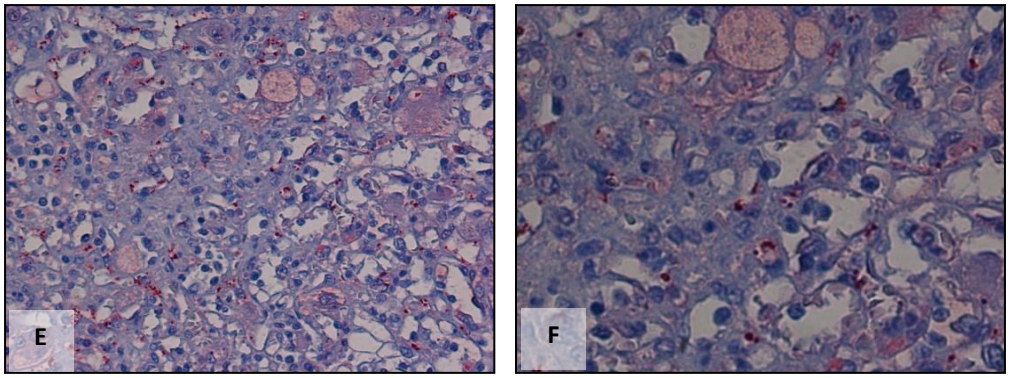
2.4.2.2.3.2. Fibrin expression in human acute liver failure secondary to other causes of acute liver failure

In contrast fibrin staining was far less prominent in acute liver failure caused by autoimmune hepatitis and acute HBV infection, and seronegative acute liver failure. In autoimmune hepatitis sections there was very sparse fibrin staining localised to macrophages only (See Figure 2-8). Acute HBV and seronegative acute liver failure demonstrated moderate levels of fibrin staining, in which fibrin was predominantly localised to macrophages with some sparse sinusoidal staining.

Figure 2-8: Fibrin staining in tissue sections from human acute liver failure secondary to seronegative, acute hepatitis B, and autoimmune hepatitis.

Varying intensity and distribution of fibrin deposition was demonstrated relating to aetiology of injury.





A: Seronegative acute liver failure: moderate fibrin deposition at low power (x 20 objective).

B: Seronegative acute liver failure sections showing fibrin accumulation in macrophages (x 40 objective).

C: Acute hepatitis B: moderate amount of fibrin deposition at low power (x20 objective).

D: Acute hepatitis B: some fibrin deposition within macrophages (x 40 objective).

E: Autoimmune hepatitis: minimal fibrin deposition seen at low power (x20 objective).

F: Autoimmune hepatitis: very sparse fibrin deposition at high power (x40 objective).

Black arrows indicate positive staining

2.4.2.3. Tissue Factor

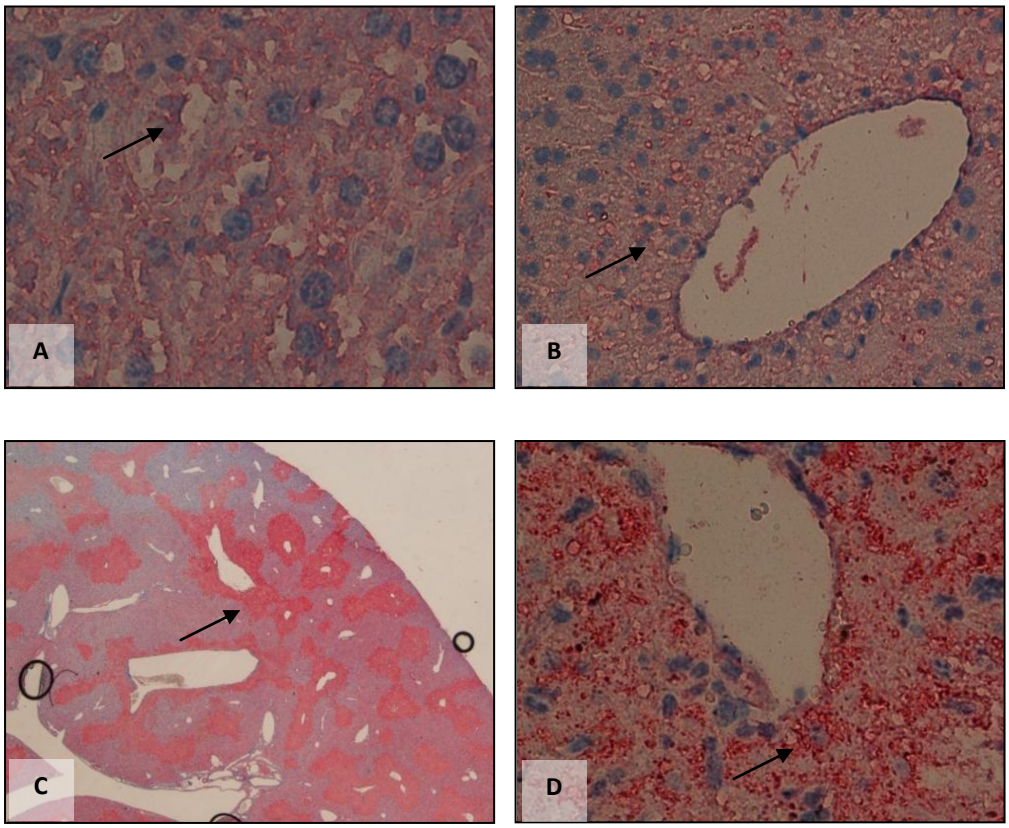
2.4.2.3.1. Tissue factor expression in normal murine livers

Positive staining for TF was demonstrated in all the sections from control mice. There was weakly positive staining in the hepatic sinusoids in all control sections. No staining around the central veins and perivenular areas was observed (See Figure 2-9).

2.4.2.3.2. Tissue factor expression in murine paracetamol induced acute liver injury

TF expression was characterised by two distinct patterns in mice treated with paracetamol. Both sinusoidal expression in viable areas and centrilobular expression in necrotic areas were demonstrated. This was in contrast to the control mice (normal livers) in which only weak sinusoidal screening was seen (See Figure 2-9). The intensity and distribution of these two distinct patterns of staining varied with the grade of injury (See Table 2-5, Figure 2-9, 2-10). Regression analysis of the relationship between grade of injury and centrilobular staining (measured by a visual analogue scale) revealed a strong correlation (Spearman correlation 0.77, $p<000.1$). Centrilobular TF expression also had a strong correlation with fibrin expression ($r = 0.731$, $p<0.001$). In contrast the amount of sinusoidal staining in viable areas did not correlate with grade of injury (r-0.4, $p>0.05$). Furthermore TF sinusoidal expression in viable areas showed no correlation with TF centrilobular expression ($r = -1.47$, $p> 0.05$) nor fibrin deposition ($r = -0.35$, $p >0.05$) (See Figure 2-10).

Figure 2-9: TF staining with varying patterns with degree of injury.



A: Control tissue (x40 objective) demonstrating only weak sinusoidal staining (stained red).

B: Grade 1 injury (x20 objective) demonstrating TF sinusoidal staining but no perivenular staining.

C: Grade 4 injury (x1.5 objective) showing widespread centrilobular staining.

D: Grade 4 injury (x40 objective) demonstrating heavy centrilobular staining**.**

Table 2-5: Tissue factor staining measured by a 4 point visual analogue scale in murine acute livery injury.

Qualified as either sinusoidal or centrilobular in distribution. Values expressed as mean (SEM). (n=16)

Grade of Injury	TF sinusoidal staining	TF centilobular staining
G0	0.5 (0.5)	0 (0)
G1	1.4 (0.3)	1.1 (0.66)
G2	0.833 (0.17)	1.2(0.16)
G3	1.0 (1.0)	2.3 (0.8)
G4	1.75 (0)	2.3 (0.3)

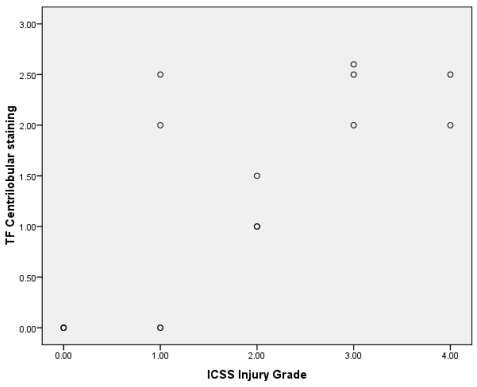
Figure 2-10: Scatterplots demonstrating TF staining in murine acute livery injury.

A: Scatterplot graph showing the visual analogue measurement of TF centrilobular staining (4 point scale) versus ICSS grade of injury (r= 0.77, p<0.001).

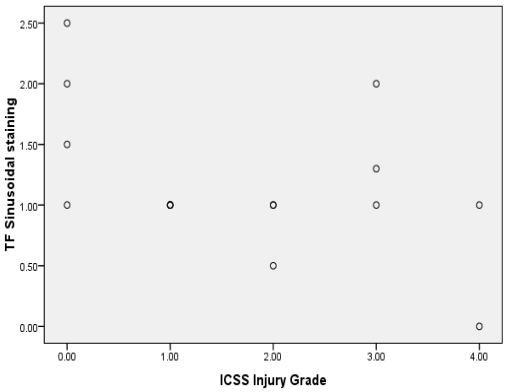
B: Scatterplot graph showing visual analogue measurement of TF sinusoidal staining versus ICSS grade of injury (r = -0.4 p>0.05).

C: Scatterplot showing visual analogue measurement of TF centrilobular staining versus fibrin staining (r= 0.71 , p<0.001)

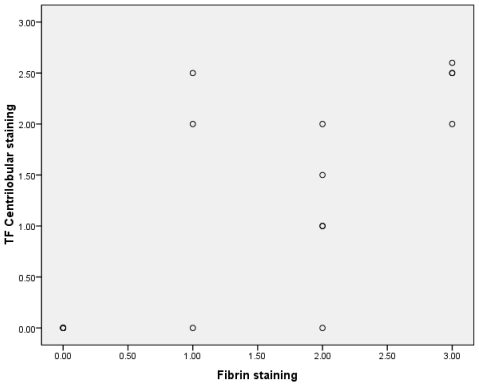
A



B



C

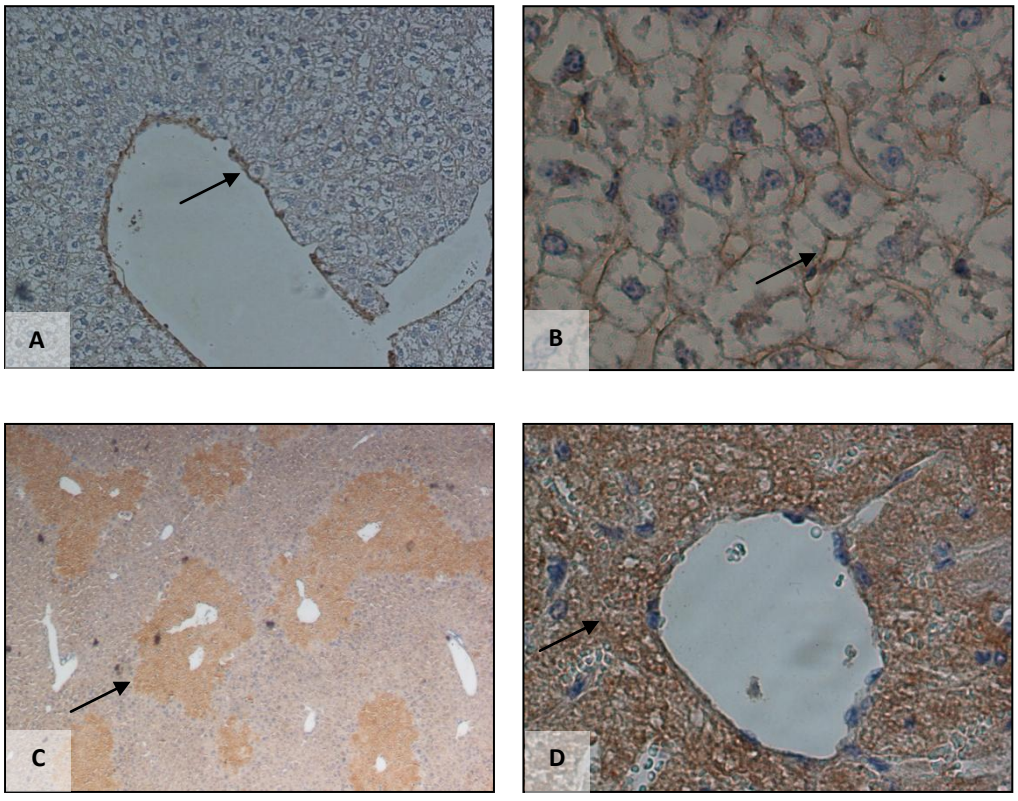


2.4.2.4. Thrombospondin-1

2.4.2.4.1. TSP-1 expression in murine paracetamol induced acute liver injury

TSP-1 was detected in all acute liver injury mice sections (grade 0 to 4) incubated with the primary antibody at a dilution of 1:200. In grade 0 and control mice tissue, TSP-1 was detected only on the cut edge of sections, with only endothelial or intravascular staining seen. In higher grades of injury there was heavy staining in a similar distribution to fibrin. At higher power TSP-1 was confined to sinusoids and areas of centrilobular necrosis (See Figure 2-11). Regression analysis demonstrated a strong correlation between the percentage of TSP-1 stained per section and grade of acute murine liver injury (Spearman correlation 0.931 $p<0.01$) (See Table 2-6, Figure 2-12).

Figure 2-11: TSP-1 staining with varying degrees of murine acute liver injury.



A: Grade 0 injury (x10 objective) demonstrating endothelial staining for TSP-1.

B: Grade 0 injury (x40 objective) demonstrating sinusoidal staining for TSP-1.

C: Grade 4 injury (x1.5 objective) showing widespread centrilobular staining.

D: Grade 4 injury (x20 objective) demonstrating heavy centrilobular staining.

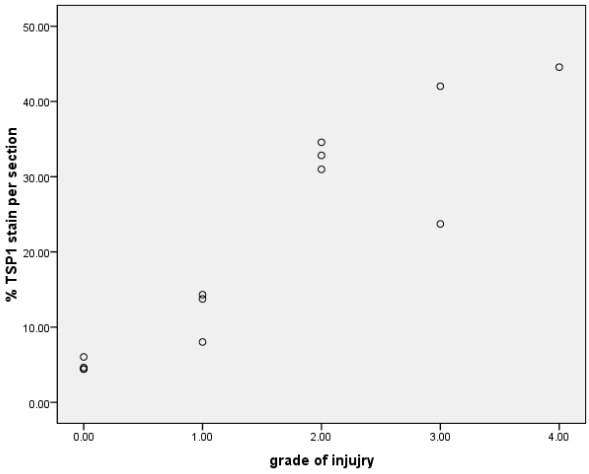
Table 2-6: TSP-1 staining measured by a 4 point visual analogue scale and digital quantification in murine acute liver injury.

(n= 13) Values expressed as mean % of section stained for fibrin (SEM).

Grade of Injury	Visual analogue score	% TSP-1 stain per section
G0	0 (0)	4.8 (0.4)
G1	1.0 (0)	12.0 (3.4.)
G2	2(0)	32.8(1.0)
G3	2.5 (0.3)	33..0 (9.1)
G4	3 (0)	44.5 (0.0)

Figure 2-12: Scatterplot graph showing digital quantification of TSP-1 staining.

Digital quantification of TSP-1 staining (% staining per section) versus Imperial College Scoring System (ICSS) . Regression analysis demonstrating a strong correlation between TSP-1 deposition and grade of injury (r= 0.931, p<0.01).

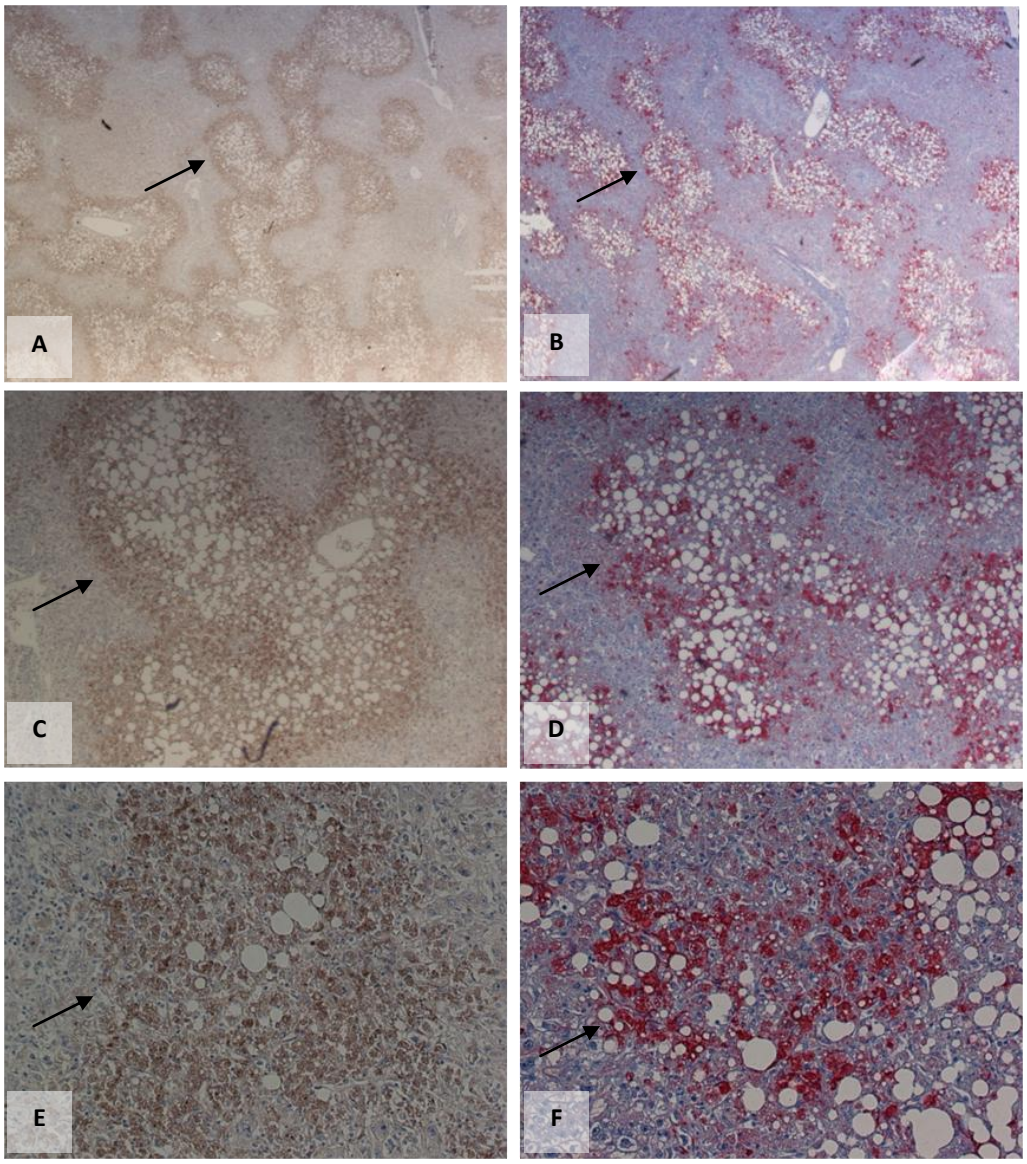


2.4.2.4.2. TSP-1 expression in human paracetamol induced acute liver injury

Tissue from explanted transplant recipients livers secondary to paracetamol overdose were stained for TSP-1. TSP-1 was expressed in all the sections (see Figure 2-13). No non specific staining was seen with omission of the primary antibody. TSP-1was heavily expressed within necrotic areas of liver sections. At both higher and lower objectives staining was demonstrated in a similar pattern of distribution to fibrin staining (Figure 2-13).

Figure 2-13: TSP-1 staining in human paracetamol acute liver injury.

TSP-1 staining confined to areas of necrosis, in a similar distribution to fibrin staining



A, B: Confluent TSP-1 deposition (A) in a similar distribution to fibrin staining (B) (x1.5 objective)

C, D: TSP-1 staining (C) confined to in necrotic areas in keeping with fibrin expression (D). (x4 objective)

E, F: TSP-1 expression (E) confined to necrotic areas in keeping with pattern of staining for fibrin (F) (x10 objective)

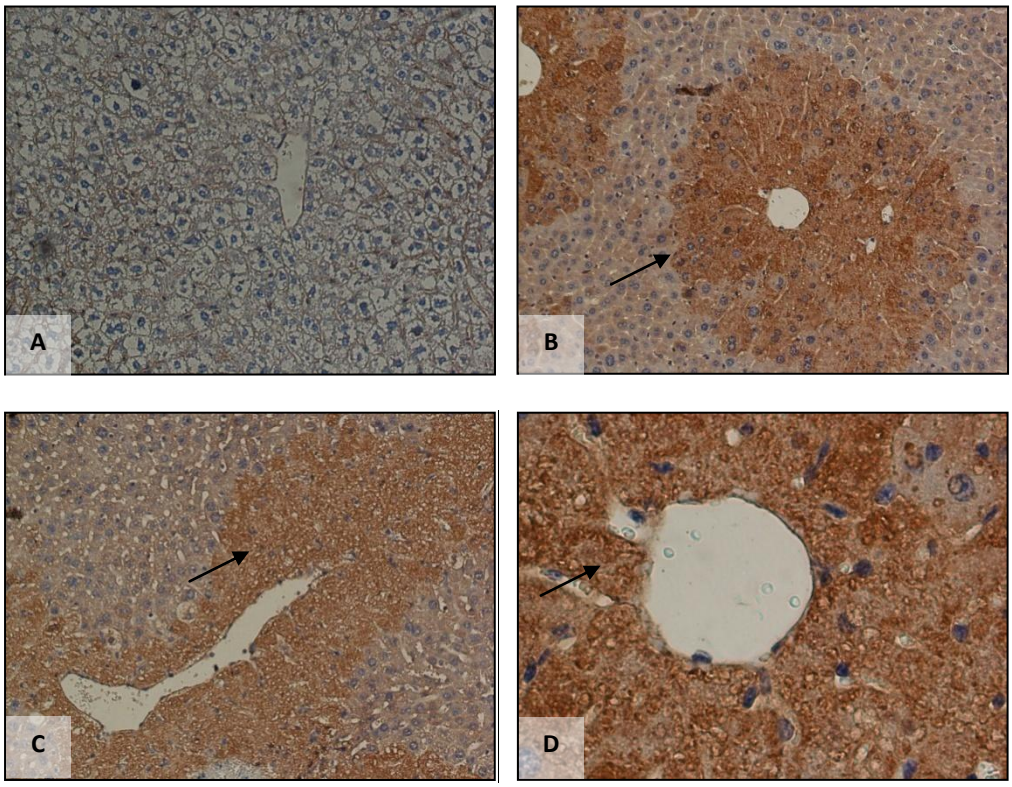
Black arrows indicate positive staining

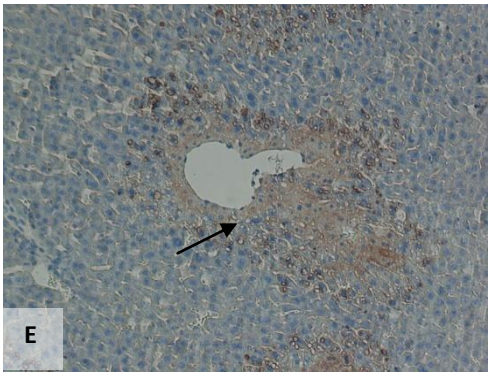
2.4.2.5. HIF-1α expression in murine paracetamol induced acute liver injury.

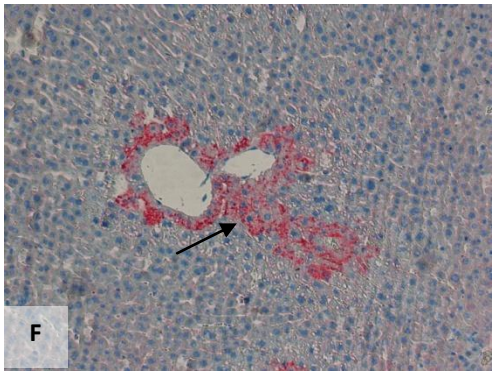
A subset of acute liver injury mice sections were for stained for HIF-1a, a marker of tissue hypoxia. In keeping with staining for fibrin and TSP-1, HIF-1a expression demonstrated a similar centrilobular pattern, with colocalization to areas of necrosis in mice with paracetamol induced acute liver injury (Figure 2-14). No staining was demonstrated in sections from control mice (see Figure 2-14 A). Furthermore when sections were stained from a subset mice culled (n=4) at earlier time points of 2 and 4 hours, staining demonstrated co-localisation of both HIF-1a and fibrin in centrilobular areas (see Figure 2-14). This demonstrates that expression of both HIF-1a and fibrin is observed early in the generation of this type of injury.

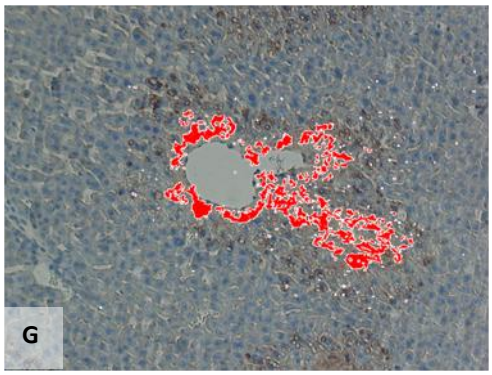
Figure 2-14: HIF1- α staining in murine acute liver injury.

Colocalised to areas of necrosis and fibrin deposition.









A: No staining for HIF-1a in liver sections from control mice at x10 objective.

B, C: HIF-1a demonstrating a centrilobular pattern of expression colocalized to areas of necrosis at (x10 objective).

D: HIF-1a demonstrating a centrilobular pattern of expression colocalized to areas of necrosis (x40 objective).

E: HIF-1a demonstrating an early weak centrilobular pattern of expression co-localized to areas of necrosis in mice culled at 2 hours post administration of paracetamol (x10 objective).

F: Fibrin deposition in a centrilobular pattern of expression co-localized to areas of necrosis and HIF-1a in mice culled at 2 hours post administration of paracetamol (x10 objective).

G: Composite image using digital mapping further demonstrating colocalization of fibrin (red) and HIF-1a (brown) in a centrilobular distribution (x10 objective).

Black arrows indicate positive staining

2.4.3. CHRONIC LIVER INJURY

2.4.3.1. H&E staining

2.4.3.1.1. H&E staining in murine liver tissue

Liver tissue sections taken from CCL4 treated mice all demonstrated moderate amounts of fibrosis, with fibrous expansion around most central veins with bridging fibrosis between central veins. Liver tissue sections taken from control mice, treated only with saline, were architecturally normal with no evidence of hepatic fibrosis.

2.4.3.1.2 H&E staining in human HCV liver tissue

Human HCV liver tissue sections demonstrated a range of grades of fibrosis in keeping with their respective Ishak fibrosis score (see Table 2-2). All biopsies selected were graded as having minimal or mild inflammatory scores (see Table 2-3).

2.4.3.2. Fibrin

2.4.3.2.1. Fibrin expression in murine chronic liver injury

Fibrin was expressed in all tissue sections from mice with CCL4 induced fibrosis. In fibrotic areas, fibrin expression was demonstrated in both the fibrous septae and blood vessels including large vessels with intravascular clot and endothelial staining of sinusoids. In perifibrotic areas there was not only a zonal staining patterm, in which there was mild cytoplasmic staining of perifibrotic hepatocytes, but also heavy sinusoidal staining. No cytoplasmic staining was seen in non fibrotic areas, where only sinusoidal staining was noted. (See Figure 2-16). Only intravascular clot and cut edge staining was noted in tissue from

control mice, treated with saline injection alone.

On performing digital image analysis there was significant up-regulation of fibrin expression within murine liver tissue treated with CCL4 when compared to normal control murine liver tissue (22.22%+/-2.13 versus 0.04%+/- 0.03, p=0.0009) (See Table 2-7; Figure 2-15).

Table 2-7: Fibrin staining measured by digital quantification in mice treated with CCL4 and normal mice.

Normal tissue (n=3) versus fibrotic murine liver tissue following exposure to 4 weeks of CCL4 (n=3). Values expressed as mean % of section stained for fibrin.

Group	Mean % Fibrin stain per section	SEM
Control mice (normal)	0.04	0.03
CCL4 mice (fibrotic)	22.22	2.13

Figure 2-15: Graph showing digital quantification of fibrin staining in murine liver tissue sections following CCL4 exposure.

Values expressed as mean % of section stained for fibrin (SEM).

Bar graph showing digital quantification of fibrin staining (mean % of sections stained for fibrin/fibrinogen) in normal control mice and fibrotic murine liver tissue sections induced by CCL4. A significant increase in fibrin expression in fibrotic versus normal control murine liver tissue (p = 0.009).

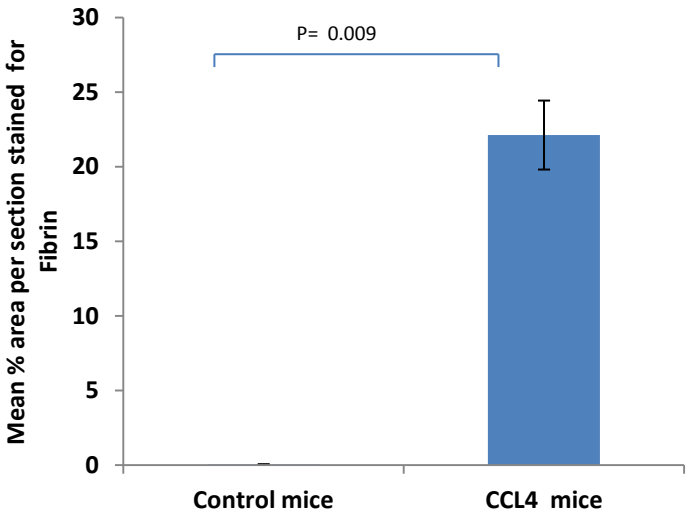
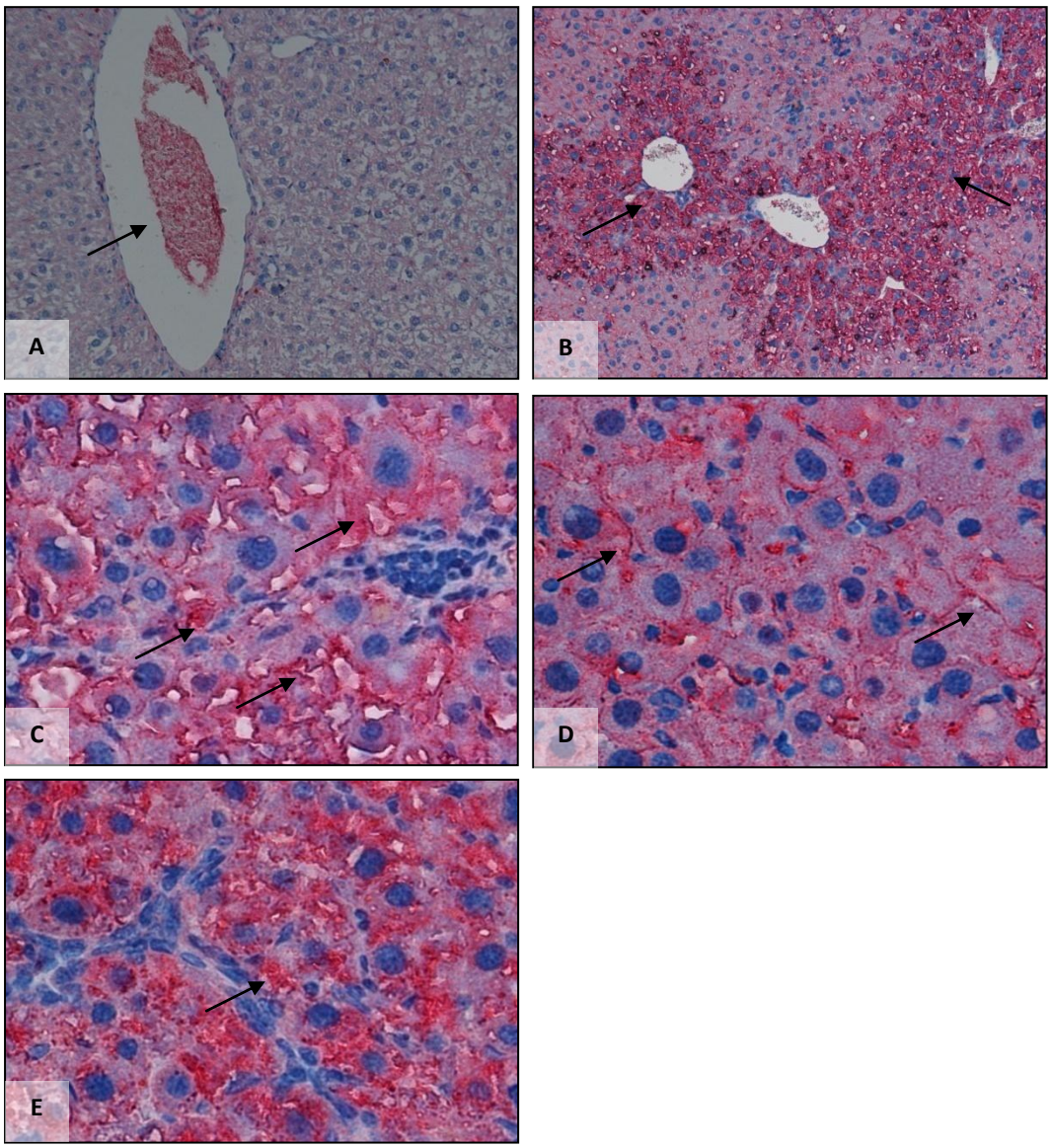


Figure 2-16: Fibrin staining in murine liver sections following CCL4 exposure.

Fibrin expression in sinusoids, perifibrotic hepatocytes and fibrotic septae.



A: Fibrin immunostaining in normal murine liver tissue, demonstrating intravascular clot (x4 objective).

B: Fibrin expressed in a fibrotic and perifibrotic tissue pattern at low power (x4 objective)

C: Fibrotic tissue: Positive staining in fibrotic septae, hepatic sinusoids and in cytoplasm of perifibrotic hepatocytes (x 20 objective)

D: Non fibrotic parenchymal tissue from CCL4 mice. Fibrin upregulated in hepatic sinusoids (x 20 objective)..

E: Fibrotic tissue: Heavy perifibrotic sinusoidal staining for fibrin (x20 objective).

Black arrows indicate positive staining

2.4.3.2.2. Fibrin expression in human HCV chronic liver injury

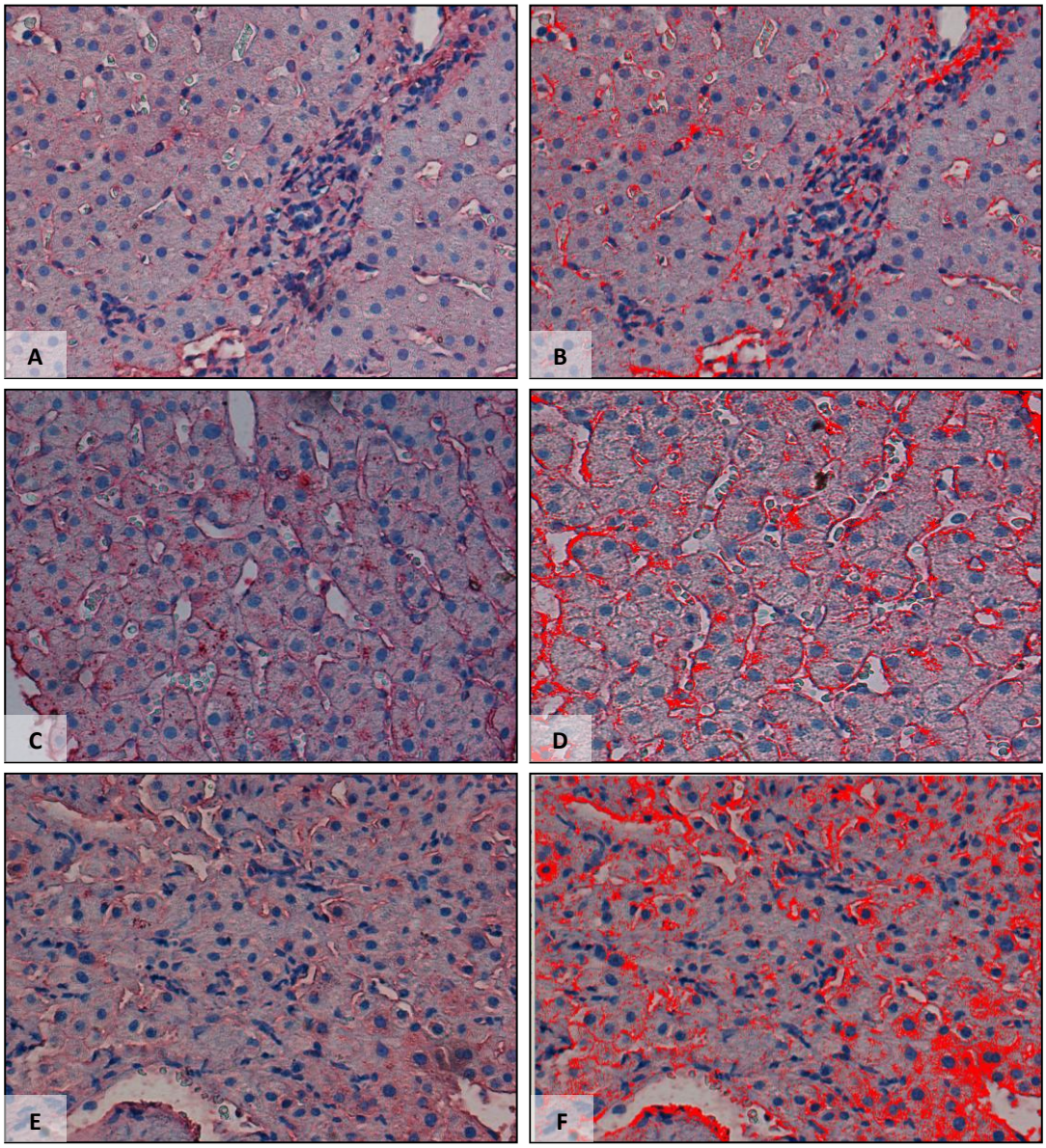
In keeping with the findings in murine fibrotic tissue, in human HCV infected liver tissue we demonstrated fibrin expression within hepatic sinusoids in both parenchymal non fibrotic areas and fibrotic areas (See Figure 2-17). There was a borderline significant correlation with grade of fibrosis (r=0.66, p=0.056) (see Table 2-8 and Figure 2-18). All negative control tissue failed to stain for fibrin as would be expected, which indicated there was no non specific staining.

Table 2-8: Fibrin staining in chronic human liver injury samples related to the degree of fibrosis. Measured by digital quantification. Values expressed as mean % of section stained for fibrin (SEM).

Group	Number	Mean % Fibrin stain per section (SEM)
Mild (Score 1-2)	2	4.08 (0.01)
Moderate (Score 3-4)	5	6.58 (1.96)
Severe (Score 5-6)	2	12.42 (2.21)

Figure 2-17: Fibrin staining in human HCV infected liver sections.

Fibrin expression in sinusoids and fibrotic septae with digitally enhanced images to highlight staining.



A, B: Fibrotic Area. Non enhanced image (A) and digitally enhanced image (B) isolating fibrin staining. Evidence of fibrin deposition within fibrotic septae (x 20 objective).

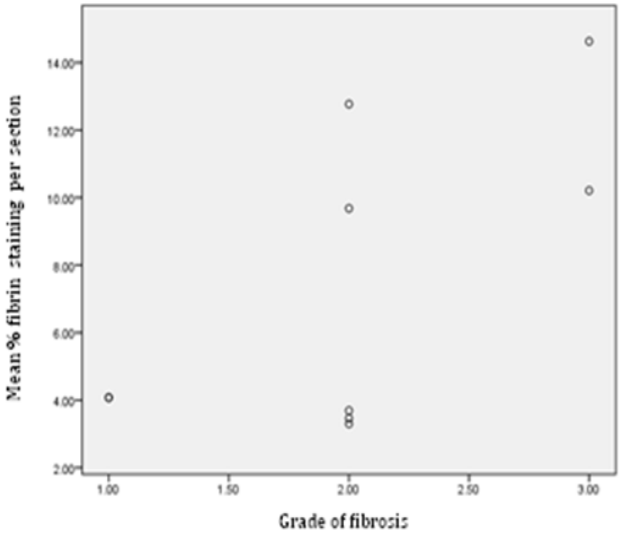
C, D: Non fibrotic area (fibrosis score 3). Non enhanced image (C) and digitally enhanced image isolating fibrin staining (D). Upregulation of sinusoidal fibrin expression in non fibrotic areas. (x20 objective).

E, F: Non fibrotic area (fibrosis score 5). Non enhanced image (E) and digitally enhanced image isolating fibrin staining (F). Increased sinusoidal fibrin expression in non fibrotic areas (x 20 objective).

Figure 2-18: Scatterplot graph showing digital quantification of fibrin staining in human HCV liver sections.

(Mean % fibrin staining per section) versus: the grade of fibrosis using the Ishak Modified Histological Activity Index (1=Mild, 2=Moderate, 3=Severe).

Regression analysis demonstrates a borderline significant correlation between fibrin staining and the fibrosis grade (r=0.66, p=0.056).



2.4.3.3. Tissue Factor

2.4.3.3.1. TF expression in murine chronic liver injury

TF was expressed in normal murine liver tissue sections. Positive staining was demonstrated in the hepatic sinusoids. No perivenular staining was seen (see Figure 2-19). TF was expressed in all the sections from mice with CCL4 induced fibrosis. Heavy TF staining was observed in both fibrotic and perifibrotic areas (see Figure 2-19). TF was staining was also observed in both hepatic sinusoids and fibrotic septae within fibrotic areas. Additionally sinusoidal areas stained for TF in non fibrotic areas. On performing digital image analysis there was a significant increase in TF expression within fibrotic murine liver tissue treated with CCL4 when compared to tissue from normal control mice (15.14% +/- 2.04 versus 2.04%+/- 0.25, p=0.002) (see Table 2-9; see Figure 2-19 & Figure 2-20).

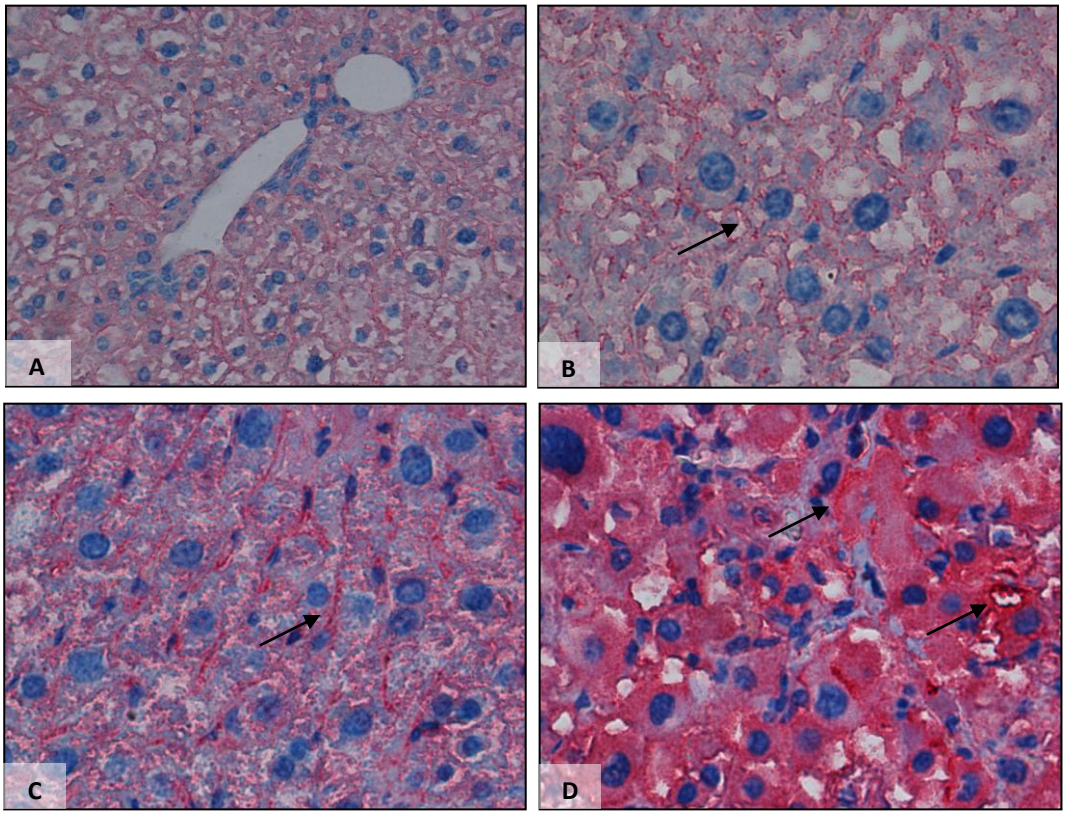
Table 2-9: TF staining measured by digital quantification in mice treated with CCL4 and normal mice.

Normal tissue (n=3) versus fibrotic murine liver tissue following exposure to 4 weeks of CCL4 (n=3). Values expressed as mean % of section stained for fibrin (SEM).

Group	Mean % TF stain per section	SEM
Control mice (normal)	2.04	0.25
CCL4 mice (fibrotic)	15.14	2.04

Figure 2-19: TF staining in fibrotic murine liver sections following CCL4 exposure.

TF expressed in sinusoids, perifibrotic hepatocytes and fibrotic septae.



A,B: TF immunostaining in normal murine liver tissue sections demonstrating weak sinusoidal staining (B, x 40 objective) but no perivenular staining (A, x20 objective).

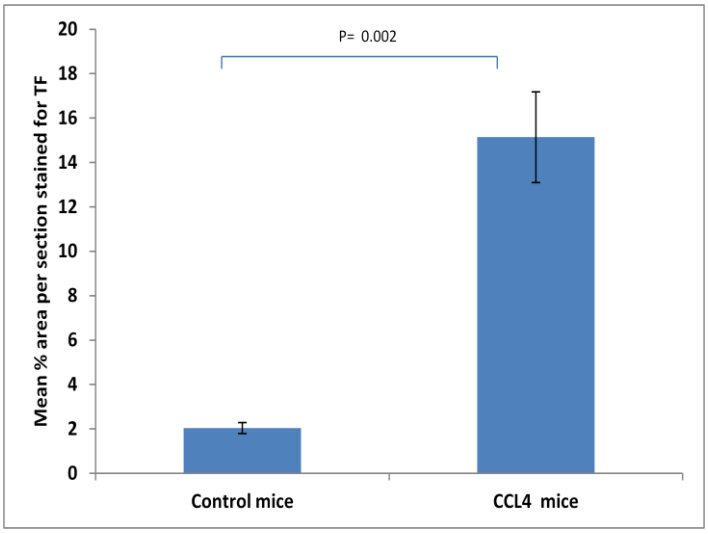
C,D: Patterns of TF immunostaining in liver tissue sections from mice treated with CCL4. Non-fibrotic areas (C) demonstrating up-regulation of sinusoidal TF in contrast to (B) normal liver tissue. Fibrotic areas (D) with heavy perifibrotic cytoplasmic and sinusoidal expression of TF (x40 objective)

Black arrows indicative positive staining

Figure 2-20: Graph showing digital quantification of TF staining in normal and fibrotic murine liver tissue sections.

Mean % per section stained for TF in normal and fibrotic murine liver tissue sections. Error bars represent standard error of mean

A significant increase in TF expression quantified by digitally in liver tissue from mice treated with CCL4 compared to normal control mice (p = 0.002).

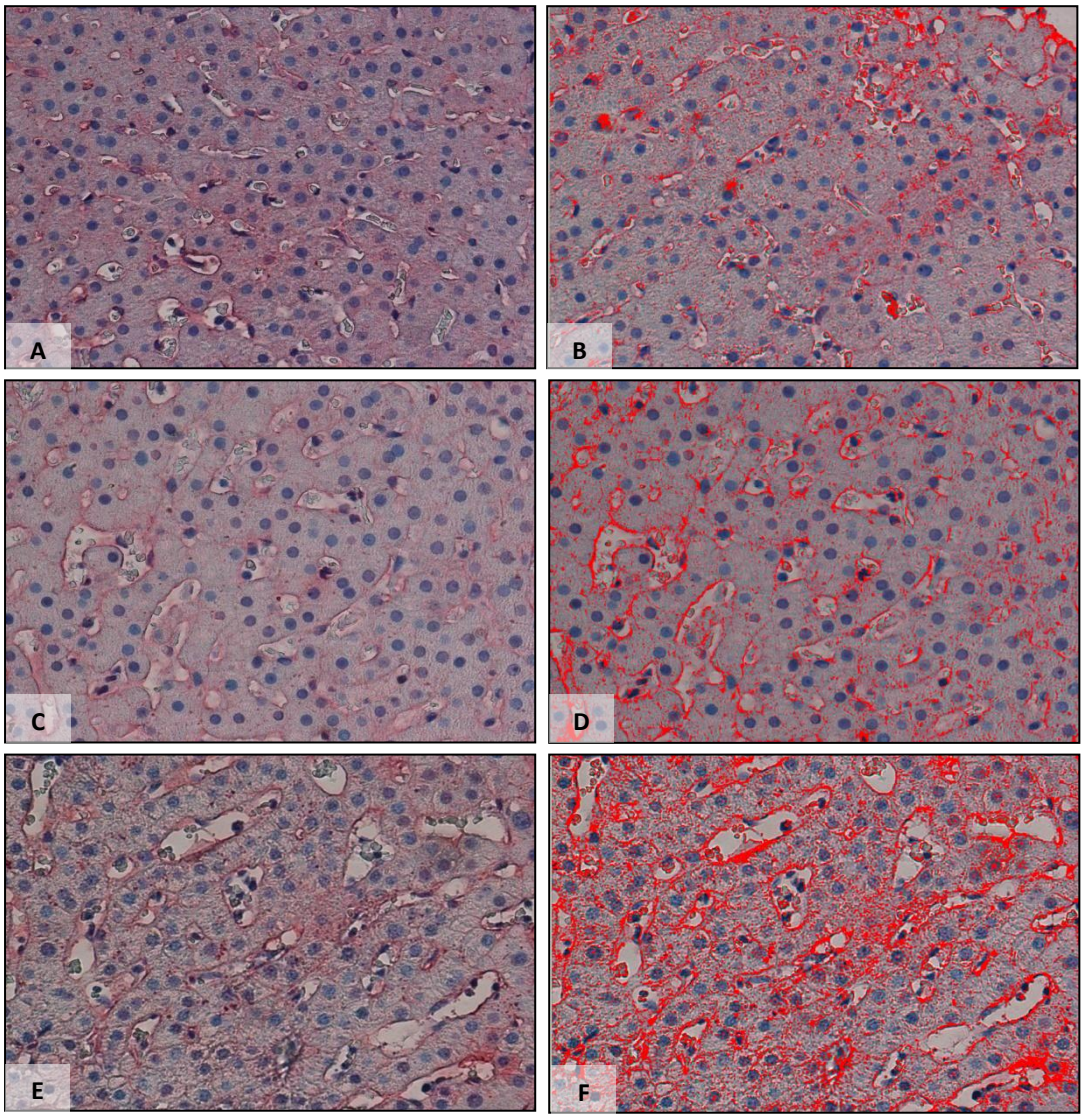


2.4.3.3.2. TF expression in human HCV chronic liver injury

In human HCV infected liver tissue a sinusoidal pattern of TF expression, with sinusoidal endothelial staining and weak staining of inflammatory cells was observed (see Figure 2-21). TF expression correlated with the degree of hepatic fibrosis (see Figure 2-22A) with regression analysis demonstrating a significant between correlation between TF expression and the grade of liver fibrosis (r=0.71; p=0.015) (see Table 2-10 and Figure 2-22A). Furthermore, there was a significant correlation between TF expression and the expression of fibrin in human fibrotic liver tissue (r=0.82, p=0.024) (see Figure 2-22B). All negative control tissue failed to stain, as would be expected, which demonstrated that staining was specific.

Figure 2-21: TF staining in human HCV infected liver sections.

Patterns of TF immunostaining varying with the degree of fibrosis in chronic human liver tissue sections.



A,B: Non enhanced image (A) and digitally enhanced image (B) isolating TF staining. Sinusoidal expression of TF in section with mild degree of fibrosis (fibrosis score 1; inflammation score 3).

C,D: Non enhanced image (C) and digitally enhanced image isolating TF staining (D). Up-regulation of sinusoidal TF expression in section with moderate fibrosis (fibrosis score 3, inflammation score 3). (x 20 objective).

E, F: Non enhanced image (E) and digitally enhanced image isolating fibrin staining (F). Increased sinusoidal TF expression in section with severe fibrosis (fibrosis score 5). (x 20 objective).

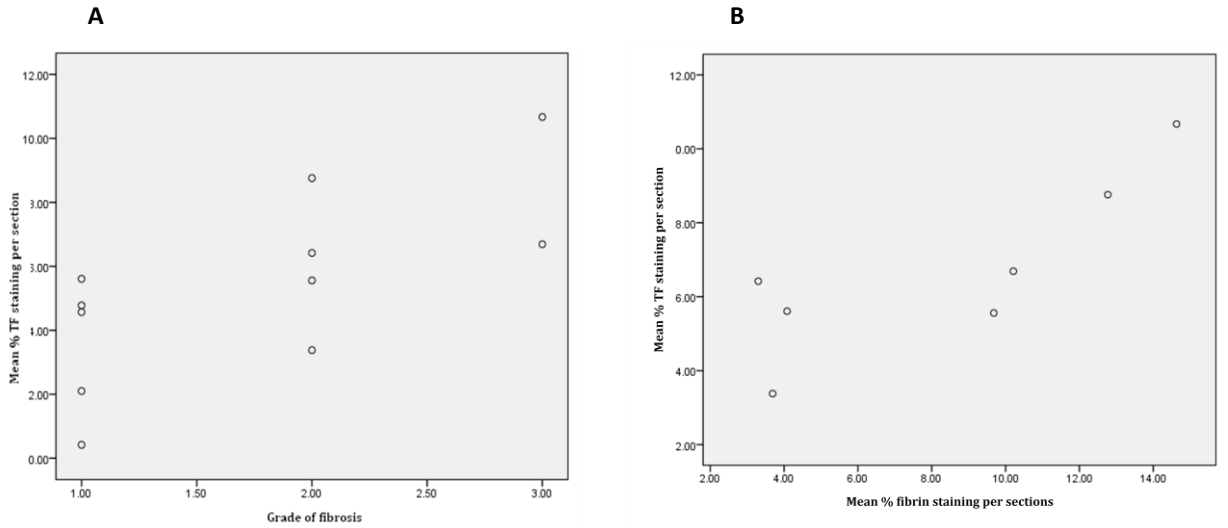
Table 2-10: TF staining in fibrotic human liver samples related to the degree of fibrosis.

Measured by digital quantification. Values expressed as mean % of section stained for fibrin (SEM).

Group	Number	Mean % TF stain per section (SEM)
Mild (Score 1-2)	5	3.50 (0.97)
Moderate (Score 3-4)	4	6.03 (1.11)
Severe (Score 5-6)	2	8.68 (1.99)

Figure 2-22: Scatterplot graphs showing digital quantification of TF staining in human HCV liver sections.

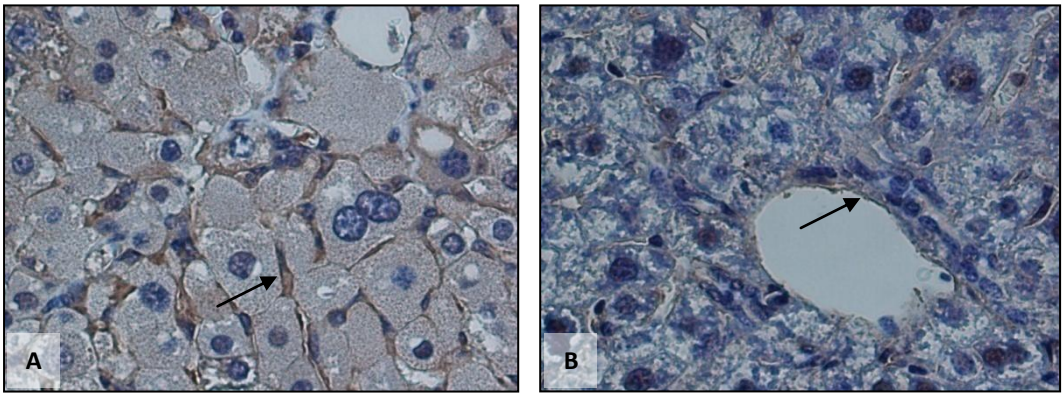
(Mean % TF staining per section) **A.** versus the grade of fibrosis using the Ishak Modified Histological Activity Index. Regression analysis demonstrates a strong correlation between TF staining and the fibrosis grade (r=0.71, p=0.015); **B**. versus fibrin staining (Mean % fibrin staining per section). Regression analysis demonstrates a significant correlation between TF and fibrin staining (r=0.82, p=0.024).



2.4.3.4. Thrombomodulin

Thrombomodulin was expressed within hepatic sinusoids in liver tissue from mice treated with CCL4. Furthermore, we showed minimal TM expression in perifibrotic and perivenular areas, in contrast to heavy staining seen with TF and fibrin (see Figure 2-23).

Figure 2-23: Pattern of TM immunostaining in chronic murine liver tissue section.



A. Sinusoidal staining pattern (x20 objective).

B. Sinusoidal staining pattern, minimal central vessel staining (x20 objective).

Black arrows indicative positive staining

2.5. DISCUSSION

2.5.1. Acute Liver Injury

Paracetamol toxicity is a common cause, in humans, of acute hepatic failure (Ostapowicz *et al.,* 2002). Numerous factors contribute to paracetamol induced injury including microchrondrial alterations, reactive oxygen and nitrogen species, and cytokines (James *et al.,* 2003; Cover *et al.,* 2005; Chiu *et al.,* 2003; Gardner *et al.,* 2002; Hinson *et al.,* 2004; Jaeschke *et al.,* 2004) resulting in hepatocellular centrilobular necrosis. Despite extensive research, the mechanisms involved in the process are not fully understood including the role of coagulation proteins. Coagulopathy may become evident in humans 24 to 72 hours after ingestion of paracetamol, including elevation of the PT. Haematological abnormalities occur partly since the liver is responsible for the synthesis of coagulation factors, and a reduction in both procoagulant factors, with the exception of factor VIII and vWF, and anticoagulants including protein C and protein S occurs (Lisman *et al.,* 2010). Evidence is now emerging which suggests increased consumption caused by activation of the coagulation cascade, may contribute to this coagulopathy. A recent study has demonstrated the consumption of coagulation proteins peripherally, with plasma TF and TAT complexes being elevated in patients with acute liver injury (Kerr, 2003). The extent of consumption or activation of coagulation factors centrally within the liver in relation to ALF is much less defined. I sought to examine this by using immunohistochemical staining techniques to quantify and qualify the expression of coagulation proteins in both mouse and human liver tissue following paracetamol induced acute injury.

2.5.1.1 The role of Fibrin and TF in acute liver injury

Using a murine model of paracetamol liver injury we have demonstrated a significant correlation between fibrin deposition and severity of acute liver injury, graded using a novel scoring system based on extent of necrosis (see Figure 2-3 and 2-4). Moreover we have demonstrated that the fibrin deposition is heavily co-localized to necrotic areas within the liver in these mice (see Figure 2-5), and confirmed this finding in human liver sections from livers extracted at the time of transplantation following acute failure induced by paracetamol (see Figure 2-6). A recent study has demonstrated fibrin and or fibrinogen staining within necrotic areas of livers from mice with an acute liver injury (Ganey *et al.,* 2007). Our data not only also supports these findings, but more specifically confirms the substantial expression of fibrin within these necrotic areas. Expression of TF was demonstrated within hepatic sinusoids in both normal tissue and viable areas of acutely injured tissue, with heavy centrilobular staining in necrotic areas of injured tissue (see Figure 2-9). A significant correlation was demonstrated between centrilobular expression of TF, grade of injury and fibrin deposition, in mice (see Figure 2-10). Up-regulation of TF with fibrin in a centrilobular pattern of expression suggest that centrilobular necrosis, the primary injury in acute liver failure induced by paracetamol, is associated with activation of the coagulation cascade and this activation is initiated by TF. This is supported by data obtained from studies in patients with acute liver failure induced by paracetamol, showing a significant reduction in serum levels of factors VII, IX, V, X and II, which are factors that are consumed as a result of TF driven activation of the coagulation cascade (Kerr, 2003). Furthermore intrahepatic activation of the coagulation cascade results in hepatic consumption of these coagulation factors, which combined with peripheral consumption, will contribute to the coagulopathy we observe in acute liver injury secondary to paracetamol.

The significant correlation between both TF expression and fibrin deposition with the grade of severity of acute liver injury, in conjunction with the co-expression of HIF-1a, a marker of tissue hypoxia, could suggest a role for these proteins in the pathogenesis of acute liver injury via microcirculatory dysfunction and ischaemia. Evidence that damage occurs to sinusoidal endothelial cells in paracetamol toxicity in both mice and humans could be central to the mechanism by which TF is released and activated in this setting. (Ito *et al*., 2003; McCuskey., *et al* 2005; DeLeve *et al*., 1997; Williams *et al*., 2003). Production of proinflammatory cytokines in the liver including TNF have been demonstrated in both animal models (Dambach, 2006) and human patients with paracetamol poisoning (Kerr, 2003). TNF induces the expression of TF and PAI-1 in cultured endothelial cells, both of which would promote thrombosis within the liver (Ganey *et al.,* 2007). A potential mechanistic pathway explaining the up-regulation of TF in paracetamol induced liver injury can be postulated. Proinflammatory cytokines cause up-regulation of TF from sinusoidal endothelial cells, which results in activation of the coagulation cascade, hepatic consumption of coagulation factors and deposition of fibrin in the microvasculature with tissue hypoxia and up-regulation of HIF-1a, driving ischaemia and necrosis. Therefore considerable intrahepatic activation of the coagulation cascade, could not only contribute to the coagulopathy of acute liver failure, it could have an important role in the initiation and propagation of liver injury in this setting (Lisman *et al.,* 2010).

The demonstration of fibrin and TF expression does not obviously prove causality. The results from our small time course study suggest that both fibrin deposition and HIF-1a expression are early events in the development of acute injury in mice since they are expressed within 2 hours following induction with paracetamol. (see Figure 2-14). This is before confluent necrosis occurs. It is however feasible that following TF release and

activation of the cascade, the resulting substantial deposition of fibrin partly results from a failure of clearance. Recent studies of macrophage function during acute liver injury demonstrate that macrophage function is not impaired (Antoniades *et al., in press*). We have demonstrated colocalization of fibrin with CD68, in human acute liver injury tissue (see Figure 2-7), which would indicate fibrin is being cleared in part by macrophages. Few studies have investigated fibrinolysis in the context of acute liver injury, and it is not clear if it is truly impaired. Evidence suggests most fibrinolytic proteins are reduced in this setting, with the exception of plasminogen activator inhibitor 1 (PAI-I) (Pernambuco *et al.,* 1993). If this is proven to be the case, fibrin deposition, even in the context of a failure of fibrinolysis, would still be of importance, since the resulting microcirulatory dysfunction, tissue hypoxia, and ischaemia would remain a consequence. These questions are of significance and require further study, since it is likely these mechanisms are involved in the pathogenesis and propagation of this type of injury. Importantly clarifying exactly at which stage coagulation may contribute to injury is of relevance when considering the optimal timing of any targeted inhibition as a therapeutic intervention. Further studies, including studies looking at very early timepoints of acute liver injury in mice are therefore still required.

2.5.1.2 Role of Thrombospondin and platelet aggregation in acute liver injury

Our data demonstrated TSP-1 was up-regulated in a murine model of acute paracetamol injury, and localized to areas of necrosis (see Figure 2-11). Furthermore we have demonstrated a correlation between grade of injury, classified by extent of tissue necrosis with colocalization of TSP-1, fibrin and necrosis (see Figure 2-11 & 2-12). These findings have been confirmed in human acute liver injury with heavy staining of TSP-1 in explanted liver from patients with acute liver failure secondary to paracetamol overdose, again in a similar distribution to fibrin deposition (See Figure 2-13).

TSP-1 is an extracellular glycoprotein released by thrombin activated platelets and inflammatory cells and is an important mediator of platelet aggregation (Bornstein *et al.,* 2001; Isenberg *et al.,* 2008). Upregulation of TSP-1 in both murine and human acute liver injury tissue within necrotic areas, is indicative of thrombin mediated platelet activation and will result in platelet aggregation. Its expression in necrotic tissue not only confirms the presence of coagulants, such as thrombin, on which its expression is dependent, but is in keeping with other studies, which have suggested increased platelet activation in this setting (Hughes *et al.,*1985). Since fibrin is heavily expressed in necrotic areas, it is not surprising that thrombin mediated platelet activation and aggregation, via TSP-1 up-regulation would be present. Furthermore since platelet aggregation is known to contribute to both tissue ischaemia via occlusion of vasculature, and to thrombus formation with fibrin deposition (Chung *et al.,* 1997; Isenberg *et al.,* 2008), thrombin mediated platelet aggregation in this setting in synergy with fibrin deposition could an important mediator for propagating tissue injury via microvascular occlusion and dysfunction.

2.5.1.3 Applicability of this data to clinical practice

It is well established that acute liver injury leads to changes in haemostasis including prolongation of the prothrombin time and thrombocytopenia, which has lead to a perceived risk of bleeding (Lisman *et al.,* 2010). Clinically clotting times, in particular the international normalized ratio (INR) and prothrombin time have been used as markers of hepatic function and prognosis in acute liver failure. The data presented above suggests that centrilobular expression of TF, fibrin deposition and up-regulation of TSP-1, a marker of thrombin mediated platelet aggregation correlate with extent of injury. Prolongation of clotting times, historically have been seen as an indicator of hepatic dysfunction and reduced intrahepatic

synthesis of coagulation factors. In acute liver injury we have demonstrated, in keeping with other studies (Ganey *et al.,* 2007), that there is substantial intrahepatic activation of the coagulation cascade, with heavy fibrin deposition in areas of necrotic injury. Our data suggests there is therefore substantial consumption of factors within in the liver, which as described above could also have a direct role in contributing to injury. We can postulate that coagulopathy is secondary to both consumption and reduced synthesis of factors, and to some extent a prothrombotic state, all be it most likely at a intrahepatic microvascular level, arises during acute liver injury. These findings have implications towards understanding the nature of coagulopathy in acute liver failure, and its management in defining bleeding risk in these patients. Recent studies clearly demonstrated in chronic liver disease that contrary to previous views and prolongation of clotting times, which can be poor markers for bleeding risk, the haemostatic balance is preserved and can even favour a prothrombotic state (Tripodi *et al.,* 2005). Few studies have explored the true haemostatic balance in acute liver failure. A single case report has suggested that acute liver injury induced paracetamol was associated with a prothrombotic state (Moulakahis *et al.,* 2007). Further studies are needed not only to define the true thrombotic risk and bleeding risk, but to determine whether targeting the coagulation cascade as a potential therapeutic intervention to prevent or treat acute liver injury, would be feasible. To date two animal studies have explored this, showing a prophylactic benefit by reducing hepatocellular damage in acute liver injury induced in mice with heparin (Ganey *et al.,* 2007), and in rats with antithrombin III (Fujiwara *et al.,* 1988). These findings have not been adequately taken forward and studied in human based studies as yet, and this needs to be carefully considered once we have a better understanding of the true haemostatic balances in acute liver injury.

A further potential clinico-pathological correlation for our data is suggested by recent evidence which demonstrates admission platelet counts may be an independent predictor of poor prognosis in patients with acute liver failure secondary to paracetamol overdose. A platelet count of less than 79 $x10^9$/mm on admission was found to be related to a significantly worse prognosis (Macphail *et al.,* 2009). Thrombocytopenia in acute liver injury secondary to paracetamol is common, and is often multi-factorial, including a direct toxic effect of paracetamol on the bone marrow, and disseminated intravascular coagulation (Schiødt *et al*., 2003). Platelet aggregation or consumption can contribute to this. Upregulation of TSP-1, which we have demonstrated, will contribute to ongoing platelet aggregation, and as described above theoretically to ischaemia, necrosis, and hepatic dysfunction. One study using prostacyclin has examined the effects of inhibiting platelet aggregation in a murine model of acute liver injury induced by paracetamol, showing beneficial effects on liver necrosis and mortality (Guarner *et al*., 1988). No similar studies have been replicated in human subjects to date, and the inhibition of platelet aggregation as a potential therapy to improve survival needs to be considered.

2.5.2. Chronic Liver Injury

The majority of liver disease results from chronic inflammation which drives liver fibrosis. Liver fibrosis represents the uniform response of the liver to toxic, infectious, and metabolic agents and is characterized by an increased synthesis and altered deposition of extracellular matrix components (Friedman, 2008) and is a complex dynamic process, involving the recruitment and activation of platelets, inflammatory cells, hepatic stellate cells and other extracellular matrix producing cells, (Calvaruso *et al*., 2008; Beaussier *et al*., 2007; Forbes *et al*., 2004). Recent evidence suggests hepatic fibrogenesis is associated with prothrombotic tendencies (Tripodi *et al.,* 2005), and activation of the coagulation system either through microthrombi deposition and the theory of parenchymal extinction (Wanless *et al.,* 1995) or the pro-fibrotic properties of thrombin, contributes to liver fibrosis. To examine these concepts further, we sought to demonstrate the intrahepatic expression of coagulation proteins using an immunohistochemically based study, in the setting of both murine CCL4 induced liver fibrosis, and human hepatic fibrosis secondary to chronic hepatitis C virus infection.

2.5.2.1. Coagulation proteins in chronic liver injury: evidence of a local procoagulant state

In murine and human chronic injury liver tissue, we have demonstrated expression of TF in the both hepatic sinusoids and fibrotic septae (see Figures 2-19 & 2-21). We have demonstrated a significant up-regulation of TF in livers of mice treated with CCL4 to induce fibrosis versus normal control mice (see Figure 2-20), and using digital analysis shown a significant correlation between the TF expression and severity of fibrosis in human HCV

fibrosis (see Figures 2-21 & 2-22). Similarly staining for fibrin demonstrated significant up-regulation within hepatic sinusoids, perifibrotic and fibrotic areas in mice treated with CCL4 (see Figures 2-15 & 2-16). Digital image analysis demonstrated a significant correlation between fibrin deposition and TF expression (see Figure 2-22), and a borderline significant relationship between fibrin expression and severity of fibrosis in human liver tissue infected with HCV (see Figures 2-17 & 2-18). TM was only weakly expressed in hepatic sinusoids, and perifibrotic areas in mice, which was in contrast to the heavier expression seen with TF and fibrin in these areas (see Figure 2-23).

Only one previous study, by Rullier et al., has investigated the expression of TF in the fibrotic livers. They found a predominantly hepatocytic expression of TF in both normal and cirrhotic human liver (Rullier *et al.,* 2006). Our findings in contrast demonstrate a predominantly endothelial expression of TF, with positive staining in sinusoids and blood vessels. This would be more in keeping with the basic principles of the function of TF and consistent with TF expression seen in non-liver tissue (Nakasaki *et al.,* 2002; Shoji *et al*., 1998; Rullier *et al.,* 2006). Moreover, the samples classed as normal liver tissue by Rullier were taken at a distance from benign liver tumours and cancer metastasis. This may have confounded what they assumed to be normal staining patterns since malignancy has been shown to up-regulate TF expression (Shigemori *et al.,* 1998; Ohta *et al.,* 2002; Wojtukiewicz *et al.,* 2001). Our data demonstrates for the first time a correlation between TF expression and severity of fibrotic injury in the liver, with a both a significant difference between normal versus fibrotic murine tissue and between different grades of fibrosis human tissue. There was no significant difference in inflammatory scores in the sections stained, with all sections graded as having minimal to mild inflammation, irrespective of fibrosis score. Since all

sections had a degree of inflammation this would account for why TF expression was demonstrated in all sections.

The significant correlation between fibrin and TF in human tissue, and up-regulation of fibrin, in a similar fashion to TF in mice supports TF driven activation of the coagulation cascade in a similar fashion. These findings would be would be in keeping with our current understanding of fibrosis and TF expression. Fibrosis is driven by inflammation (Friedman 2008), and inflammation is known to cause up-regulation of intravascular TF (Esmon *et al.,* 2004). TF up-regulation in fibrotic tissue is therefore likely to be driven initially by inflammation, and would result in activation of the coagulation cascade, and thrombin and fibrin production.

Demonstrating fibrin in fibrotic tissue is not unique to this study. Neubauer et al. demonstrated fibrin deposition within the fibrous septa in long-term liver damage in a rat model of CCl_4 (Neubauer *et al.,* 1995) and our findings in a mouse model of CCl_4 concur. However no previous studies have attempted to demonstrate fibrin deposition immunohistochemically in human fibrotic liver tissue, and our findings confirm intrahepatic activation of the coagulation cascade in this setting. The fact that fibrin staining only had a borderline correlation with severity of fibrosis most likely relates to our small sample size; increasing sample numbers would maximise the effect and potentially demonstrate a significant correlation.

In contrast to TF and fibrin staining, TM was only weakly expressed in the hepatic sinusoids and in the central blood vessels in mice (see Figure2.23). Its sinusoidal expression conforms to TM being an endothelial receptor for protein C (Moore *et al.,* 1987; Fukudome *et al.,* 1994) and is consistent with one other study that has demonstrated TM expression in

sinusoidal endothelial cells following chronic liver injury (Zeniya *et al.,* 1995). TM, in complex with protein C, degrades factor Va and VIIIa, resulting in decreased generation of thrombin (Esmon *et al.,* 2004; Dittman *et al.,* 1990). Even though our findings indicate some up-regulation of TM locally within the liver following chronic liver injury, it is likely that the presence of TM in this setting fails to inhibit thrombosis, since fibrin is also co-expressed in these areas and with more intensity. This is probably due to the fact that thrombin and other procoagulants are activated to a higher degree than TM, which is known to be down regulated even by mild inflammation.

Importantly these findings not only add further support to the recent concept that advanced hepatic fibrosis is a procoagulant state (Tripodi *et al.,* 2005; Søgaard *et al.,* 2009), but indicate that even in early or milder fibrotic states some activation of the coagulation cascade occurs. This local activation of coagulants within the liver may also be an important mechanism in driving fibrogenesis, which would explain the correlations between severity of fibrosis and expression of coagulants that we demonstrated

2.5.2.2. A role for coagulation proteins in hepatic fibrosis

It is evident from the above data that activation of the coagulation system occurs not only in fibrosis induced by CCL4 in mice, but in fibrosis secondary to HCV in human. In conjunction with previous studies it adds further weight to the growing body of evidence that suggests a role for coagulation proteins in the pathogenesis of liver fibrosis.

Two mechanisms have been postulated as possible mechanisms by which activation of the coagulation system results in generation of hepatic fibrosis. The first was described by

Wanless *et al,* (1995), is termed parenchymal extinction, and is based on a hypothesis for liver tissue remodelling and fibrosis, in which microthrombi deposition is central. They demonstrated microthrombi within branches of the hepatic vein and portal vein in cirrhotic livers from a range of pathologies including HCV. Based on these observations they proposed that hepatic necroinflammation causes local intimal injury and occlusive thrombi generation within branches of the hepatic and portal vein, which leads to sinusoidal congestion and tissue ischaemia. This in turn leads to hepatocyte death, collapsing the region between the central veins and their adjacent portal tracts. The collapsed tissue would be replaced by fibrous tissue. They postulated that cirrhosis results from multiple areas of parenchymal extinction becoming confluent (Wanless *et a*l., 1995). Similarly in Budd-Chiari syndrome, the underlying occlusion of the hepatic vein by thrombus is well recognised to result in hepatic congestion and subsequent hepatic fibrosis (Tanaka *et al*., 1998). Our findings lend some support to the hypothesis formulated by Wanless and colleagues. We have demonstrated fibrin deposition at a microvascular level, involving hepatic sinusoids, which would support their observation of microthrombi generation. What cannot be inferred is the pathological significance of fibrin generation with regards to fibrosis, and whether it contributes to injury or is just a by-product of activation of the coagulation cascade following production of thrombin. More recently an alternative pathway, promoting the pro-fibrotic effects of coagulation proteins independent of their pro-coagulant action, in part via PAR receptor ligation, has been suggested. Thrombin is best known for the proteolytic cleavage of fibrinogen to form fibrin (Coughlin 2000), but it has recently been demonstrated to activate hepatic stellate cell, the principal cell involved in hepatic fibrosis. The cellular actions of thrombin are in part mediated by PAR signalling (Rullier *et al*., 2007). Hepatic stellate cells have been shown to express PAR1, and a substantial body of evidence from in vivo and in vitro studies is now accumulating to suggest PAR-1 activation leads to

stellate cell activation and hepatic fibrogenesis (Fiorucci *et al*., 2004; Marra *et al*., 1998; Gaca *et a*l 2002). The advent of studies demonstrating the role of thrombin via PAR-1 activation, has questioned the significance of microemboli and the role they play in generation of liver fibrosis. In reality both mechanisms are not likely mutually exclusive (Anstee *et al.,* 2009) and ultimately, whether the presence of fibrin contributes to fibrosis or not, our findings do indicate activation of the coagulation cascade in liver fibrosis, which supports evidence for fibrosis being a prothrombotic condition at an intrahepatic level, with generation of thrombin, which is a known mediator of stellate cells. A number of epidemiological studies have shown that prothrombotic conditions promote liver fibrosis (Wright *et al.,* 2003; Papatheodoridis *et al.,* 2003) and anticoagulants may slow fibrosis in animal models (Anstee *et al.,* 2008; Assey *et al.,* 2007). Therefore, if we take our findings in conjunction with these previous studies, it is highly probable that the activation of coagulation that we have demonstrated also contributes to the pathogenesis of the injury.

2.5.3. Limitations of the study

The main limitation of the study relates to the quantification of the coagulation proteins in the liver samples. The quantification of TF, fibrin and TSP-1 is semi-automated and therefore not fully objective. Since the observer determines for the programme what to classify as positive staining, there is still a degree of subjectivity. However, since observers were analyzing all sections in a blinded manner, any perceived error would occur in all sections and be minimized. Quantification is reliant on intensity of staining. Intensity of staining can be related not only to its protein expression, but also variations in incubation times, technique used, counterstaining and tissue section thickness. These effects were minimized by strict adherence to staining protocols for all runs.

As with any immunohistochemical study contribution of non-specific binding can confound the true intensity of staining. Initially, staining produced a degree of non-specific binding in the human liver sections. This was rectified by omitting the blocking reagents in these sections. Additionally, negative control tissue did not stain and positive control tissue stained for coagulation proteins in the patterns expected. This confirmed that the staining patterns seen were of a true nature and not due to non-specific binding.

Another limitation in our study is the small sample size. Despite small sample sizes the results were significant, with the exception of fibrin expression and grade of fibrosis in human HCV tissue. Future studies with larger sample sizes are required to validate our results.

A further limitation of these findings relates to proving causality. The demonstration of the expression of different coagulation proteins in acute and chronic liver injury tissue provides support for the hypothesis that coagulation proteins are upregulated in both acute and chronic liver injury. However, this does not definitively prove a causal relationship. As described above if we take our findings in conjunction with the growing body of evidence suggesting a role for coagulation proteins in liver injury, it is highly probable that both paracetamol acute liver injury and HCV related chronic liver injuries not only involve activation of the coagulation cascade, but this activation contributes to injury. Further studies evaluating the expression of these proteins in chronic liver disease secondary to other aetiologies would be required to see if these findings are not unique to HCV chronic liver injury.

2.5.4. Conclusions

The results generated by this study provide strong support for intrahepatic activation of the coagulation cascade in both acute liver injury secondary to paracetamol in mice and humans, and chronic liver injury induced by CCL4 in mice, or related to HCV infection in humans.

This activation is not only likely to contribute to the respective haemostatic imbalances in each type of injury, but in conjunction with existing evidence suggests activation of the coagulation cascade contributes to the pathophysiological mechanisms involved in generating these injuries. A strong case can be made for needing to explore these mechanisms further, including novel therapeutic strategies modulating the coagulation system using animal models, and considering the therapeutic benefit of anticoagulation in clinical trials of human liver injury.

3. MODELS OF FIBROGENESIS AND THERAPEUTICS: THE ROLE OF THE COAGULATION SYSTEM

3.1. INTRODUCTION

Hepatic fibrosis is a complex dynamic process, in which there is an interaction between genetic and environmental factors (Anstee *et al.,* 2008). In vitro and vivo models of hepatic fibrogenesis permit researchers to explore and manipulate these pathophysiological mechanisms and to test potential anti-fibrotic therapies. Hepatic stellate cell culture lines allow us to investigate mechanisms of action and the direct effects of potential therapies on the principal cell type in hepatic fibrogenesis. In contrast, murine models in environmentally or genetically controlled conditions, allow us to investigate the pathogenesis of liver fibrosis in intact organisms whose physiological and pathological responses mimic those of humans. In conjunction, both techniques are vital in proving the potential viability of novel antifibrotic therapies prior to testing in the setting of a human clinical trial.

3.1.1. The role of coagulation in the pathogenesis of liver fibrosis

The role of the coagulation system in liver fibrosis is now becoming well established. We have demonstrated, using immunohistochemical techniques up-regulation of TF and fibrin intra-hepatically in liver fibrosis in both mice and humans (See Chapter 2). The theories of parenchymal extinction and microemboli deposition rely on the activation of the coagulation system (Wanless *et al.,* 1995). There has been a recent interest in the role of individual coagulation proteins, especially thrombin, in fibrogenesis. Activation of the coagulation cascade generates thrombin from its prescursor prothrombin. In addition to its role in activating fibrinogen, thrombin mediates cellular activation of macrophages, platelets and

hepatic stellate cells via the protease-activated receptor, PAR-1 (Rullier *et al.,* 2007; Friedman 2003). Conversely, experimental thrombin antagonists have previously demonstrated some anti-fibrotic properties in animal models of liver fibrosis (Duplantier *et al.,* 2004).

Factor Xa (FXa), a protease which is activated upstream of thrombin in the coagulation cascade, has recently been shown to promote connective tissue growth factor, and activate lung fibroblasts via PAR receptors (Chambers *et al.,* 2000; Scrotton *et al.,* 2009). To date, however, no study has examined the effects of FXa on hepatic stellate cells. In lung fibrosis, a paradigm for hepatic fibrosis, direct inhibition with an experimental FXa inhibitor has recently been shown to reduce fibrosis significantly in a bleomycin mouse model (Scrotton *et al.,* 2009). An understanding of the effect of FXa on hepatic stellate cells and fibrogenesis, above and beyond its role in generating thrombin is therefore needed, and the effects of direct FXa inhibition in the setting of hepatic fibrosis needs to be evaluated to assess whether it would offer additional efficacy as an antifibrotic over direct thrombin inhibition alone.

3.1.3. Novel anticoagulants

Until very recently, anticoagulants available for clinical use have included vitamin K antagonists including warfarin; heparins including low molecular weight heparins; and more recently parenterally administered direct thrombin inhibitors. Although these drugs have been proven to be very effective anticoagulants, they have some drawbacks that limit their usage (Turpie, 2007). Warfarin is administered orally but has to be monitored regularly due to a narrow therapeutic window, inter and intra-individual variability, a slow onset of action,

and food and drug interactions. Monitoring can be inconvenient to patients and has added cost implications (Turpie 2007). Heparins have a more rapid onset of action, but unfortunately have to be given parenterally, which is often performed by a healthcare professional if the patient is unwilling. Long term use of heparins can be associated with osteopenia, thrombocytopenia and idiopathic hepatitis (Ansell *et al.,* 2011). The need to develop novel anticoagulants with better ease of use and safer side effect profiles has led to the recent development of several newer anticoagulants which are now commercially available. Unlike previous anticoagulants, they inhibit single coagulation proteins such as activated factor X or thrombin (Perzborn *et al.,* 2005).

3.1.3.1. Direct FXa inhibitors

Coagulation FX is a vitamin K dependent plasma protein that occupies a crucial position at the convergence point between the intrinsic and extrinsic pathways of the coagulation cascade. Activated FX (FXa) is vital in converting prothrombin to thrombin. Indeed one molecule of FXa can generate more than 1000 thrombin molecules or a 'thrombin burst' (Mann et al., 2003). Clinically, severe and often life threatening bleeding can occur in patients with inherited FX deficiencies, which highlights the importance of its role in clot formation. FXa is therefore seen as a logical therapeutic target to prevent thromboembolic disease (Perzborn *et al.,* 2005). Recent evidence also suggests that FXa is involved in a variety of non haemostatic disease mechanisms. These include fibrogenesis, as detailed above as well as tissue remodelling, angiogenesis and tumour metastasis (Perzborn *et al.,* 2005). Accordingly, there has been a significant drive to developing viable oral FXa inhibitors, in view of their potential benefits.

3.1.3.1.1. Rivaroxaban

Rivaroxaban (Bayer Heathcare, Germany) is a novel oral direct FXa inhibitor that has been recently developed. It selectively inhibits both free FXa and FXa prothrombinase activity, resulting in vivo and vitro inhibition of thrombin generation, prolongation of clotting times and potent antithrombotic effects (Biemond *et al.,* 2007). It is now licensed in the European Union for the prevention of thromboembolic disorders, following hip or knee surgery, having been demonstrated to be superior to low molecular weight heparins (LMWH) in reducing thromboembolic events with no increase in the frequency of bleeding events (Turpie *et al.,* 2009). It is currently administered at a dose of 10mg per day orally in adults for a period of 35 days post surgery. Common side effects include bleeding and hence it is theoretically contra-indicated in patients who have a significantly increased risk of bleeding. It requires no dose monitoring nor loading regimen. Studies have demonstrated a high degree of similarity in bioavailabilty and metabolism of this drug in human, dogs and rodents with good correlations between plasma concentration, inhibition of FXa activity and prolongation of PT (Weinz *et al.,* 2009). In addition to prevention of thromboembolic disease, Rivaroxaban has been successfully evaluated in a phase II study for the treatment of acute thromboembolic disease, using daily doses up to 60mg per day for a maximum 3 month period (Agnelli *et al.,* 2006; Buller *et al.,* 2006). Longer periods of use are currently being evaluated and results are awaited, to ensure it is safe to use as a lifelong anticoagulant in humans.

3.1.3.2. Direct thrombin antagonists

Thrombin is produced by the proteolytic cleavage of prothrombin, and is a multifunctional serine protease as detailed previously (see section 1.2). Synthetic small molecule direct thrombin inhibitors have been recently developed as a new therapeutic class of drugs that offer an alternative to traditional anticoagulants, inactivating both fibrin bound and free thrombin (Wienen *et al.,* 2007).

3.1.3.2.1. Dabigatran

Dabigatran (Boehringer Ingelheim Pharma, Germany) is a synthetic antagonist with a high affinity and specifically for thrombin (Hauel *et al.,* 2002; Ries *et al.,* 2003; Wienen *et al.,* 2007). It is currently licensed in the European Union as Dabigatran etexilate, its orally active prodrug, for the prevention of venous thrombo-embolism after total knee or total hip arthroplasty and treatment of acute venous thromboembolism, with a similar efficacy and bleeding profile to low molecular weight heparins (Hankey *et al.,* 2011). It is currently administered at oral doses of 220mg once daily for prophylaxis or 150mg twice daily for treatment of acute thromboembolism. It has been recently evaluated for the prevention of embolization in non-valvular atrial fibrillation, and found to be of similar efficacy when compared to warfarin, but is currently awaiting approval for long term use for this indication in the UK (Connolly *et al.,* 2009). Primates and rodents have demonstrated similar dose and time dependent prolongation of clotting times (Wienen *et al.,* 2007).

3.1.4. Aims and Objectives

In keeping with the body of work thus far, this study aimed to further our understanding of the contribution of the individual coagulation proteins in pathogenesis of liver fibrosis and to evaluate the anti-fibrotic potential of novel oral anticoagulants using cell line and murine models of liver fibrosis.

The specific aims were:

1. To evaluate the effect of FXa and thrombin and their direct inhibition on hepatic stellate cell activity in vitro.

2. To evaluate the impact of direct FXa inhibition on hepatic fibrosis induced by thioacetamide or CCL4 in male C57BL/6 mice.

3. To evaluate the impact of direct thrombin inhibition on hepatic fibrosis induced by thioacetamide or CCL4 in male C57BL/6 mice.

3.2. METHODS

3.2.1. Cell Culture Experiments

The activated human HSC line LX2 is a useful tool to examine hepatic stellate cell biology (Hermann *et al.,* 2007). LX2 cells (kindly provided by J. Humpries, Manchester University, UK) were cultured in Dulbecco's minimal essential medium (DMEM) (Invitrogen, UK) supplemented with 10% (v/v) foetal bovine serum (FBS) and 2 mM L-glutamine. Cells were maintained at 37°C in a humidified 5% (v/v) CO2 atmosphere.

3.2.1.1. HSC LX2 cell culture and α-SMA expression

HSC LX2 α-SMA expression was analyzed by using an immunohistochemsitry technique. LX2 cells were harvested and centrifuged at 700rpm for 5 min to remove trypsin. Cells were then re-suspended in serum free growth media at a density of 50,000 cells/well in a 4 well chamber glass slide, and then allowed to quiesce overnight. The multi chamber slides were then incubated for 24 hours at 37°C in a humidified environment of 5% CO_2 with control media alone (control) or with control media as follows: 0.5 U/ml FXa (Enzyme Research Lab, USA) with or without 0.23μM Rivaroxaban; 10 nM thrombin (Sigma, UK) with or without 1μM Dabigatran; 0.5 U/ml FXa and 10nM thrombin with or without 0.23μM Rivaroxban and 1μM Dabigatran; 0.23μM Rivaroxaban or 1μM Dabigatran alone. Dosages for anticoagulants were calculated after referencing published in vitro drug profiling data, using doses equivalent to those used in vitro to inhibit the respective factors (Perzborn *et al.,* 2005; Weinz *et al.,* 2009; Weinen *et al.,* 2007). Rivaroxaban and Dabigatran were pre-incubated with FXa and thrombin respectively for 10 minutes before adding to the wells. After incubation the media was gently removed and 200μl of paraformaldehyde pipetted into

each well and incubated at room temperature for 10 minutes. Each well was then gently rinsed twice with PBS for 3 min, and then the wells were lifted off the slide. The fixed cells were immunohistochemically stained for αSMA by technicians in St Mary's Hospital histopathology department as described below. All wells were examined with a light microscope and digital images taken.

3.2.1.2. HSC LX2 Gel Contractility Assay

Hepatic stellate cell contractility assays were performed as previously described with some minor modifications (Sohail *et al.,* 2009). Individual wells of a 24-well culture dish were incubated with PBS containing 1% BSA (500 µl per well) for 1 hour at 37°C and then washed twice with PBS and allowed to air dry. Collagen gels were prepared by mixing 7 parts of collagen type 1 (c3867 Sigma, UK) diluted in 20mM of Acetic acid, with 1 part 10XMEM (Gibco, UK), 1 part DMEM (Gibco, UK) and 1 part 0.22M NaOH, to achieve a final collagen concentration of 2.4mg/ml. 500µl of the gel mix was added to each culture well and incubated for 1 hour at 37°C. LX-2 cells were then layered on top of the collagen lattice at a concentration of 200,000 cells/ml and serum starved for 24 hours.

Gel contraction was induced by incubation of duplicate wells with 10% FBS, which acted as the positive control, or the proposed agonists, FXa or thrombin with or without their respective antagonists, Rivaroxaban or Dabigatran, in the following combinations: 0.5 U/ml FXa (Enzyme Research Lab, USA) with or without 0.23µM Rivaroxaban; 10 nM thrombin (Sigma, UK) with or without 1µM Dabigatran; 0.5 U/ml FXa and 10nM thrombin with or without 0.23µM Rivaroxban and 1µM Dabigatran; 0.23µM Rivaroxaban or 1µM Dabigatran alone. Rivaroxaban and Dabigatran were pre-incubated with FXa and thrombin respectively for 10 minutes before adding to the wells. Additional wells containing a cell free lattice and

wells containing a lattice with LX2 cells without any agonist or antagonist, acted as a controls for spontaneous gel shrinkage.

Gels were released from wall and bottom using a micro-pipetting tip (representing time point zero). The gels were photographed at 6 hours. Surface area of the collagen gels was measured using digital image analysis software, and images were processed using digital image analysis. Relative contraction of the gels was expressed as a percentage of the surface area contraction of each experimental gel in comparison to gels incubated with media and LX2 cells alone. All experiments were performed in triplicate.

3.2.2. Murine models of liver fibrosis

3.2.2.1. Animals and Animal Husbandry

All research using live animals was approved by the local ethics committee and was carried out in accordance with the Animal (Scientific Procedures) Act 1986. Procedures were performed under the authority of animal licence PPL 70/6943, "Mouse models of hepatic fibrosis and therapeutics".

90 male C57BL/6J mice, aged 8 weeks old, were purchased from Jackson Laboratories, USA. C57BL/6J mice were used in keeping with previous work done in this field (Anstee *et al.,* 2008). Animals were housed 5 per cage. Throughout the experiment, care was taken to minimise any distress caused to the animals. Animals were housed under standard conditions (12 hour light/dark cycle, temperature 21+/- 2°C, humidity 55+/-10%) in a specific

pathogen free facility and provided with a standard diet and access to drinking water *ad libitum*. Ear notching was performed to identify individual animals in each cage, and a unique study identification number was assigned. All mice were weighted on a two weekly basis.

3.2.2.2. Chemical induction of liver fibrosis

3.2.2.2.1. Thioacteamide (TAA) Exposure

45 mice were treated with TAA to induce liver fibrosis. TAA was administered in drinking water at a dose of 300mg/l for a period of 8 weeks. The animals were divided into three subgroups (n=15) and treated by oral gavage with either Rivaroxaban (FXa inhibitor group), Dabigatran (thrombin antagonist group) or standard drinking water (control group). At 8 weeks, all surviving animals from each experimental group were culled by intraperitoneal injection of 0.1ml Pentoject (Pentobarbitone Sodium Ph.Eur. 200mg/ml, Animal Care Ltd., UK) followed by cervical dislocation. Liver tissue was collected and preserved in 10% formalin for histological examination or snap frozen in liquid nitrogen and stored at -80°C as a resource for subsequent RNA and protein analysis (See Figure 3-1).

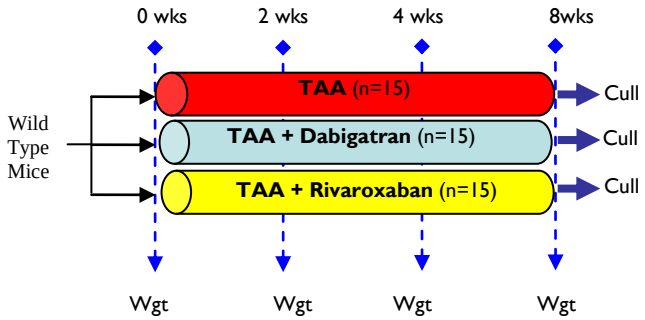


Figure 3-1: Design of murine thioacteamide experiment.

Schematic representation of murine study showing control group (TAA alone), Dabigatran treated mice, and Rivaroxaban treated mice. Mice from each group were culled at 8 weeks after study was commenced.

3.2.2.2.2. Carbon Tetrachloride (CCL4) Exposure

The remaining 45 mice were treated with carbon tetrachloride (CCl4) diluted in a corn oil vehicle and administered by intraperitoneal injection on alternate week days for a period of 6 weeks. The dose of CCl4 administered was increased weekly in a stepwise fashion as previously described (Anstee *et al.,* 2008). A maximum dose of 1 ml CCl4 per kg body weight was reached after 3 weeks (See Appendix A). The animals were divided into three subgroups (n=15) and treated by oral gavage with either Rivaroxaban (FXa inhibitor group), Dabigatran (thrombin antagonist group) or standard drinking water (control group). At 6 weeks, all surviving animals from each experimental group were culled by intraperitoneal injection of 0.1ml Pentoject (Pentobarbitone Sodium Ph.Eur. 200mg/ml, Animal Care Ltd., UK) followed by cervical dislocation. Liver tissue was collected and preserved in 10% formalin for histological examination or snap frozen in liquid nitrogen and stored at -80°C as a resource for subsequent RNA and protein analysis. (see Figure 3-2).

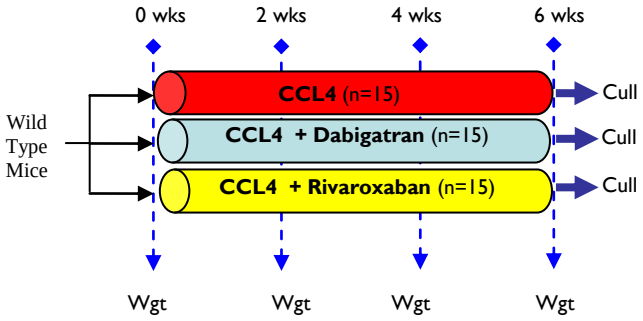


Figure 3-2: Design of murine tetrachloride experiment.

Schematic representation of murine study showing control group (CCL4 alone), Dabigatran treated mice, and Rivaroxaban treated mice. Mice from each group were culled at 6 weeks after study was commenced.

3.2.2.3. Anticoagulation administration

3.2.2.3.1. Direct FXa inhibition

Rivaroxaban (Bayer Heathcare, Germany), the direct FXa inhibitor was administered via oral gavage daily to both subgroups of CCL4 and TAA treated mice, to evaluate the effects of FXa inhibition on chemically induced liver fibrosis. Each 10mg tablet of Rivaroxaban was crushed with a pestle and mortar to a fine powder. Distilled water was then added to form a fine suspension. A dose of 40mg/kg was attained to cause prolongation of the prothrombin time to between 1.5 to 2 times (See Appendix B), as measured using a handheld coagulation meter, following blood sampling from animals tail veins and referencing previous rodent data (Weinz *et al.,* 2009). The dose was administered based on the weight of each mouse, as per a pre-formulated weight/dose chart. Prior to administration of the drug, the vial containing the active suspension was shaken vigorously to ensure each dose had the appropriate amount of suspension per calculated dose. The gavaged suspension was not to exceed 1ml in total to prevent risk of aspiration.

3.2.2.3.2. Direct Thrombin inhibition

Dabigatran (Boehringer Ingelheim, Germany), the direct thrombin antagonist was administered by oral gavage daily to determine if specific inhibition of thrombin alone would affect the development of chemically induced hepatic fibrosis. Each 75mg tablet was crushed to a fine powder to which distilled water was added to form a suspension. A dose of 100mg/kg was then administered to cause prolongation of the prothrombin time to between 1.5 to 2 (See Appendix B), as measured using a handheld coagulation meter, following blood sampling from animals tail veins and referencing previous rodent data (Weinen *et al.,* 2007).

The dose administered was based on each individual animal as per a pre-formulated weight/dose chart. The gavaged suspension was not to exceed 1ml per mouse, as with the administration of the Rivaroxaban suspension, to prevent risk of aspiration.

3.2.2.4. Histology

3.2.2.4.1. Fibrosis scoring

Tissue collected at each time point was fixed in 10% formal saline, processed into paraffin wax and sections were stained with both H&E and Picro-Sirius red by experienced technicians at St Mary's Hospital cellular pathology department. Picro-Sirius red is a commonly used stain for collagen and fibrosis (Junqueria *et al.,*1979). All sections were examined using a light microscope by a histopathologist (Dr Adam Levene) blinded to the nature of the specimen. The extent of hepatic fibrosis observed in each sample was assessed and a semi-quantitative score was used to record key features in a manner previously described in mouse models of fibrosis (Anstee *et al*., 2008). This scoring system is similar to the scoring of diagnostic specimens in patients with chronic HCV and adapted from the (Ishak) Modified Histological Activity Index fibrosis score (Ishak *et al.,* 1995) to take into account histological changes secondary to chemically induced fibrosis.

3.2.2.4.2. Digital Image Analysis and Collagen Proportionate Area (CPA)

Digital image analysis allows semi-automated quantification of fibrous tissue deposition within histological sections without the need to determine morphological distribution. Tissue collected was fixed in 10% formal saline, sections cut and stained using Picro-Sirius red. Using an Olympus BX50 microscope at x1.5 objective under fixed lighting conditions,

multiple digital images of each section stained per animal was taken. Using the Nikon NIS Elements Software Viewing System (Nikon, Japan) the digital images acquired from each section were 'stitched' to produce a single image representative of the entire section. This image was analysed, using Nikon NIS digital image analysis software to calculate the percentage area of staining for Picro-Sirius red in each section. The mean percentage area of fibrosis for each section or collagen proportionate area was calculated to generate a measure of fibrotic tissue deposition in each animal.

3.2.2.4.3. Alpha smooth muscle actin expression

Tissue collected was fixed in 10% formal saline, sections cut and stained for αSMA (Carpino *et al.,* 2005). Expression of αSMA is a sensitive marker of hepatic stellate cell activation to a fibroblast-like phenotype therefore αSMA levels were measured as an indicator of HSC activation. Formalin fixed tissue was immunohistochemically stained for αSMA by technicians in St Mary's Hospital histopathology department. The primary antibody was a mouse IgG2a isotype monoclonal antibody raised against a synthetic decapeptide that has the same sequence as the NH2-terminal of alpha-smooth muscle actin (Clone 1A4, Dako Inc, USA). This was then detected using a horse radish peroxidise conjugated goat anti-mouse secondary antibody, stained with diaminobenzidine (DAB) and counterstained with haemtoxylin.

All sections were examined with a light microscope and the number of stellate cells stained positive for αSMA were counted per field of vision at x20 objective. Six fields per section were randomly chosen, and an average calculated, which represented the mean number of activated stellate cells for each individual mouse per field.

3.2.3. Statistical Analysis

Data was collated using Microsoft Excel (Microsoft Inc, USA), and statistical analysis was performed using SPSS v16 (SPSS, USA). Normally distributed continuous variables were compared by Student's t-test, as represented as mean (+/-SEM), Variables which were not distributed normally were compared using the non parametric tests, Mann-Whitney or Kruskall-Wallis. Quantitative data were expressed as mean +/- standard error of mean, unless otherwise stated. Statistical significance was accepted at $p<0.05$.

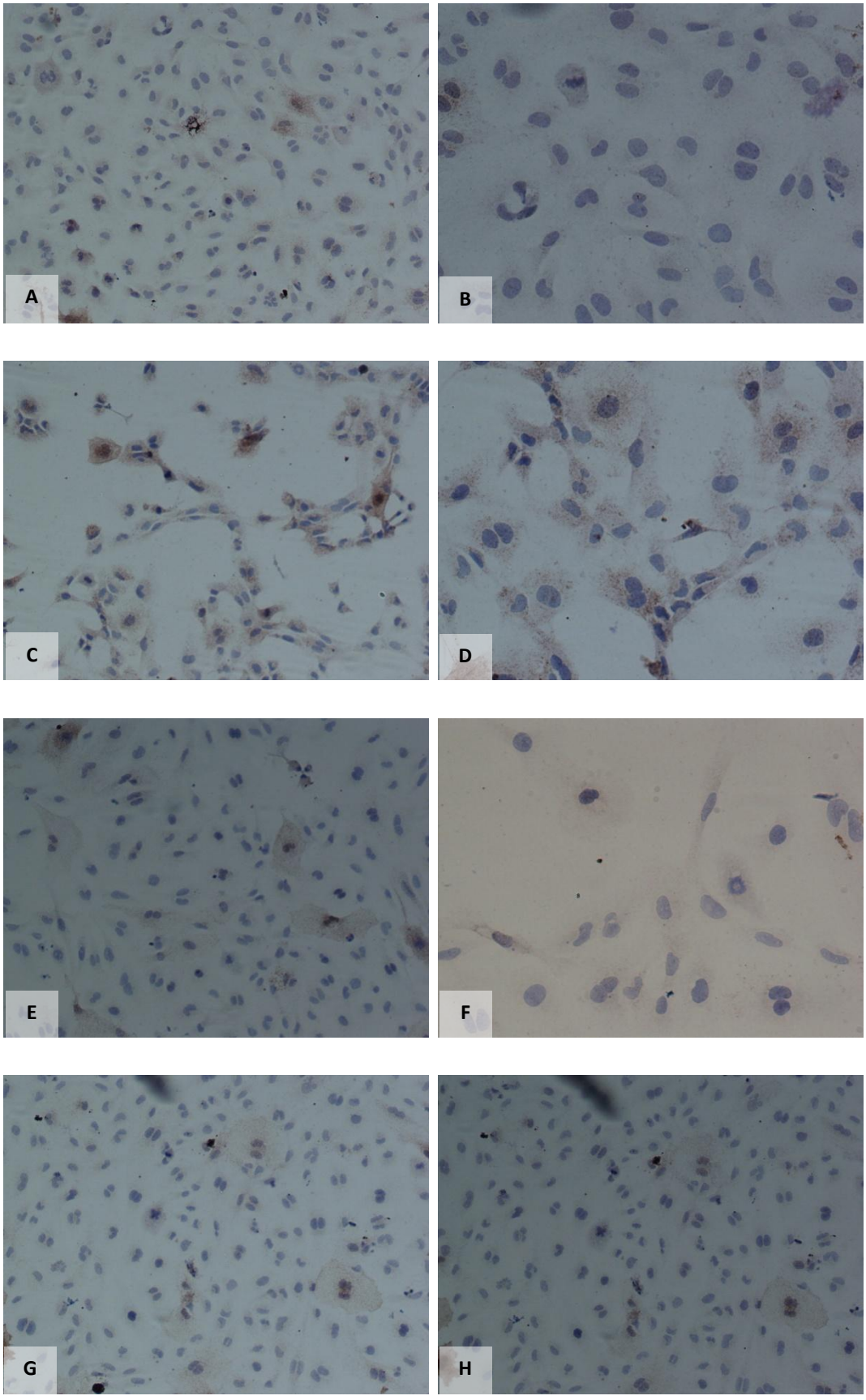
3.3. RESULTS

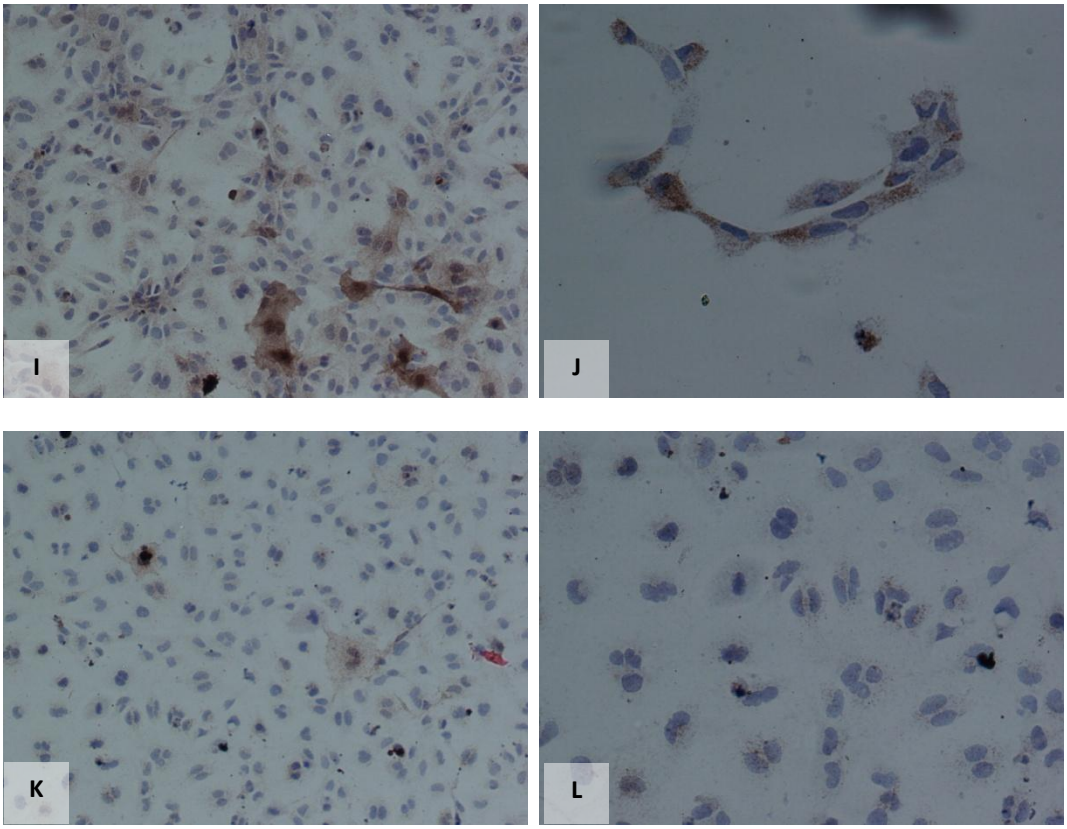
3.3.1. LX2 hepatic stellate cell line

To determine the effect of individual coagulation proteins on the activity of hepatic stellate cells, the principal cell of hepatic fibrosis, a LX2 cell line was used. Expression of α -SMA and a change in cell morphology, to a contracted myofibroblast, were used as markers of stellate cell contractility and activation.

Incubation of LX2 cells with medium alone on a glass surface resulted in minimal staining for α -SMA (See Figure 3-3A-B). Incubation of LX2 cells with FXa at a doses of 0.5iU resulting in activation of LX2 cells as demonstrated by positive staining for α-SMA and change in cell morphology to the contracted myofibroblast phenotype (See Figure 3-3 C-D). Incubation with thrombin, similarly demonstrated some positive staining for α -SMA in LX2 cells (see Figure 3-3E-F). FXa and thrombin together resulting in heavy staining for α-SMA, with a contracted myofibroblastic phenotype (see Figure 3-3 I-J) In contrast, FXa and thrombin alone and in combination together, when given with their respective direct inhibitors, Rivaroxaban and Dabigatran resulted in a reduction in α -SMA staining and stellate cell activation (see Figure 3-3 G-H & K-L).

Figure 3-3: Effect of FXa, thrombin and their direct inhibition on LX2 cells in vitro.





A & B: Incubation of cells with control medium alone resulting in minimal staining for α-SMA (x10 and x20 objective)

C & D: Incubation of LX2 cells with FXa at a dose of 0.5U/ml resulted in activation of LX2 cells with positive staining for α -SMA and a contracted phenotype (x10 and x20 objective).

E & F: Incubation of LX2 cells with Thrombin at a dose of 10nM resulted in some activation of LX2 cells with positive staining for α -SMA and a contracted phenotype (x10 and x20 objective).

G & H: Incubation of LX2 cells with FXa (0.5 U/ml) and the direct inhibitor FXa, Rivaroxaban (0.23μM), resulted in a quiescent phenotype and minimal staining for α –SMA **(G).** Addition of thrombin (10nM) in combination with the direct thrombin inhibitor, Dabigatran (1μM) **(H)** resulted with a quiescent phenotype with minimal staining for α –SMA (both x10 objective).

I & J: Addition of FXa (0.5 U/ml) in combination thrombin (10nM) resulted in activation of LX2 cells with heavy positive staining for α -SMA and a contracted phenotype (x10 and x20 objective).

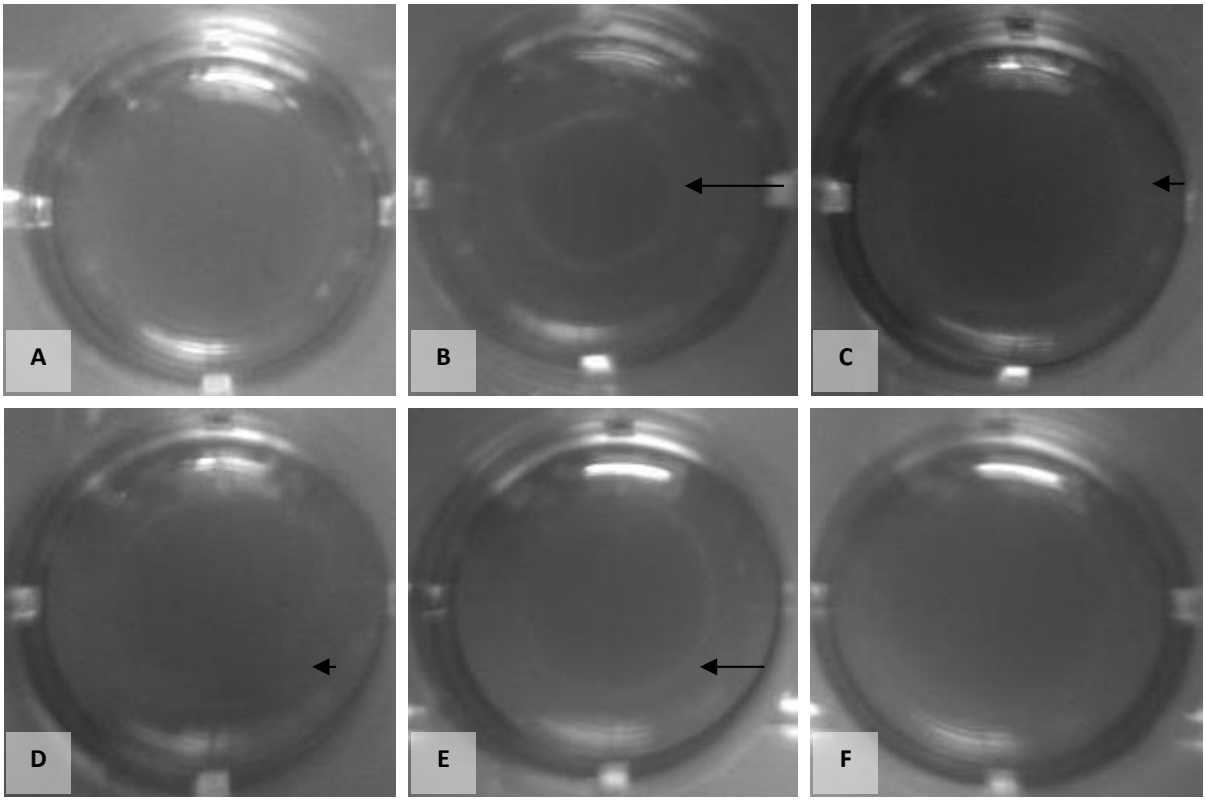
K & L: Incubation with Addition of FXa (0.5 U/ml) in combination thrombin (10nM) and Rivaroxaban (0.23μM) with Dabigatran (1μM) resulted with a quiescent phenotype with minimal staining for α –SMA (x10 and x20 objective).

3.3.2. HSC Contractility assay

The use of contractility gel assays allowed for a quantitative measurement of hepatic stellate cell contractility. Incubation of LX2 cells with medium alone resulted in a small degree of spontaneous gel shrinkage at 6 hours (see Figure 3-4A), and was used as a control. The mean surface area of experimental gels were expressed as a percentage of the surface area of mean control gels and subtracted from 100 to calculate the percentage gel contraction. This allowed for any correction of spontaneous gel shrinkage that may have occurred.

Incubation with FBS, which acted as a positive control, resulted in a significant contraction of the gel (66.71% +/- 3.19) (See Figures 3-4B & 3-5). The addition of FXa and thrombin individually resulted in contraction of gels (See Figures 3-4 & 3-5). FXa treated gels demonstrated more contraction than thrombin treated gels (26.90% +/- 8.90 versus 13.10% +/- 9.84) at 6 hours, but the difference did not reach significance ($p>0.05$) (see Figures 3-4D, 3-4C & 3-5). The addition of FXa and thrombin together however demonstrated a potentiated effect on cell contraction (43.48% +/- 4.12), with significantly more contraction compared to incubation with FXa ($p=0.02$) and thrombin alone ($p<0.001$) (see Figures 3-4E & 3-5). The addition of Rivaroxaban, a direct FXa inhibitor in combination with FXa resulted in a significant reduction in gel contraction (15.95%+/- 5.92) compared to FXa alone ($p = 0.03$) (see Figure 3-5). The addition of both Rivaroxaban and the direct thrombin antagonist, Dabigatran, in combination with FXa and thrombin resulted in a significant reduction in gel contraction (8.94% +/- 3.04) compared to gels incubated with both FXa and thrombin (46.48% +/- 4.12), ($p <0.001$) (see Figures 3-4F & 3-5).

Figure 3-4: Effect of FXa, thrombin and their direct inhibition on LX2 hepatic stellate cell contractility assays.



A: Incubation of LX2 cells with medium alone (negative control) at 6 hours.

B: Incubation of LX2 cells with medium and 10% FBS (positive control) at 6 hours.

C: Incubation of LX2 cells with medium and Thrombin (10nM) at 6 hours.

D: Incubation of LX2 cells with medium and FXa (0.5U/ml) at 6 hours.

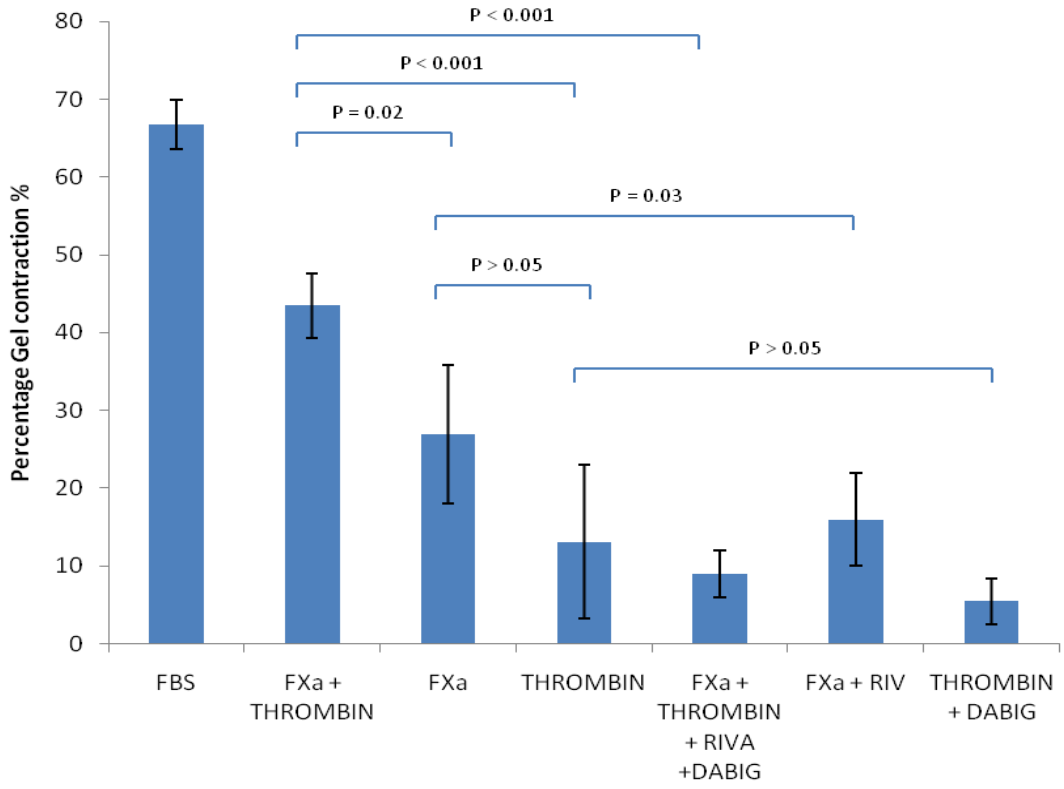
E: Incubation of LX2 cells with medium, FXa (0.5U/ml) and Thrombin (10nM) at 6 hours.

F: Incubation of LX2 cells with medium, FXa (0.5U/ml), Thrombin (10nM), Rivaroxaban (0.23μM) and Dabigatran (1μM)

Black arrows indicate shrink of gel margin from wall of gel plate

Collagen lattices with LX2 cells, photographed 6 hours after addition of coagulation proteins with or without their direct antagonists. Control cells demonstrated a minimal amount of shrinkage **(A)** in contrast to FBS which resulting in a significant contraction of the gel **(B).** The addition of FXa and thrombin individually resulted in a small amounts of gel contraction **(C & D)** but given together had a potentiated effect on cell contraction **(E).** The combined addition of direct thrombin and FXa inhibitors, with FXa and thrombin resulting in inhibition of stellate cell contraction **(F).**

Figure 3-5: Bar graph showing percentage gel contraction for FXa and/or thrombin with or without direct inhibitors.



Gel contraction expressed as mean percentage of contraction. Error bars represent standard error of mean. Incubation with FXa and thrombin together resulted in a significant increase in gel contraction compared to FXa and thrombin alone ($p=0.02$ and $p<0.001$ respectively). The addition of Rivaroxaban, the direct FXa inhibitor with FXa significantly reduced gel contraction compared to FXa alone ($p=0.03$).The addition of both Rivaroxaban with Dabigatran, a direct thrombin inhibitor, with FXa and thrombin significantly reduced gel contraction compared to gels incubated with FXa and Thrombin ($p<0.001$).

3.3.1. Murine models of liver fibrosis

3.3.1.1. Animal Husbandry

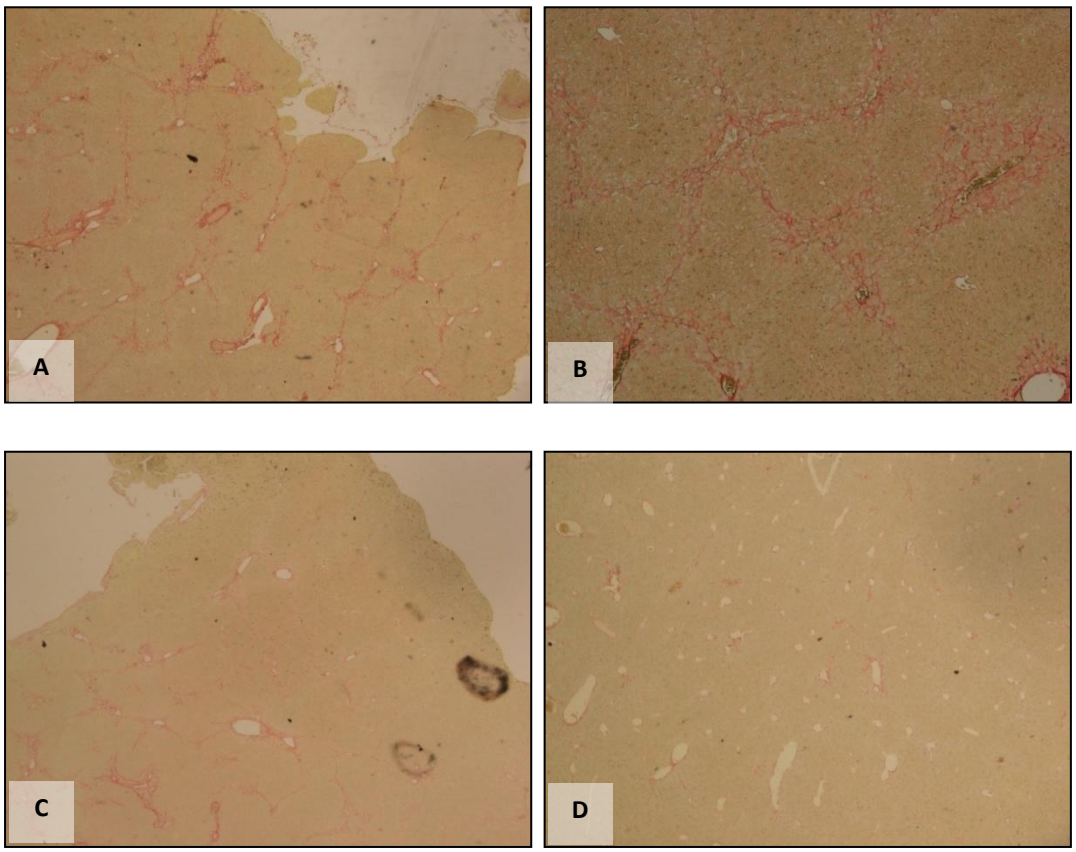
Early mortality was confined to the thioacteamide group and affected both treatment subgroups and the control subgroup equally. Two mice were lost in both the thioactemide alone group and thioacetamide with Rivaroxaban group. Three animals were lost in the Thioacteamide with Dagibatran group. All mortality occurred within the first week of induction of fibrosis, and was not ascribed to haemorrhage in the anticoagulant group animals. All surviving animals were weighted every second week. No animal recorded a reduction in overall weight at the end of the study, in comparison to its starting weight. No bleeding events were demonstrated in any of the anticoagulated mice.

3.3.2. Histology

3.3.2.1. Thioacteamide

After 8 weeks of exposure with TAA via drinking water (300mg/L), control mice demonstrated bridging fibrosis with occasional nodule formation (see Figure 3-6). Mice treated with the direct thrombin antagonist, Dabigatran (100mg/kg), exhibited frequent extensive bridging fibrosis, similar to control mice, but only very occasional nodule formation (see Figure 3-6). In comparison, the mice treated with the direct FXa inhibitor Rivaroxaban (40mg/kg) demonstrated milder fibrosis, predominantly confined to fibrous expansion around the central veins, with less frequent bridging and the absence of nodule formation (see Figure 3-6).

Figure 3-6: Histological images using Picro-sirius red staining of liver tissue for fibrosis in mice treated with 8 weeks of thioacteamide with or without FXa or Thrombin inhibition.



A & B: Control mice treated with Thioacetamide alone demonstrated bridging fibrosis with occasional nodule formation (x 1.5 and x4 objective).

C: Mice treated with thioactemide and the thrombin inhibitor, Dagibatran (100mg/kg) exhibited frequent bridging fibrosis similar to control mice, but only very occasional nodule formation (x 1.5 objective).

D: Mice treated with thioactemide and the FXa inhibitor, Rivaroxaban (40mg/kg) had milder fibrosis, with fibrous expansion round the central veins, less extensive bridging and the absence of nodule formation (x 1.5 objective).

3.3.3.2. CCL4 group

After 6 weeks of exposure with intraperitoneal CCL4, control mice exhibited marked bridging fibrosis with occasional nodule formation. Similar histological patterns were seen in both the Dabigatran and Rivaroxaban treated groups of mice.

3.3.4. Fibrosis Scoring and Digital Image Analysis

3.3.4.1. Thioacteamide group

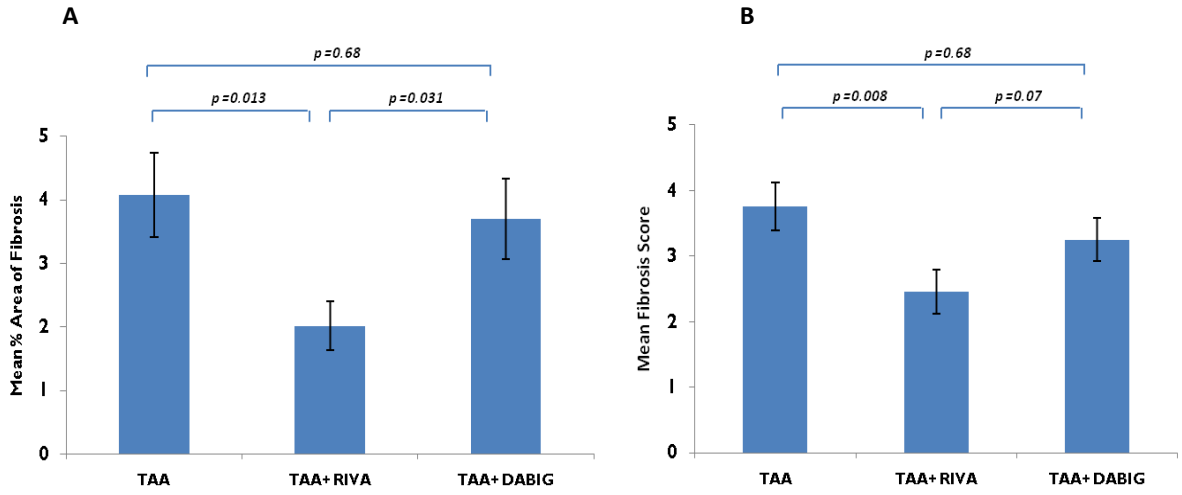
Mean fibrosis scores in mice treated with the FXa inhibitor Rivaroxaban (40mg/kg) were significantly reduced (p=0.008) in comparison to the mice in the control group (2.46 ± 0.33 versus 3.76 ± 0.36). In accordance with this finding, there was a significant reduction (p=0.012) in mean percentage area of fibrosis, measured by digital image analysis , between the Rivaroxaban (2.02% ± 0.39) and control groups (4.08± 0.66), (Table 3-1 and Figure 3-7). In contrast, mice treated with the direct thrombin antagonist, Dabigatran, had a mean fibrosis score of 3.25 ± 0.63 (SEM). This represented only a 13% reduction in fibrosis scores in comparison to the control group, and did not reach statistical significance (p=0.68). Consistent with this finding, the mean percentage area of fibrosis in the Dabigatran group was 3.70% ± 0.63 (SEM), representing only a 9% reduction compared to the control mice, which again was not statistically significant (p=0.68) (Table 3-1 and Figure 3-7).

When comparing direct FXa inhibition to direct thrombin antagonism, direct FXa inhibition demonstrated a significant reduction in mean percentage area of fibrosis (p=0.031), and a non significant reduction in mean fibrosis scores (p=0.07) (See Table 3-1 and Figure 3-7).

Table 3-1: Mean percentage area of fibrosis and mean histology scores following 8 weeks of TAA exposure.

Group	Number	Mean % area of fibrosis	SEM	Mean Visual Fibrosis Score	SEM
TAA (300mg/l)	13	4.08	0.66	3.76	0.36
TAA (300mg/l) + Rivaroxaban (40mg/kg)	13	2.02	0.39	2.46	0.33
TAA(300mg/l) + Dabigatran (100mg/kg)	12	3.7	0.63	3.25	0.33

Figure 3-7: Mean percentage area of fibrosis and mean fibrosis scores following 8 weeks of exposure with TAA.



Bar graphs showing mean percentage area of fibrosis **(A)** and mean visual fibrosis score **(B)**, following 8 weeks of TAA exposure. Mean fibrosis scores and percentage area of fibrosis in mice treated with the FXa inhibitor Rivaroxaban were significantly reduced in comparison to control mice (p=0.008; p=0.012). In contrast mice treated with Dabigatran, the direct thrombin antagonist failed to show a significant difference compared to controls, and demonstrated a significantly greater mean area of fibrosis compared to FXa treated mice (p=0.031).

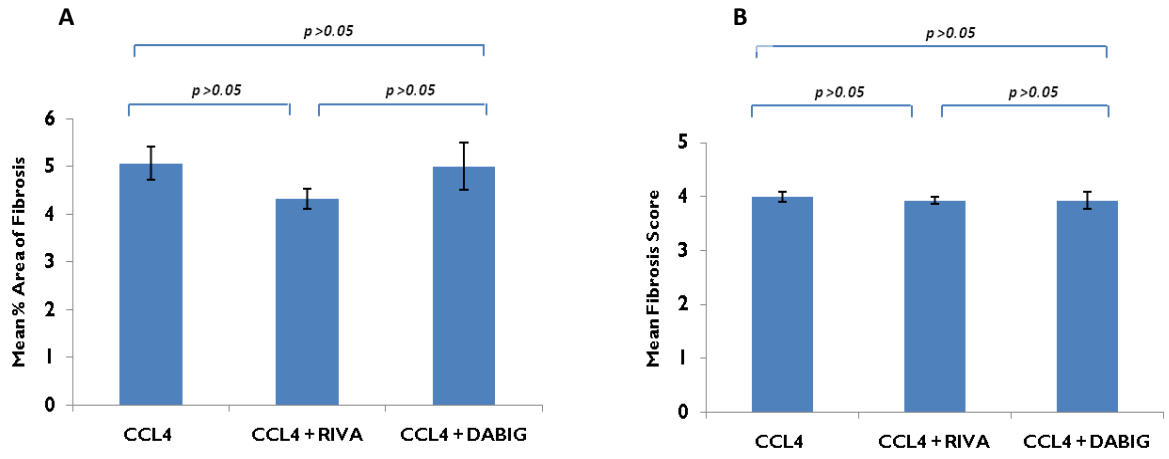
3.3.4.2. CCL4 group

No significant differences were demonstrated between the treatment subgroups or the control groups in mean fibrosis scores nor mean percentage area of fibrosis (see Table 3-2 and Figure 3-8). Mice treated with the FXa inhibitor, Rivaroxaban, demonstrated a reduction in mean area of fibrosis (4.32% ± 0.39) in comparison to both control group mice (5.07% ± 0.55) and Dabigatran treated mice (5.01% ± 0.50), but neither comparisons reached statistical significance (see Table 3-2 and Figure 3-8).

Table 3-2: Mean area of fibrosis and mean histology scores following 6 weeks of CCl4 exposure.

Group	No.	Mean % area of fibrosis	SEM	Mean Visual Fibrosis Score	SEM
CCL4 (variable dose)	15	5.07	0.35	4.00	0.09
CCl4 (variable dose) + Rivaroxaban	14	4.32	0.21	3.93	0.07
CCL4 (variable dose) + Dabigatran	15	5.01	0.50	3.93	0.16

Figure 3-8: Mean percentage area of fibrosis and mean fibrosis scores following 6 weeks of exposure with CCL4.



Bar graphs showing mean percentage area of fibrosis **(A)** and mean visual fibrosis score **(B)**, following 6 weeks CCL4 exposure. No significant reduction was demonstrated in mean fibrosis scores between the control mice and the mice treated with neither Rivaroxaban nor Dabigatran. Mice treated with Rivaroxaban demonstrated a reduction in mean percentage area of fibrosis in comparison to both control mice and mice treated with Dabigatran, but this did not reach statistical significance ($p>0.05$).

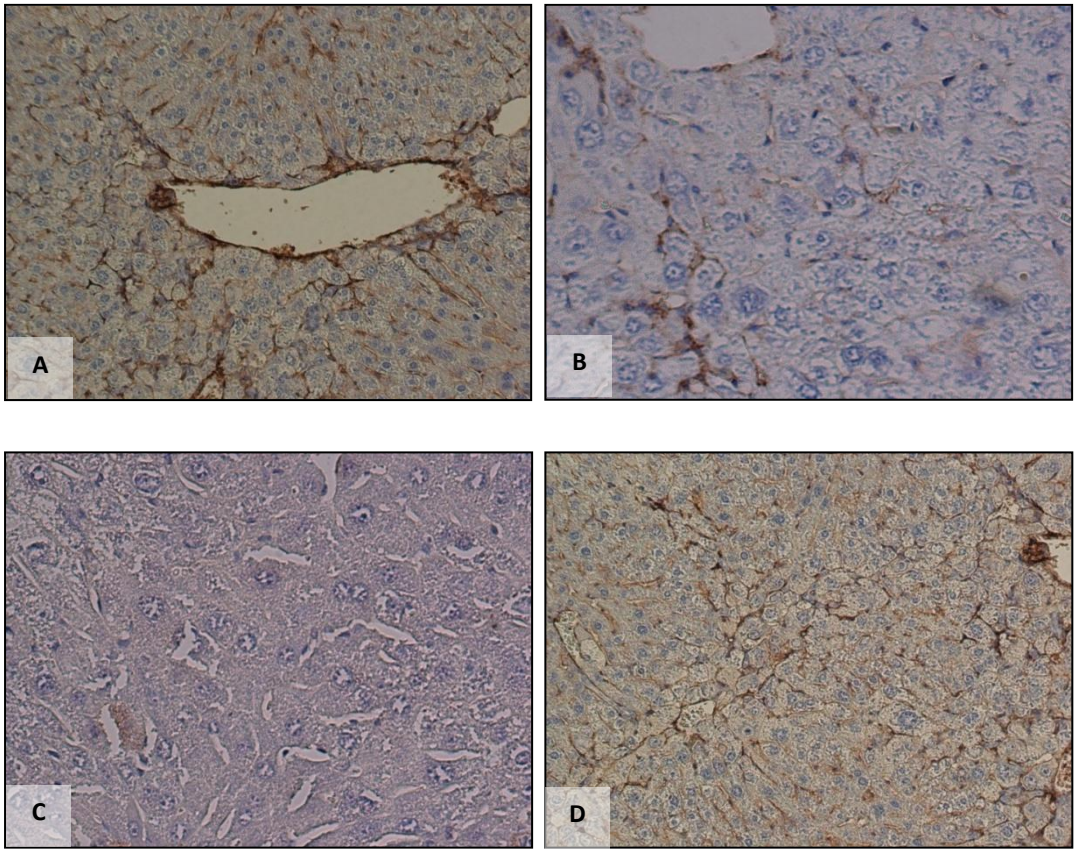
3.3.5. Alpha smooth muscle actin expression

Alpha smooth muscle actin expression was performed only on tissue from subgroups exposed to TAA, since no significant difference in the fibrosis scoring was noted between the CCL4 treated control and treatment groups.

Following 8 weeks exposure to TAA, mice treated with the FXa inhibitor Rivaroxaban at a dose of 40mg/kg demonstrated a significant reduction in the mean number of αSMA positive hepatic stellate cells per field of vision compared to control group mice (5.23 ± 1.06 versus 10.62±1.92; $p= 0.02$). Direct thrombin antagonism with Dabigatran (100mg/kg) resulted in a

non significant reduction in αSMA positive hepatic stellate cell expression compared to the control group (7.29±1.39 versus10.62±1.92; p>0.05). FXa inhibition with Rivaroxaban (5.23 ± 1.06) in comparison to direct thrombin antagonism with Dabigatran (7.29±1.39), resulted in a reduction of αSMA expression but this was not significant. (See Table 3-3 and Figures 3-9 & 3-10).

Figure 3-9: Immunohistochemical staining of liver tissue for αSMA following exposure to thioacetamide.

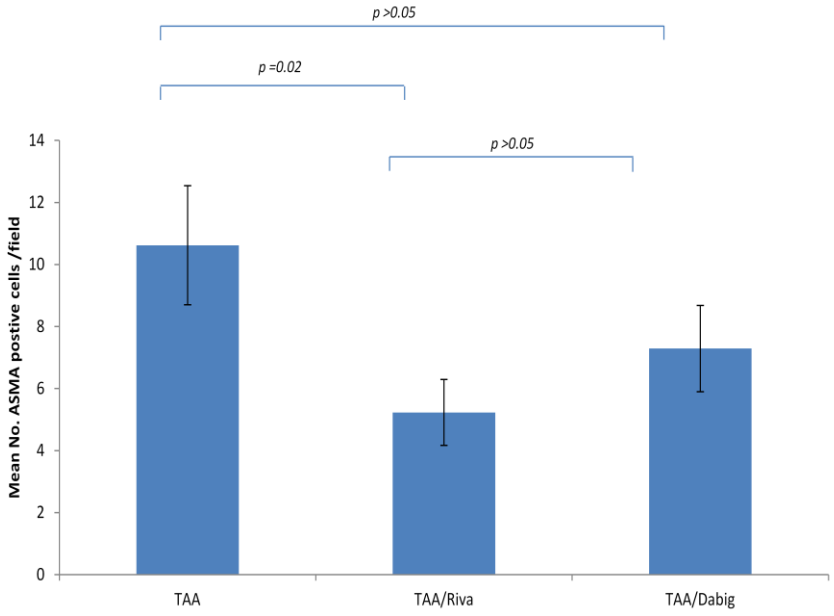


Relative to control mice treated with TAA alone (**A,B**), reduced numbers of αSMA positive stellate cells were demonstrated in mice treated with Rivaroxaban, the FXa inhibitor (**C**) but not with Dabigaran, the direct thrombin antagonism (**D**).

Table 3-3: Mean number of αSMA HSC cells per field following 8 weeks TAA exposure.

Group	Number	Mean number of αSMA cells per field	SEM
TAA (300mg/l)	13	10.62	1.92
TAA (300mg/l) + Rivaroxaban (40mg/kg)	13	5.23	1.06
TAA(300mg/l) + Dabigatran (100mg/kg)	12	7.29	1.39

Figure 3-10: Mean number of αSMA positive stellate cells per field following 8 weeks of exposure with TAA.



Bar graph showing αSMA expression (mean number of αSMA positive stellate cells per field of vision). Compared to control mice, a significant reduction in number of positive αSMA stellate cells was demonstrated with FXa inhibition (p=0.02), but not Thrombin antagonism (p>0.05).

3.4. DISCUSSION

Previous evidence, including data described in Chapter 2, suggests that not only is fibrosis associated with activation of the cascade but activated coagulation proteins may be important mediators of hepatic fibrosis. Hence with this study we sought to firstly examine the effects of FXa and thrombin and their direct inhibition on hepatic stellate cells in vitro, and secondly to evaluate the anti-fibrotic potential of direct FXa and thrombin inhibition using novel commercial available agents in murine models of chemically induced fibrosis.

3.4.1. Effects of FXa and Thrombin on LX2 hepatic stellate cells in vitro: a role in activation

We have demonstrated using immunohistochemistry and a cell culture line of LX2 hepatic stellate cells that incubation with FXa or thrombin, independently and in combination, up-regulate αSMA expression on stellate cells (see Figure 3-3). The stellate cell is the principal cell type involved in hepatic fibrogenesis and the expression of αSMA is a marker of its activation (Carpino *et al.,* 2005). On stimulation the hepatic stellate cell contracts, a state that is characterised by the increased expression of alpha smooth muscle actin, which is a contractile filament protein (Rockey *et al*., 1992). Therefore demonstrating up-regulation of αSMA in stellate cells with FXa and thrombin supports the hypothesis that these proteins independently mediate activation of stellate cells and fibrogenesis. This was further supported by the results of the gel contraction assay. Gel contraction assays are an established method of quantifying stellate cell contraction and activation (Sohail *et al.,* 2009). We have demonstrated contraction of stellate cell gel assays, when incubated with FXa or thrombin alone, with a non significant increase in contraction with FXa compared to

thrombin (see Figure 3-4). A highly significant increase in contraction was demonstrated when FXa and thrombin were administered in combination compared to alone (See Figures 3-4 & 3-5).

Both upregulation of αSMA and contraction of gel assays confirm that FXa and thrombin independently mediate hepatic stellate cell activation and in synergy this stimulation is significantly increased. Thrombin is known to be a mediator of stellate cell activation (Duplantier *et al.,* 2004). It is now established that cellular actions of thrombin are in part mediated by protease activated receptor (PAR) signalling (Rullier *et al*.,2007). PAR receptors are a family of widely expressed G-protein-coupled receptors, that transduce transmembrane signalling and four PARs have been identified (Coughlin., 2000). PAR 1 and 3 are both preferentially activated by thrombin. PAR4 has reduced affinity and PAR2 is resistant to thrombin activation. A substantial body of evidence from in vivo and in vitro studies is now accumulating to suggest PAR-1 activation leads to stellate cell activation and hepatic fibrogenesis (Fiorucci *et al*., 2004; Marra *et al*., 1998; Gaca *et a*l., 2002). In addition to thrombin activating stellate cells via PAR 1, indirect activation via other PAR receptors has been demonstrated. PAR mediated platelet degranulation results in the release PDGF and PAR mediates the activation of TGF-beta. Both PDGF and TGFb stimulate stellate cell activation (Friedman, 2003).

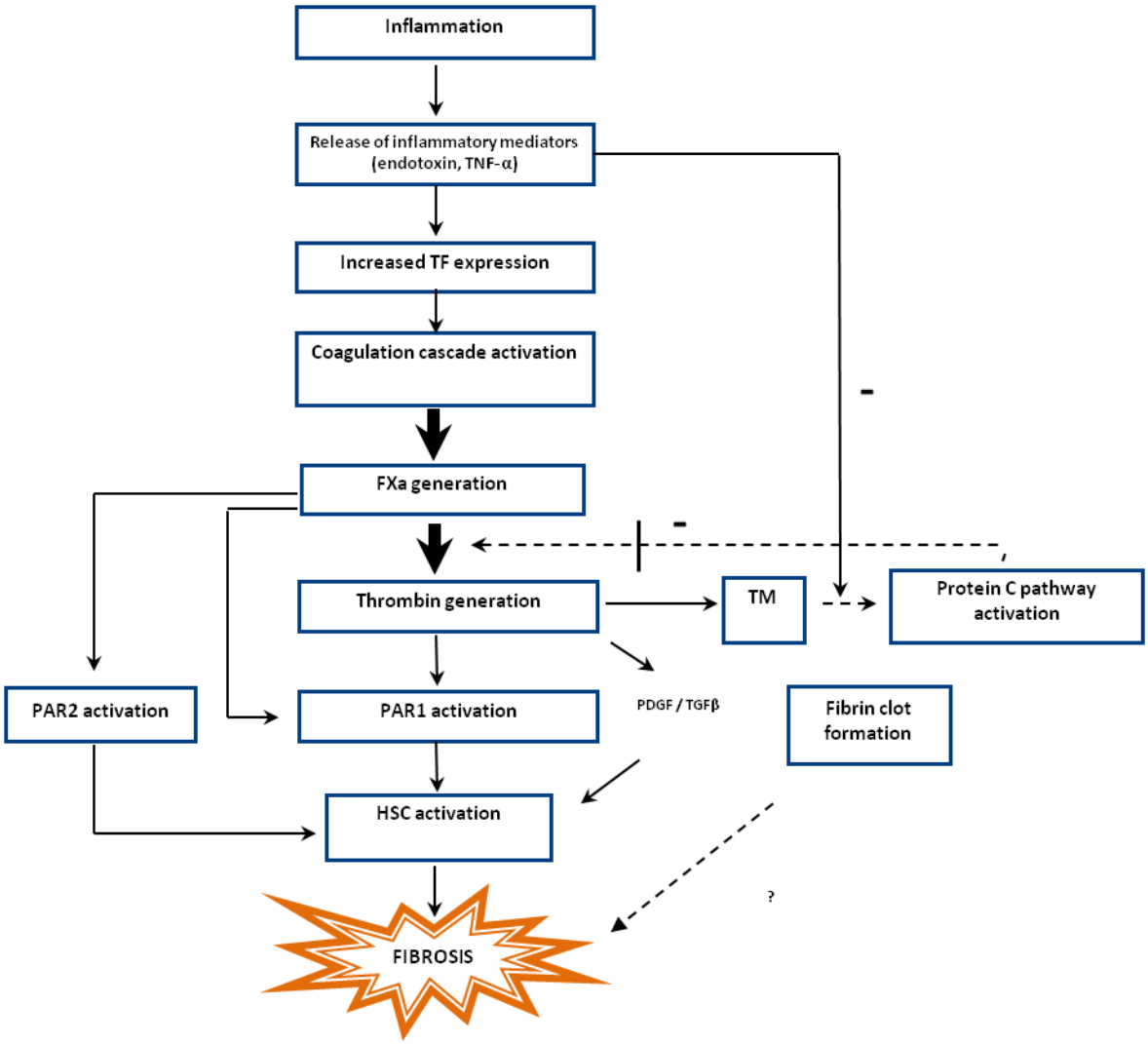
Whilst thrombin PAR1 mediated ligation results in stellate cell activation, the mechanism by which fibroblasts are activated by FXa has been less well studied. FXa, is a coagulation factor generated at the point of convergence of the intrinsic and extrinsic coagulation pathways. It is an essential component of the prothrombinase complex, which is responsible for the conversion of prothrombin to thrombin by proteolytic activity. Recent evidence suggests that FXa activates PAR 1, in a similar fashion to thrombin, as well as PAR 2 (Ruf *et*

al., 2003; Borensztajn *et al.,* 2008). In pulmonary fibrosis FXa mediated PAR1 activation has been demonstrated (Scrotton *et al.,* 2009). FXa activation of PAR2, has been demonstrated to result in the release of pro-inflammatory cytokines in a number of cell lines, including endothelial, mesangial, epithelial and fibroblastic cells (Pan *et al.,* 2008; Tanaka *et al.,* 2008; Wilson *et al.,* 2009). PAR2 expression has recently been demonstrated on both hepatic stellate cells and hepatocytes (Borensztajn *et al.,* 2010; Fiorucci *et al.,* 2004; Gaca *et al.,* 2002), and shown to be up-regulated in fibrotic livers (Borensztajn *et al.,* 2010). PAR 2 agonists demonstrate hepatic stellate cell proliferation and collagen synthesis in rats (Fiorucci *et al.,* 2004; Gaca *et al.,* 2002), and PAR2 activation results in the release of the profibrotic cytokines, PAI-1 and TGF-beta from fibroblasts (Borensztajn *et al.,* 2008). Therefore FXa mediated stellate cell activation in contrast to thrombin, involves both PAR1 and PAR2. The added role of PAR2 mediated activation explains the increased effect on stellate cell contraction and activation demonstrated with FXa or FXa with thrombin, compared to thrombin alone. In vivo FXa is also central in converting prothrombin to thrombin, with one molecule of FXa generating a 'thrombin burst' of over 1000 thrombin molecules (Mann *et al.,* 2003). Therefore FXa is pro-fibrotic by two pathways. Firstly it drives thrombin and subsequent fibrin production and secondly, independent of its procoagulant activity, it activates stellate cells by PAR 1 and PAR 2 mediated mechanisms.

Therefore in conjunction with the data presented in Chapter 2, we can suggest a mechanistic pathway by which FXa contributes to fibrosis. Inflammation within the liver parenchyma upregulates TF, leading to activation of the coagulation cascade. The generation of both FXa and thrombin, result in PAR ligation and HSC activation. Fibrin deposition occurs, which may contribute to microvascular dysfunction. Inflammatory mediators down-regulate TM and the protein C pathway, resulting in a failure of thrombin-TM negative feedback and further driving activation of the cascade (See Figure 3-11)

Figure 3-11: PAR mediated actions of thrombin and FXa on stellate cell activation.

A proposed mechanistic pathway outlining the pro-fibrotic properties of FXa and Thrombin.



Inflammation within the liver parenchyma increases expression of tissue factor (TF), leading to activation of the coagulation cascade, FXa and thrombin generation, PAR ligation and hepatic stellate cell (HSC) activation. The released inflammatory mediators down-regulate thrombomodulin (TM) and the protein C pathway, resulting in a procoagulant imbalance, caused by failure of the thrombin-thrombomodulin negative feedback. PDGF: platelet-derived growth factor. TGFβ: Tissue growth factor – beta. (Adapted from Anstee *et al.,* 2009).

3.4.2. The potential efficacy of FXa and Thrombin inhibition as antifibrotic therapies

From the pathway described above, we can hypothesise that targeting the coagulation cascade upstream should have a more potent anti-fibrotic effect than inhibiting thrombin alone. We sought to study this using anticoagulants both in vitro and in vivo using mice.

In vitro we have demonstrated the addition of Rivaroxaban, the direct FXa inhibitor, in combination with FXa resulted in a significant reduction in stellate gel contraction and reduced αSMA expression compared to incubation with FXa alone (see Figures 3-3 through 3-5). Furthermore the addition of both Rivaroxaban and the direct thrombin antagonist, Dabigatran, in combination with FXa and thrombin resulted in both reduced LX2 cell αSMA expression (see Figure 3-3) and a significant reduction in gel contraction compared to incubated with both FXa and thrombin without their respective inhibitors (see Figure 3-4 & 3-5). These findings would be in keeping with the highly selective affinity for both these drugs for FXa and thrombin respectively (Perzborn *et al.,* 2005; Wienen *et al.,* 2007), and the expected effect in vitro of inhibiting activation of hepatic stellate cells in their presence.

In vivo using a murine model of liver fibrosis induced by 8 weeks exposure to thioacteamide we have demonstrated a significant reduction in mean percentage area of fibrosis determined by digital image analysis, mean fibrosis scores and αSMA expression in mice treated with Rivaroxaban, at a dose of 40 mg/kg versus control mice. In contrast the thrombin inhibitor Dabigatran at a dose of 100mg/kg resulted in a non significant reduction in these parameters compared to control mice, and a significantly higher mean percentage area of fibrosis compared to Rivaroxaban treated mice, despite an equivalent level of anticoagulation (see Figures 3-7 & 3-10).

The concordance between our in vitro and in vivo data highlights the importance of the coagulation proteins, thrombin and in particular FXa in the generation of hepatic fibrosis. In vitro targeted inhibition of both these proteins had a demonstrable effect on preventing stellate cell contraction and reducing αSMA expression, both markers of stellate activation. In vivo the administration of the FXa inhibitor, Rivaroxaban, not only inhibits FXa, but will prevent the downstream production of the thrombin 'burst' (Perzborn *et al.,* 2005). Accordingly the significant reduction in fibrosis and αSMA expression seen in mice exposed to thioacetamide and treated with Rivaroxaban, could be explained by both the direct reduction in FXa mediated PAR1 and PAR2 stellate activation, as well as the indirect reduction in thrombin mediated stellate cell activation via PDGF, TGFb and PAR1 ligation. This would also explain why direct thrombin inhibition with Dabigatran was a less effective anti-fibrotic intervention compared to FXa inhibition with Rivaroxaban, since inhibition of thrombin alone would fail to block FXa PAR mediated activation of stellate cells and fibrogenesis. Parallels can be drawn from previous unpublished data, in which Ximelagatran, a thrombin antagonist, which had now been withdrawn from use, failed to exhibit a significant anti-fibrotic effect in wild type mice treated with CCL4 over a 4 week period (Dr Quentin Anstee, *PhD Thesis* 2006).

Whilst a reduction in fibrosis was seen in the thioacetamide model of fibrosis with FXa inhibition and to a lesser degree with direct thrombin inhibition, a comparable reduction was not observed in the CCL4 model. A possible explanation for why we failed to see a significant reduction in fibrosis in the mice exposed to CCL4 is that we employed an increasing dose weight based regimen of administration of CCL4 over a period of 6 weeks. This was adapted from a protocol previously used by our group (Anstee *et al.,* 2008). In the original protocol a 4 week regimen was employed. We decided to employ a 6 week

regimen, meaning our mice had an extra 14 days of treatment with CCL4. This additional period of exposure to CCL4 may have counteracted any benefits seen with treatment with the anticoagulants we used. Further studies using a 4 week period of CCL4 exposure could be considered. Another explanation could be that CCL4 is known to result in considerably more inflammation, necrosis and fatty infiltration than TAA (Kreft *et al.,* 1998). As demonstrated by data presented in Chapter 2, there is also significant intrahepatic activation of coagulation in CCL4 exposed mice. Due to experimental design, including concerns about excessive gavage volumes, we decided to aim for a minimum INR of 1.5. It is conceivable that CCL4 administration results in substantially more intrahepatic upregulation of pro-coagulant proteins than TAA, due to the nature of injury it induces. This could signify that higher levels of anticoagulation would be required to counteract this before a beneficial effect is seen. Previous animal studies investigating the anti-fibrotic effects of warfarin on CCL4 induced liver fibrosis, demonstrated a benefit with prolongation of the prothrombin times to achieve an INR of between 2 to 3 (Anstee et al., 2008). Therefore a failure to show efficacy for these treatments in CCL4 induced murine fibrosis, is until proven otherwise most likely secondary to experimental design rather than a failure of potential therapies, since we did demonstrate efficacy in the thioacetamide mice. Indeed thioacetamide is seen as a more suitable model than CCL4, when evaluating potential antifibrotic therapies in that it has closer histological similarities with human viral hepatitis and cirrhosis, including fibrous enlargement of the portal tracts, with portal-portal and portal-central septae developing resulting in regenerative nodules and cirrhosis (Zimmerman *et al.,* 1987; Li *et al.,* 2006; Muller *et al*., 1988); a longer period of resolution, when compared to CCL4 models (Zimmerman *et al.,* 1987, Dashti *et al.,* 1989); and less heterogencity between study animals than CCL4 (Li *et al*., 2006).

Both the in vitro and in vivo data presented above support previous animal studies that have shown targeting the coagulation cascade can ameliorate fibrosis (Anstee *et al.,* 2008; Duplantier *et al.,* 2004; Scrotton *et al.,* 2009). Our data supports the hypothesis that targeting the coagulation cascade earlier would not only reduce thrombin, but control pro-fibrotic signalling secondary to FXa, hence maximising the antifibotic effect.

3.4.3. The potential implications for human studies

A large body of evidence from both in vitro and animal studies now exists which suggests modulating coagulation has potential benefits as an anti-fibrotic intervention in experimental liver fibrosis. Data presented in this study, which is the first that provides a head to head comparison of the anti-fibrotic properties of a FXa inhibitor and a direct thrombin antagonist, suggests that targeting the coagulation system upstream to inhibit both FXa and thrombin is a more effective anti-fibrotic strategy than targeting thrombin alone in animal studies. This has important implications when deciding on appropriate agents to take forward to clinical trials in human liver fibrosis. The recent development of Rivaroxaban, a novel factor Xa inhibitor which is now readily available commercially, provides us the opportunity to intervene earlier in the coagulation cascade. In view of the data from this study, demonstrating its efficacy in significantly reducing fibrosis induced in TAA exposed mice, with no bleeding related complications, it may be the logical choice to trial in human patients to prove the anti-fibrotic potential of anticoagulation in liver disease. Rivaroxaban has however only recently been approved by the relevant regulatory authorities for the prevention of thromboembolic disorders, and is currently being evaluated for treatment of thromboembolic disease to ensure it is safe to use as a lifelong anticoagulant in humans. Until the relevant approvals are granted for both its treatment dose and long term use in

humans, it would be unfeasible to design a clinical study to evaluate its anti-fibrotic potential in liver disease. Suitable alternative agents that are readily available with well defined side effect profiles and act on various coagulation factors include heparin and warfarin.

Heparin is an indirect thrombin inhibitor which complexes with the cofactor antithrombin, converting it from a slow to a rapid inactivator of thrombin, factor Xa, and to a lesser extent, factors XIIa, XIa, and IX (Hirsch *et al.,* 2008). However long term heparin use is not without limitations, including side effects such as thrombocytopenia, heparin induced hepatitis and osteopenia (Ansell *et al.,* 2004). These in conjunction with a parental route of administration and the need for therapeutic monitoring if given for prolonged periods, do not make heparin an ideal candidate to trial as a long term anti-fibrotic therapy. A more appropriate candidate would be warfarin. Warfarin inhibits the vitamin K-dependent gamma-carboxylation of coagulation factors II, VII, IX, and X (Ansell *et al.,* 2008). Therapeutic use of warfarin anticoagulation to prevent fibrosis in humans is not without precedent. Kubo et al in a clinical trial of warfarin in idiopathic pulmonary fibrosis reduced mortality at three years by 50% (Kubo *et al.,* 2005). In this trial, patients in the treatment group had their INR raised to between two and three with no associated morbidity which is in line with other reports of complication rates at this level of anticoagulation. There are some issues with the need for long term monitoring, but this is widely available and with the low cost of warfarin and the fact it is taken orally, it is currently the most appropriate drug to use in a trial to evaluate the anti-fibrotic potential of anticoagulation in patients with liver fibrosis. Therefore, warfarin needs to be evaluated in hepatic fibrosis using well designed clinical trials with relevant endpoints.

3.4.4. Conclusions

The results generated by this study provide further evidence to support the hypothesis that coagulation proteins, specifically FXa and thrombin, promote hepatic stellate cell contraction, activation and fibrogenesis. We have shown that FXa inhibition significantly reduced the rate of hepatic fibrosis and hepatic stellate cell activity in a TAA model of liver fibrosis, being a more effective anti-fibrotic than direct thrombin inhibition alone. This effect is likely due to thrombin inhibitors failing to block PAR-mediated stellate cell activation by FXa, and highlights the importance of inhibiting the coagulation cascade earlier when planning therapeutic approaches to hepatic fibrogenesis. FXa inhibiton would be a potential novel anti-fibrotic approach, but may be difficult to trial in liver patients at present without data about long term use and therapeutic dosing. Warfarin anticoagulation is currently a more appropriate candidate and now warrants further investigation as an anti-fibrotic in human chronic liver injury.

4. ANTICOAGULATION IN LIVER FIBROSIS: A PILOT STUDY

4.1. INTRODUCTION

Convincing evidence exists outlining a role for the coagulation system in the pathogenesis of liver fibrosis. In vivo and in vitro studies have suggested a role for thrombin and FXa in activating hepatic stellate cells (see Chapter 3) and epidemiological studies have demonstrated that prothrombotic states accelerate liver fibrosis (Wright *et al.,* 2003).

Hence if prothrombotic states accelerate liver fibrosis, conversely anticoagulation should slow liver fibrosis. Animal studies have confirmed this (Anstee *et al.,* 2008; Duplantier *et al.,* 2004), and confirmed the beneficial effect of inhibiting the coagulation cascade earlier (see Chapter 3). The number of patients with HCV infection on anticoagulants is small and there is no published case series. Similarly there are problems assessing disease progression using patients with haemophilia and HCV infection. The therapeutic use of anticoagulation to prevent fibrosis in humans is not without precedent, and warfarin has demonstrated a survival benefit in pulmonary fibrosis (Kubo *et al*., 2005).

The antifibrotic potential of warfarin anticoagulation needs to be formally assessed in the setting of a clinical trial using patients with documented liver fibrosis. Most previous human studies of antifibrotics have taken place in patients with chronic HCV infection who have failed anti-viral therapy, as they are a model of progressive fibrosis (Anstee *et al.,* 2009).

4.2. STUDY AIMS

1. To evaluate if any potential effect on the progression of liver fibrosis in patients infected with Hepatitis C virus, with moderate severity liver fibrosis is demonstrable with anticoagulation.

2. To evaluate the safety of anticoagulation in patients infected with Hepatitis C virus infection, with moderate severity liver fibrosis.

4.3. METHODOLOGY

4.3.1. Patients

The study was approved by the St. Mary's Hospital Ethics committee and conducted in accordance with the principles of the Declaration of Helsinki. Potential participants were identified via the departmental Hepatitis C database. All potential candidates were screened for the inclusion and exclusion criteria. Patients were eligible for inclusion if they were aged greater than 17 years of age, had evidence of active Hepatitis C viral replication (HCV RNA PCR positive), ALT of greater than 40 iu/ml, a modified histology activity index fibrosis score (Ishak *et al.*, 1995) of greater than 2 but less than 5 on liver biopsy within the last five years, and had failed antiviral therapy for Hepatitis C in the last 5 years. The exclusion criteria were: patients requiring anticoagulation for existing clinical indications; standard contraindications to anticoagulation (active peptic ulcer disease, past history of haemorrhagic stroke, thrombocytopaenia, platelets count < 100 x109 /L); clinical evidence of portal hypertension; known cerebrovascular abnormalities; HIV antibody positive; alcohol abuse (> 40 units/week); menhorragia and pregnancy. Potential qualifying subjects were initially contacted by telephone to be informed about the study and arrange a formal screening visit. During the screening visit, entry criteria were confirmed and all patients who agreed to participate were required to give written informed consent.

Table 4-1: Inclusion and exclusion criteria for pilot study.

Inclusion Criteria	Exclusion Criteria
Age > 17 years	Patients requiring anticoagulation for existing clinical indications
HCV RNA positive	Active peptic ulcer disease
ALT > 40 iu/ml	Past history of haemorrhagic stroke
Ishak Fibrosis score >2 and <5 in last 5 years	Thrombocytopaenia (platelets count < 100 x109 /L)
Failed antiviral therapy for HCV in last 5 years	Clinical evidence of portal hypertension
	Known cerebrovascular abnormalities;
	HIV antibody positive;
	Alcohol abuse (> 40 units/week)
	Menhorragia
	Pregnancy

4.3.2. Study Design

The study employed a single centred, prospective, open labelled design. Practical as well as safety concerns dictated that the study could not be conducted in a blinded fashion, since patients taking anticoagulation require monitoring. The study consisted of two 8 week phases: Phase 1 and Phase 2 (see Figure 4-1).

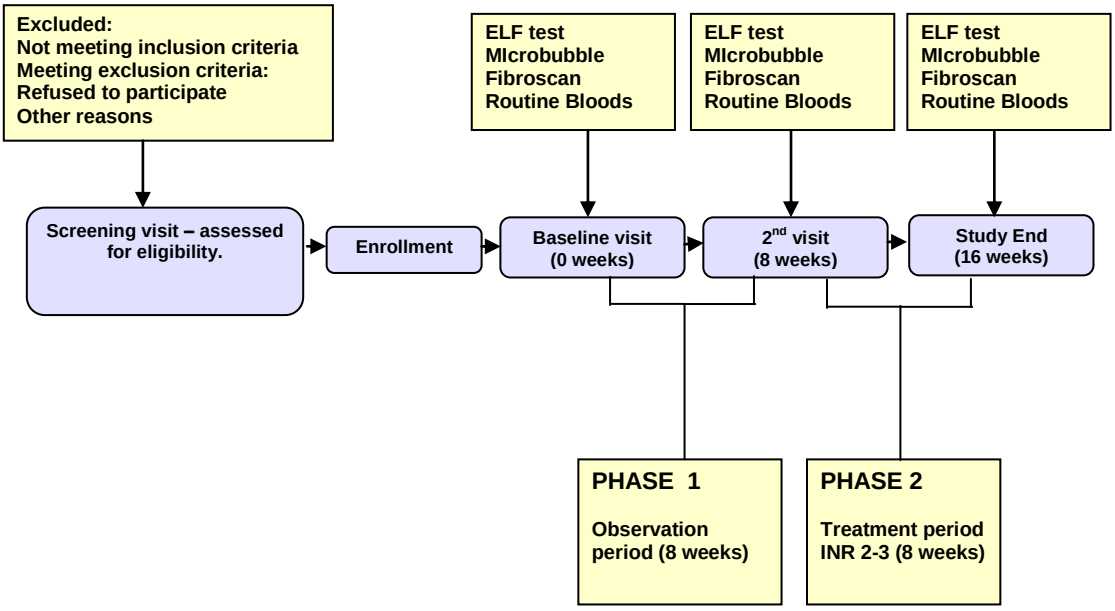


Figure 4-1: Anticoagulation in liver fibrosis: pilot study design

An open labelled study, including 2 eight week phases: Phase 1 (observation phase, 0 to 8 weeks) and Phase 2 (treatment phase with warfarin anticoagulation, 8 to 16 weeks). Study completed at end of Phase 2.

4.3.2.1. Phase 1 (Week 0 to Week 8)

Phase 1 of the study consisted of 8 weeks of observation, which commenced following a baseline visit at week 0. At the baseline study routine blood tests and non-invasive markers of fibrosis were performed. No placebo was given during the observation period. At week 8 patients underwent their second study visit during which routine blood tests and evaluation with non-invasive markers of fibrosis were repeated. The week 8 study visit marked the completion of Phase 1, following which patients entered Phase 2 of the study.

4.3.2.2. Phase 2 (Week 8 to Week 16)

Phase 2 of the study consisted of 8 weeks of anticoagulation with warfarin. In previous animal studies (Anstee *et al*., 2008), warfarin anticoagulation to achieve a whole blood clotting of twice the normal range was sufficient to retard fibrosis significantly, hence the international normalised ration (INR) was aimed to be maintained between 2 to 3 during the treatment period. Patients were given a standard induction regimen of warfarin in keeping with the outpatient warfarin loading protocol of the hospital's anticoagulation clinic. Warfarin was supplied by the hospital pharmacy. Routine INR monitoring and warfarin dosing was undertaken by the anticoagulation clinic on a weekly basis. Patients were monitored at these visits for any adverse events related to the treatment. At week 16, following 8 weeks of anticoagulation, a further study visit was organised. Routine bloods tests and non-invasive markers of fibrosis were performed at this visit, which marked the completion of each patient's participation in the study.

4.3.3. Evaluation of Patients

Patients were evaluated by physical examination, routine bloods test and non invasive markers of fibrosis at study visits performed at Week 0 (baseline), Week 8 (completion of observation period) and Week 16 (completion of anticoagulation period). (see Figure 4-1)

4.3.3.1. Routine Blood tests

At each study visit, patients underwent venesection for blood samples to test for serum biochemical markers of liver function, renal function, routine haematological investigations, and Hepatitis C viral load (see Figure 4-1). Liver function tests and platelet count were used to calculate the APRI score for each subject timepoint as described in Chapter 1.

4.3.3.2. Non invasive markers of liver fibrosis

Liver biopsies could not be justified ethically or clinically in this study (see section 1.5). Therefore non-invasive markers of fibrosis, the Enhanced Liver Fibrosis test, Hepatic transit times, and Liver Stiffness measurements were employed and performed on all patients at the Week 0, 8 and 16 study visits.

4.3.3.2.1. Enhanced Liver Fibrosis Test (ELF test)

Serum samples were obtained from patients at Week 0, 8 and 16 study visits for ELF testing. Serum was collected in a single serum separator tube. The sample was then separated and sent for analysis to a commercial third party, iQur Ltd (iQur Ltd, Southampton, UK) to generate a numeric test score derived from the ELF algorithm (Rosenberg *et al*., 2004).

4.3.3.2.2. Hepatic Transit Times

Hepatic vein transit times (HTTs) were performed on all patients at Week 0, 8 and 16 study visits. HTTs were measured with ultrasound by a trained ultrasonographer (Miss Nayna Patel) following an intravenous injection of microbubble contrast agent, SonoVue ® (Bracco, Milan, Italy) using a previously described method (Lim *et al*., 2005). An intravenous cannula was inserted into the antecubital fossa of the left arm of each patient and via a three way tap, 1ml of the reconstituted microbubble contrast reagent was injected, followed by 8mls of normal saline. The time taken for the contrast agent to arrive in the hepatic artery and then the hepatic vein was recorded using scanner contrast agent software and vascular recognition imaging with ultralow acoustic power (Toshiba Medical Systems Europe, Zoetermeer, Netherlands). The HTT was calculated as the hepatic vein arrival time minus the hepatic artery arrival time in seconds. The HTT was measured twice for each subject at each visit and the recorded HTT was taken as the mean of the two measurements.

4.3.3.2.3. Liver Stiffness Measurement

Liver stiffness measurements (LSM) using transient elastrography was measured using a Fibroscan® machine (EchoSens, Paris, France) at the Week 0, 8 and 16 study visits. Only experienced operators, who had performed a minimum of 50 previous examinations using the standard previously described technique (Zoil *et al*., 2005, Foucher *et al.,* 2006) performed the tests (Dr Ameet Dhar, Dr Jeremy Cobbold). With the patient lying in dorsal decubitus with the right arm in the maximal abduction, an adequate section of liver tissue in the right lobe free of large vascular structures and the gallbladder was identified via an intercostal space. The tip of the FibroScan transducer was covered with a drop of gel and

placed perpendicularly in the intercostal space. Liver Stiffness was measured on a cylinder of hepatic tissue of 1 cm in diameter and 2–4 cm in length. As previously described, 10 measurements were taken, requiring an interquartile range (IQR) over a median ratio lower than 30% were considered valid.

4.3.4. ANALYSIS

Data was collated using Microsoft Excel (Microsoft Inc, USA). Statistical analysis was performed using SPSS version 14 (SPSS, USA). Quantitative data is expressed as mean ± standard error of mean. The Wilcoxon signed rank test and paired t–test were used for comparisons as appropriate. Statistical significance was accepted at $p<0.05$. Graphs were generated using Microsoft Excel and SPSS (Microsoft Inc, USA) with error bars representing standard error of mean or range where appropriate.

4.3.4.1. Primary Endpoint:

The primary endpoint was the proportion of patients with a reduction in liver stiffness measurements following the 8 week period of anticoagulation compared to 8 week period of observation using a Wilcoxon signed rank test.

4.3.4.2. Secondary Endpoints:

The secondary endpoints were the proportion of patients with a reduction in serum markers of fibrosis and HTTs following the 8 week period of anticoagulation compared to the 8 week period of observation and the safety of anticoagulation defined by the number of adverse events.

4.4. RESULTS

4.4.1. Patient Disposition and Demographics

A total of 14 patients were enrolled in the study. Ten patients completed the study. Four patients withdrew from the study, all during the observation period due to personal reasons, and have not been included in the analyses (See Figure 4-2).

The mean age was 50.4 years (range 42 to 62 years), and the majority of patients were male (80%). All patients were Caucasian. The majority of patients had HCV genotype 3 (70%) and a baseline Ishak fibrosis score of 3 (80%) (See Table 4-1).

Figure 4-2: Consort flow demonstrating the disposition of patients through the anticoagulation pilot study.

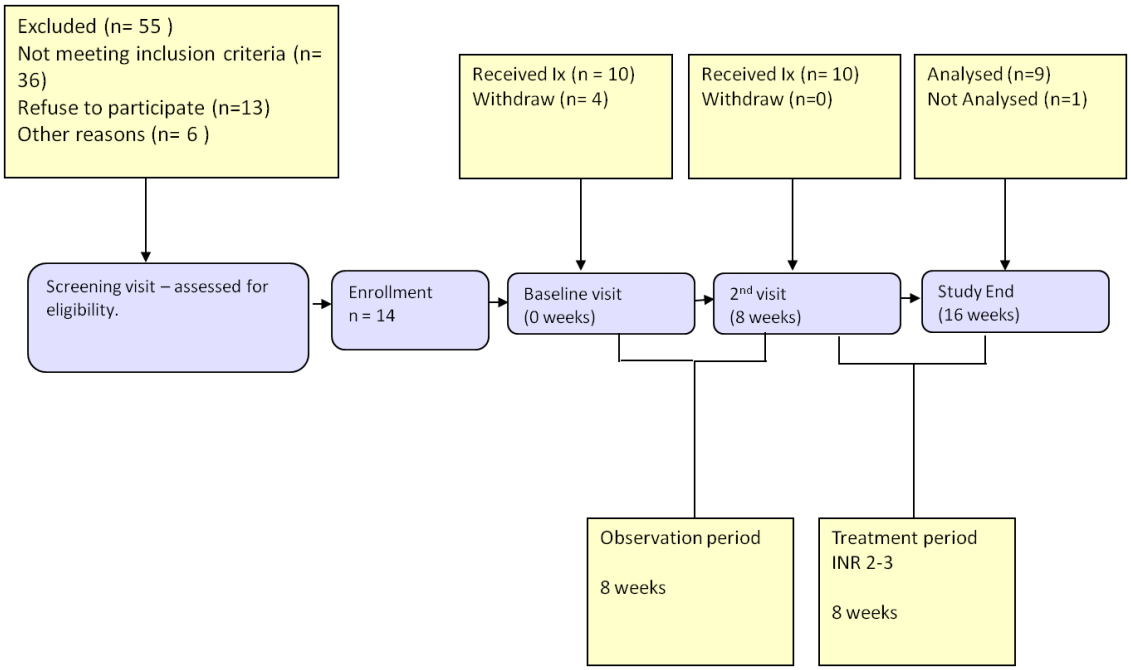


Table 4-2: Baseline demographics and select baseline characteristics for patients enrolled in warfarin anticoagulation for liver fibrosis; a pilot study.
(n=10)

Characteristic	
Age, years. mean (range)	49.5 (42-62)
Sex, n (%)	
Male	6 (60)
Female	4 (40)
Race, n (%)	
Caucasian	10 (100)
HCV genotype, n(%)	
1	2 (20)
2	0 (0)
3	7 (70)
4	1 (10)
Baseline Ishak score (in the last 5 yrs), n(%)	
3	8 (80)
4	2 (20)

4.4.2. Warfarin dosing and monitoring.

With the standard warfarin loading protocol, the time to reach therapeutic range (defined as an INR >2) took a mean of 10.6 ± 1.5 days (range 7-14 days). The mean number of total weeks each patient remained in therapeutic range was 5.9 ± 0.4 (range 5-7 weeks).

4.4.3. Withdrawals and Adverse Events

All patients who completed the observation phase of the study completed the anticoagulation phase of the study. Four patients withdrew from the study during the observation phase citing personal reasons. Data points from these four patients were not included in the study analyses. No adverse events were reported during the observation phase of the study. No patients withdrew after commencing the treatment phase. A single adverse event was noted in one subject during the anticoagulation period. This was classed as minor in severity and related to an episode of minor haemorrhage secondary to an episode of gingivitis, which resolved following antibiotics and did not require blood transfusion, but did require cessation of the anticoagulation for a short period of 2 days. This patient was not included in the analysis. No other subjects required cessation of treatment during the anticoagulation period.

4.4.4. Serum indices and body mass index

No significant differences were seen in mean values for transaminases, APRI score, albumin, total bilirubin, platelet count nor HCV viral load between the baseline timepoint (week 0), completion of the observation period (week 8) and completion of the period of anticoagulation (week 16) (Table 4-2).

Table 4-3: Routine serum indices and body mass indices for patients enrolled in warfarin anticoagulation for liver fibrosis; a pilot study.

At baseline (week 0), completion of observation period (8 weeks) and completion of treatment period (16 weeks). (n=9). Values expressed as mean (SEM). No significant differences were seen in mean parameters between any of the three study time points.

	0 weeks	8 weeks	16 weeks	p values*
ALT (iu/L) [0-40]	133.8 (24.5)	127.8 (37.4)	126.0 (34.9)	NS
AST (iu/L) [0-40]	78.5 (16.0) [+]	90.7 (23.4)	89.4 (19.3)	NS
APRI Score	1.1 (0.2) [+]	1.3 (0.1)	1.2 (0.3)	NS
BMI (kg/m^2)	24.9 (1.9)	24.8 (1.8)	24.9 (1.8)	NS
Albumin (g/L) [35-50]	42.6 (0.9)	42.6 (1.4)	40.5 (1.2)	NS
Total Bilirubin (µmol/L) [0-20]	8.6 (1.1)	9.0 (1.6)	9.0 (1.3)	NS
Platelets $x10^9/L$	191 (13.2)	185 (11.5)	184 (13.0)	NS
HCV viral load ($x10^6$copies/ml)	1.9 (1.2)	1.5 (0.9)	1.3 (0.6)	NS
ELF score	9.34 (0.2)	9.25 (0.2)	9.22 (0.2)	NS

Reference ranges included in square brackets. Abbreviations: ALT, alanine aminotransferase; AST, asparate aminotransferase; HCV, hepatitis C virus. ELF, Enhanced Liver fibrosis test; APRI, asparate aminotransferase to platelet ratio index.

* P values for 0 weeks versus 8 weeks, 0 weeks versus 16 weeks, and 8 weeks versus 16 weeks analyses. NS, non-significant ($p>0.05$).

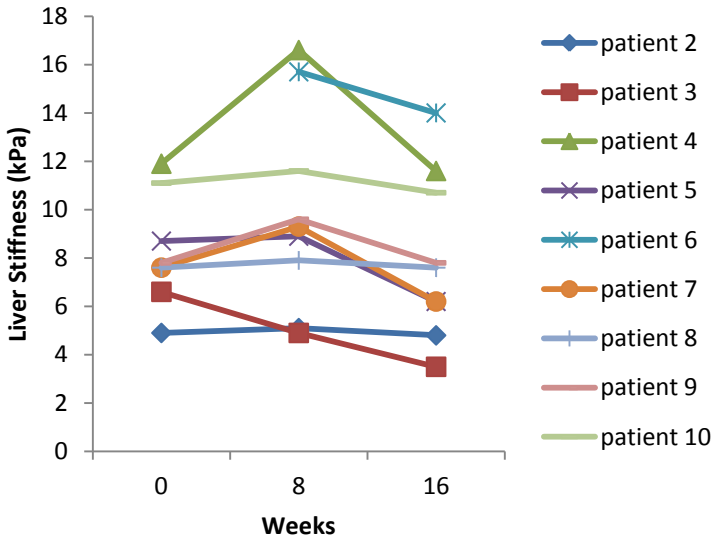
[+] Denotes n=7

4.4.5. Non invasive markers of Fibrosis

4.4.5.1. Liver Stiffness Measurement (LSM)

A significant reduction in the median liver stiffness values was demonstrated on completion of the period of anticoagulation at week 16 (6.90 kPa) versus completion of the observation period at week 8 (9.60 kPa; p=0.008), and start of the observation period at week 0 (7.70 kPa; p= 0.028) (Figure 4-4). There was no significant difference in liver stiffness values between the start and end of the observation period (Figure 4-4). Furthermore individual data showed concordance in liver stiffness measurements between subjects following anticoagulation (Figure 4-3). The mean success rate overall for LSM measurements was 88.7% +/-2.59 with a mean IQR ratio of 16.0%+/-1.06, which were both within valid parameters.

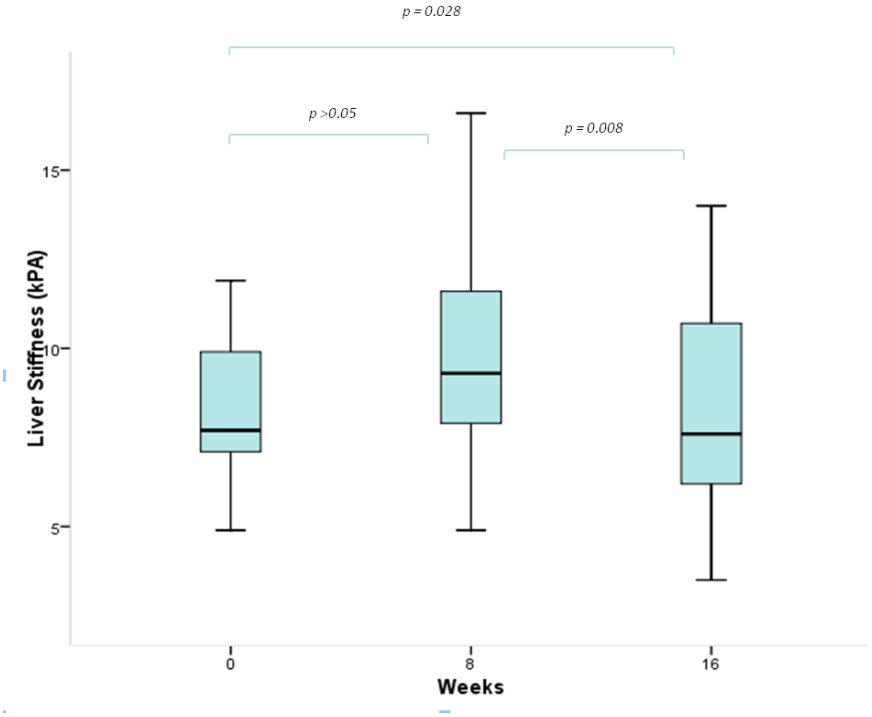
Figure 4-3: Individual liver stiffness values at baseline, 8 weeks and 16 weeks.
Concordance is seen in liver stiffness values in response to treatment with anticoagulation. (n=9)



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Figure 4-4: Liver stiffness values plotted against study time points.

Represented as a box and whisker plot showing median (horizontal line); IQR (box) and range (whiskers). Significant reduction in liver stiffness measurements following anticoagulation was demonstrated following warfarin anticoagulation (8 vs 16 weeks: p=0.008; 0 vs 16 weeks: p=0.028).



4.4.5.2. Hepatic Transit Times (HTT):

No significant differences in mean HTTs measured by dynamic contrast ultrasound, were demonstrated at any of the timepoints. There was a trend towards prolongation of the mean HTT at completion of anticoagulation period (11.7 ±1.2 secs) in comparison to the mean HTT at completion of the observation period (11.2 ± 1.5 secs). However this trend did not reach statistical significance (Figure 3-4). Individual data showed no concordance in HTT values in response to treatment with warfarin anticoagulation.

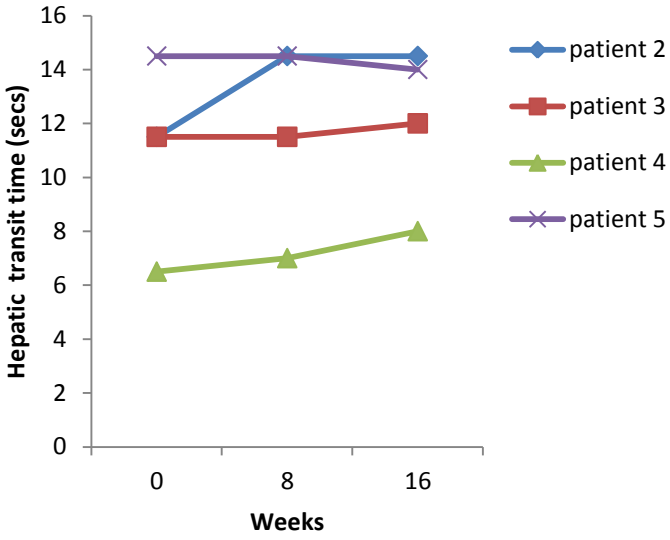


Figure 4-5: Individual patients liver HTT at baseline, 8 weeks and 16 weeks.
No concordance is seen in individual HTT values in response to treatment with anticoagulation. (n= 4)

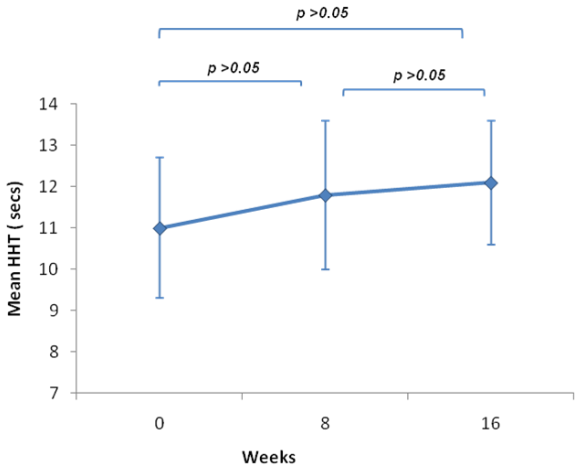


Figure 4-6: Mean HTT values plotted against study time points.
In seconds at each time point showing a non–significant trend to a prolongation following anticoagulation. Error bars represent standard error of mean.

4.4.5.3. Enhanced Liver Fibrosis (ELF) Test scores:

No significant changes in the mean ELF test scores were demonstrated between timepoints. No concordance was demonstrated between patients (Figure 4-7). Anticoagulation failed to demonstrate a significant improvement in ELF test scores in comparison to week 0 and week 8 results (9.22± 0.2 versus 9.34 ± 0.2 and 9.22± 0.3) (Figure 4-8).

Figure 4-7: Individual patients ELF test scores at baseline, 8 weeks and 16 weeks.

No concordance is seen in ELF test values in response to treatment with anticoagulation. (n= 9)

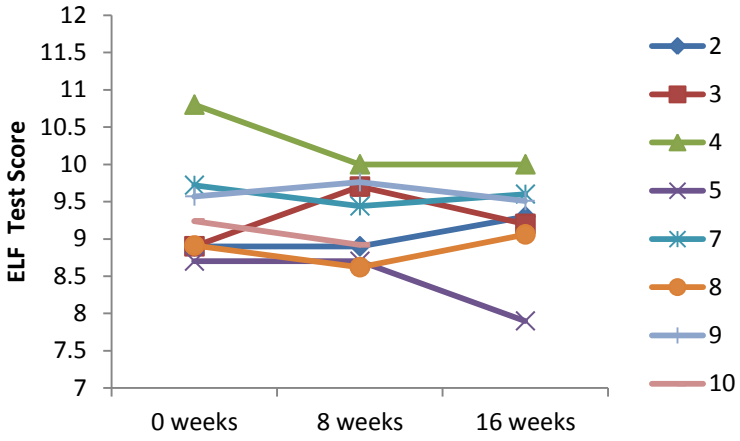
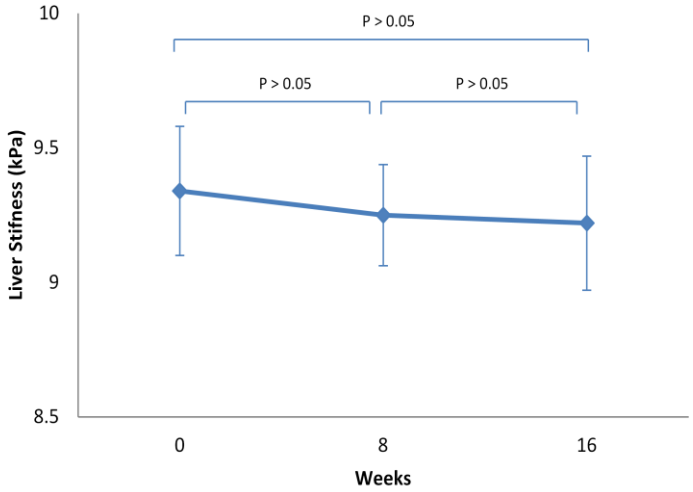


Figure 4-8: Mean ELF test scores plotted against study time points.

No significant change in the ELF test score was demonstrated following completion of the anticoagulation period (16 weeks) compared to completion of the observation period (8 weeks). Error bars represent standard error of mean.



4.5. DISCUSSION

Hepatic fibrosis is a dynamic and complex process with interactions between host genetic factors and environmental influences (Anstee et al., 2009). A strong body of evidence suggests a role for the activation of coagulation proteins in the generation of liver fibrosis. Upregulation of coagulation proteins in fibrotic liver tissue (see Chapter 2), the role of thrombin and FXa in stellate cell activation (see Chapter 3) and epidemiological evidence suggesting an association between prothrombotic tendencies and rapid fibrosis in HCV (Wright et al., 2003), all support this theory. Based on the strength of this scientific rationale, we hypothesised that inhibition of the coagulation cascade should result in an antifibrotic state. Indeed several animal studies have previously supported this, including data presented in this thesis (see Chapter 3), but as yet this hypothesis has not been adequately studied in the human setting of liver fibrosis. To this avail, this pilot study used an open labelled design to evaluate the impact of an eight week period of treatment with warfarin anticoagulation on non invasive markers of fibrosis in subjects with Hepatitis C virus infection with previously documented moderately severe liver fibrosis.

The results demonstrate warfarin anticoagulation significantly reduced median liver stiffness values compared to values at the start and end of the observation period, with no significant difference in liver stiffness values between the start and end of the observation period (see Figures 4-3 & 4-4). A non significant trend towards prolongation was seen in mean hepatic transit times following anticoagulation but no significant differences between ELF test scores, serum ALT values and APRI scores were demonstrated following anticoagulation (see Figures 4-5 to 4-8).

4.5.1. Safety of Warfarin Anticoagulation

Anticoagulation with warfarin is widely used in medicine as a treatment and prophylaxis of thombo-embolic disease. There is a vast wealth of experience when monitoring and dosing warfarin in the outpatient setting, with a network of anticoagulation monitoring clinics based in hospitals and the community (Choudry *et al*., 2006). Despite the proven benefits of anticoagulation, a number of clinicians (and patients) have reservations about the use of these drugs particularly if they have witnessed a significant adverse event (Choudary *et al.,* 2006), which probably accounts for the under use of anticoagulation where clinically indicated (Rashid *et al.,* 2006). It is therefore natural for hepatologists to have safety concerns in view of the risks of bleeding from oesophageal varices in advanced liver disease.

No studies have previously investigated the tolerability of warfarin anticoagulation as an anti-fibrotic in pre-cirrhotic patients infected with hepatitis C. In this small pilot study of patients with underlying moderate HCV related hepatic fibrosis, no serious adverse events were reported during the anticoagulation period. One patient had a minor adverse event, requiring temporary cessation of warfarin treatment, and was excluded from the analysis. Treatment was otherwise tolerated well such that no periods of treatment cessation were required in any other patient who was started on warfarin. The safety data from this study is in keeping with various other studies have which have demonstrated an acceptable safety profile for anticoagulation in patients with advanced hepatic fibrosis studied in the setting of extrahepatic portal vein thrombosis. Prolonged periods of anticoagulation administered in several series have not been associated with an increased risk of bleeding and considered relatively safe in this setting even in the presence of cirrhosis (Romero-Gomez *et al.,* 2002; Amitrano *et al.,* 2010; Francoz *et al.,* 2005). Furthermore, a single small study has demonstrated a role for heparin anticoagulation as a potential prophylactic agent against portal vein thrombosis in patients with Child B and C cirrhosis. In addition to a significant

reduction in the incidence of portal vein thrombosis and episodes of decompensation, importantly there was no increased risk of bleeding in cirrhotic patients given heparin for a period of 24 months compared to control group patients treated with placebo alone (Zechini *et al.,* 2010). A similar safety profile has been demonstrated in the post-transplant setting. Burroughs et al (*unpublished data*) have audited a group of 80 patients who received liver transplants at the Royal Free Hospital and who were anticoagulated for standard clinical indications such as Budd Chiari syndrome. In this group of patients, no anticoagulation associated adverse events occurred. Therefore prolonged anticoagulation is not without precedent in liver patients and from a safety perspective, can be considered in patients with or at risk of developing hepatic fibrosis, whether it be in the setting of an anti-fibrotic clinical trial or treatment for thrombo-embolic disease.

4.5.2. Non Invasive markers of Fibrosis: The effect of anticoagulation on liver stiffness

A number of non invasive markers have been developed, including serum and imaging tests. In this study we employed a combination of three non invasive tests to evaluate the impact of warfarin anticoagulation as an antifibrotic: Hepatic Transit Times, measured by microbubble contrast ultrasonography; Liver Stiffness measurements by transient elastrograpghy (Fibroscan®) and the Enhanced Liver Fibrosis (ELF) test, a serum panel of three direct markers of fibrosis (HA, TIMP-1, and PNIIIP).

We have demonstrated that an 8 week period of warfarin anticoagulation significantly reduced median liver stiffness values compared to values at the start and end of the

observation period. Additionally, as would be expected, there was no significant difference in liver stiffness values between the start and end of the observation period.

Fibrosis is a dynamic process, the result of a net balance of matrix accumulation over degradation. Any proven antifibrotic over a reasonable period of time, could theoretically not only prevent fibrosis, but also induce regression of scar tissue, by tipping the balance away from accumulation towards degradation (Freidman 2008). Liver stiffness measurements are a surrogate marker of hepatic fibrosis, and a reduction in their values post anti-coagulation could potentially represent regression of liver fibrosis. This is an unlikely assumption, since an 8 week period of anticoagulation or indeed any potential antifibrotic agent would not be of sufficient duration to cause significant regression. A potential explanation for the reduction in liver stiffness demonstrated, would be the actions both indirect and direct of warfarin anticoagulation on hepatic stellate cell activation.

Hepatic stellate cells lie in the subendothelial space of Disse. On activation, the hepatic stellate cell contracts, a state that is characterised by the increased expression of alpha smooth muscle actin, a contractile filament protein (Rockey *et al*., 1992). This results in impedence of sinusoidal blood flow increasing portal resistance (Freidman 2008). Since liver stiffness not only depends on the extracellular matrix of the organ, which transfers the external forces through the liver, but also intrahepatic resistance to blood flow (Mueller *et al.,* 2010) and has been shown to correlate with hepatic venous pressure gradients (Carrion *et al.,* 2006), hepatic stellate cell activation directly influences liver stiffness measurements. Indeed Dechêne et al have demonstrated a positive correlation between liver stiffness, the degree of liver cell damage, and alpha smooth muscle actin expression, a marker of stellate cell activation and contractility in liver injury (Dechêne *et al.,* 2010). Therefore if it is

accepted that stellate cell activation and subsequent contraction can result in increased liver stiffness, then conversely stellate cell relaxation could explain in part the reduction in liver stiffness values demonstrated with warfarin anticoagulation.

Evidence presented in this thesis (see Chapter 3) suggests that both factor Xa, and thrombin when given to cultured human stellate cells stimulate activation, upregulate alpha smooth muscle actin expression and cause significant cell contraction. Furthermore these effects are potentiated when both are given in combination and reversed when the respective direct factor Xa and thrombin inhibitors are added to culture medium. Warfarin inhibits the effective synthesis of biologically active forms of the vitamin K-dependent clotting factors II, VII, IX and X, as well as the regulatory factors protein C and protein S. The net effect is a reduction not only in the generation of the individual factors, but decreased generation of fibrin and hence any potential microemboli deposition in the hepatic microcirculation. More importantly however is the potential reduction in stellate cell activation via factor Xa and thrombin mediated PAR 1 and PAR 2 ligation, and hence the maintenance of the stellate cell in a quiescent non fibrotic, non contractile relaxed state. This potential for anticoagulation to reduce stellate cell activation and contractility could account for the observed reduction in liver stiffness values seen in patients post anticoagulation.

Various factors are recognised to influence liver stiffness in addition to fibrosis, sinusoidal resistance and hepatic portal pressures. There is evidence that inflammation and steatosis can contribute to liver stiffness measurements (Cobbold *et al.,* 2007) and hence we have to consider whether changes in either could account for our findings. Since there was no significant difference in transaminases nor BMI measurements (see Table 4-3) between any

of the timepoints, we can assume that these factors have not confounded our findings and have not significantly influenced liver stiffness measurements between timepoints.

4.5.3. Non Invasive markers of Fibrosis: The effect of anticoagulation on other non invasive markers

Despite a reduction in liver stiffness measurements following anticoagulation, a non significant trend towards prolongation was seen in mean hepatic transit times following anticoagulation (see Figures 4-5 & 4-6) and no significant differences between ELF test scores (see Figures 4-7 & 4-8), serum ALT values nor APRI scores (see Table 4-2) were demonstrated following anticoagulation.

Despite diagnostic accuracy in differentiating mild from severe fibrosis, there is evidence that there is substantial overlap when differentiating patients with mild to moderate fibrosis with non invasive markers of fibrosis (Ziol *et al.,* 2005; Cobbold *et al.,* 2007, Lim *et al.,* 2005). Regarding the ELF test, it is unclear whether the serum markers used in this test are sufficiently sensitive to detect the small size of effect on fibrosis that would have occurred after only 8 weeks of anticoagulation. It is also unclear if there was a significant difference in each of the component markers of the ELF test, since the test was performed commercially and due to contractual issues, individual component scores were not disclosed to us. It would be important to explore whether any significant effect was seen in the individual components, but nullified when entered into the ELF algorithm.

Surprisingly there was no significant change in HTTs in patients while anticoagulated, despite a trend towards prolongation following warfarin. Changes in hepatic transit times are thought to be related to changes in intrahepatic vascularity (Sugimoto *et al*., 2002) and have

previously been shown to demonstrate a correlation with even mild fibrosis scores (Lim *et al*., 2005). We would have expected the anticoagulant action of warfarin to cause an alteration in intravascular dynamics within the liver possibly secondary to effects on hepatic intravascular resistance. The failure to demonstrate a significant effect in this marker may be explained by the fact all patients recruited had documented evidence of only moderate fibrosis (Ishak fibrosis stage 3 or 4). If we had recruited patients with more severe fibrosis, a more profound effect in the HTTs may have been demonstrated. Secondly the small number of patients in the study who underwent hepatic transit time assessment (n=5) will have had a significant effect on our results. A larger study number would have reduced the impact of inter-patient variability, and possibly allowed for the trend we demonstrated to reach statistical significance. Finally, the method by which we recorded transit times may have influenced the effect demonstrated. After intravenous injection of the microbubble contrast, the HTT was calculated, as the difference between the arrival time of the contrast to the hepatic artery and hepatic vein. More recently, an alternate method of assessing regional hepatic perfusion has been suggested, using microbubble contrast enhanced ultrasound (Berzigotti *et al.,* 2011). Since microbubble contrast remains entirely within the intravascular space it is possible to destroy the microbubbles in the area being scanned by ultrasound using high emission power and then to measure the replenishment curve of a region of interest using specifically designed computer software and to calculate regional perfusion. This technique has recently been demonstrated as a reliable method for estimating hepatic perfusion in cirrhotics and a surrogate marker for hepatic function and stage of fibrosis. It may be that this alternative method would have been more sensitive when attempting to demonstrate more subtle changes in intrahepatic blood flow and resistance (Berzigotti *et al.,* 2011).

4.5.4. Contribution of study design to results and the potential of warfarin anticoagulation as an antifibrotic in liver disease

The small number of patients in the study will have significantly affected our results. Despite this, we still demonstrated a significant reduction in liver stiffness measurements following anticoagulation without any significant adverse events. The effect on the other non invasive markers of liver fibrosis needs to be evaluated further since a larger study group would have reduced inter-patient variability. Another consideration is the small size of effect on fibrosis that would be expected in this cohort of patients with 8 weeks of anticoagulation. The progression of fibrosis in HCV patients has been studied extensively. The median time from Hepatitis C infection to cirrhosis is 30 to 35 years (Poynard *et al.,* 1997, Wright *et al.,* 2003 B) with the rate of fibrosis in non-transplanted patients with HCV infection usually less than 0.2 fibrosis units/year (Wright *et al.,* 2003 B). Therefore to demonstrate significant changes in surrogate markers of fibrosis and fibrosis stage itself, an intervention in a pre-cirrhotic HCV patient group would need to be given for a minimum of five years (Poynard *et al.,* 1997, Wright *et al.,* 2003 B). In our study the intervention period was only 8 weeks, and a treatment period of over 5 years is not feasible due to potential ethical and recruitment issues.

A more appropriate patient group and intervention period therefore needs to be considered to evaluate more accurately the role of warfarin anticoagulation as an antifibrotic agent. An alternative would have been to identify and recruit patients specifically with rapid fibrosis or with advanced fibrosis or cirrhosis into the study. At the time of planning and carrying out this study, this would have been problematic on ethical grounds due to the perceived risks of haemorrhage secondary to portal hypertension in cirrhotic patients. This could however now be justified, since we have demonstrated a beneficial effect on liver stiffness

measurements in patients with pre-existing moderate fibrosis and an acceptable safety profile for anticoagulation in patients with both moderate and severe fibrosis has been demonstrated by this and other recent studies (Romero-Gomez *et al.,* 2002; Amitrano *et al.,* 2010; Francoz *et al.,* 2005).

A more suitable study group would be patients transplanted with HCV, where re-infection of the graft is inevitable and leads to recurrent hepatitis and fibrosis. Fibrosis post-transplant is frequently accelerated relative to the rates of progression pre-transplant and up to 20% of patients develop Ishak stage three or higher fibrosis within the first year post-transplant (Berenguer *et al.,* 2002). There is currently no licensed anti-fibrotic treatment for patients with accelerated fibrosis and early graft loss after liver transplantation. Trials of therapeutic intervention to reduce the rate of fibrosis in patients with HCV infection who have been transplanted are therefore urgently required and ethically justified. Furthermore, as transplant recipients receive annual liver biopsies under standard protocol, the histological monitoring of liver fibrosis is feasible. Hence this patient group represents a more appropriate cohort in which to evaluate the anti-fibrotic properties of warfarin anticoagulation. The WAFT-C study (Warfarin anticoagulation for liver fibrosis in patients transplanted for Hepatitis C infection), is a UK based multi-centred investigator-led non commercial trial, which has been set up to this avail, and will be discussed in further detail in the next chapter of this thesis.

4.6. CONCLUSIONS

The results generated from this small pilot study of patients with HCV and moderate fibrosis, following 8 weeks of warfarin anticoagulation, demonstrated a significant reduction in liver stiffness measurements. A possible explanation for this is hepatic stellate cell relaxation and reduction of intrahepatic resistance, but this needs to be studied further. No significant adverse events were reporting in this patient group while treated with anticoagulation. A non significant prolongation in hepatic transit times and no significant difference in serum markers of fibrosis were observed following anticoagulation, which could result from inherent limitations within the design of the study, including small numbers, a small effect and the chosen methods of assessment. The anti-fibrotic potential of anticoagulation therefore needs to be further evaluated and a large multi-centred study studying the effect of warfarin in patients transplanted for HCV infection is currently being undertaken, and will be described in the next chapter.

5. WARFARIN ANTICOAGULATION FOR LIVER FIBROSIS IN PATIENTS TRANSPLANTED FOR HEPATITS C VIRUS INFECTION (WAFT-C STUDY)

5.1. INTRODUCTION

To date there are no specific antifibrotic therapies licensed in the UK or elsewhere in the world. A number of possible candidates have been subjected to clinical trials, but none as yet have been given regulatory approval. A new candidate for fibrosis inhibition is therefore urgently required.

5.1.1. Evidence for anticoagulation as an anti-fibrotic for patients with liver fibrosis

A compelling body of evidence now exists to consider appropriate evaluation of anticoagulation as an anti-fibrotic in liver patients. In an immunohistochemical based study, we have demonstrated up-regulation of coagulation proteins, supporting existing evidence which suggests liver fibrosis is procoagulant state (see Chapter 2). Using in vitro and animal studies we have demonstrated a role for thrombin and FXa in hepatic stellate cell activation, and proven that targeting coagulation proteins earlier in the cascade is a more effective anti-fibrotic strategy than inhibiting thrombin alone (see Chapter 3). Taking these findings on, a short pilot study in humans with chronic HCV demonstrated that warfarin anticoagulation is safe in patients with pre-existing moderate fibrosis and results in a significant reduction in liver stiffness measurements, possibly due to hepatic stellate cell relaxation (see Chapter 4). These studies in conjunction with previous evidence, including epidemiological data which demonstrates pro-thrombotic conditions accelerate hepatic fibrosis, highlight the urgent

need for a well designed clinical trial with robust endpoints in an appropriate patient population to evaluate further the anti-fibrotic potential of anticoagulation.

5.1.2. Evaluating anti-fibrotics in liver patients: an appropriate patient group

Chronic HCV infection affects over 200 million people worldwide and approximately 400,000 people in the UK. Infection leads to chronic inflammation and progressive fibrosis. To date, human studies of chronic progressive liver disease have been conducted in patients with HCV infection since this is an expanding cohort of patients with a progressive fibrotic liver who may benefit from alternative strategies targeted at delaying disease progression (Anstee *et al.,* 2008). The progression of fibrosis in HCV patients has been studied extensively. The slow rate of progression of liver fibrosis in non transplanted HCV patients would make anti-fibrotic trials challenging due to associated study duration, issues with patient compliance and study costs.

HCV is now one of the leading indications for liver transplantation. In patients transplanted with HCV, re-infection of the graft is inevitable leading to recurrent hepatitis and fibrosis. Fibrosis post-transplant is frequently accelerated relative to the rates of progression pre-transplant and up to 20% of patients develop Ishak stage three (out of six) or more fibrosis within the first year (Berenguer *et al.,* 2002*)*. Accelerated fibrosis has been associated with steatosis, donor age and preservation injury but these putative factors remain controversial and fail to provide an explanatory mechanism in the majority of cases (Samonakis *et al.* 2004, Toniutto *et al*., 2005; Berenguer *et al.*, 2002). Prospective studies which can determine factors that influence fibrosis in HCV patients post transplantation are therefore required. No study to date has prospectively investigated the impact of prothrombotic tendencies on

fibrosis in this patient group. Indeed, endothelial damage with activation of coagulation occurs in both acute and chronic liver graft rejection (Olszewski *et al*., 2005), and up-regulation of various coagulation factors has been reported in the post-transplant liver with HCV infection (Smith *et al*., 2006). Therefore the incidence and significance of prothrombotic tendencies needs to be defined in patients in the setting of disease progression.

There is currently no adequate treatment for HCV patients with accelerated fibrosis and early graft loss after liver transplantation. Interferon based regimens to eliminate the virus are successful in less than one third of patients (Yedibela *et al.*, 2005). Re-transplantation is rarely an option due to donor shortages and because the second graft may become cirrhotic in less than one year. Trials of therapeutic intervention to reduce the rate of fibrosis in patients with HCV infection who have been transplanted are therefore urgently required and ethically justified. Furthermore as transplant recipients receive annual liver biopsies under standard protocol the accurate monitoring of liver fibrosis is feasible. HCV patients post liver transplantation therefore represent a suitable patient group to trial anti-fibrotic therapies since their clinical need is high and the requirement for interval liver biopsies adds no additional burden to their routine clinical care.

5.2. AIMS

1. To evaluate the impact of anticoagulation on the progression of liver fibrosis in patients who have received a liver transplant for hepatitis C related disease.

2. To determine whether stellate cell activation is diminished in patients who are anticoagulated.

3. To determine if the presence of thrombophilia promotes fibrosis in patients after liver transplantation.

4. To prospectively determine factors associated with post transplantation liver fibrosis in patients transplanted for HCV infection.

5. Evaluation of serum markers of liver fibrosis in patients after liver transplantation.

5.3. METHODOLOGY

The study has been by approved by Royal Free Medical School and Hospital Ethics Committee and the relevant local ethics committees for each participating site (Reference number: 07/Q0501/79). Clinical trial authorisation has been granted by the Medicines and Health Regulatory Authority (EudraCT number: 2006-006221-77). The study is currently ongoing and is being conducted in accordance with the principles of the Declaration of Helsinki (ISRCTN: 12504151).

5.3.1. Study Design

This study is a non commercial multi-centred open-labelled randomised controlled trial of anticoagulation in patients transplanted for HCV infection. The treatment / observation period is two years (see Figure 5-1). Recruited patients are allocated to the anticoagulation or control arms of the study using computer generated random number allocation. Stratification by centre and gender is performed as the impact of anticoagulation is more profound in males in animal studies (Anstee *et al*., 2007). Patients allocated to the treatment arm are given a standard induction dose of warfarin and monitored by measurement of their INR. Anticoagulation commences between 2 weeks and 4 months after the transplant procedure. In mouse studies anticoagulation to achieve a whole blood clotting time of twice the normal range was sufficient to retard fibrosis significantly. INR is maintained between 2 and 3. Patients will continue on warfarin for two years. Anticoagulated patients receive standard post-operative liver transplant care with regard to immunosuppression, treatment of opportunistic infections and treatment of episodes of acute rejection. Patients allocated to the control group are given standard post-operative liver transplant care. Immunosuppression is maintained with tacrolimus, azathioprine and prednisolone. A

screening visit takes place within the first four months following transplantation to confirm entry criteria and obtain consent. A baseline visit takes place following this and patients in the treatment group commence anticoagulation therapy immediately after the baseline visit.

5.3.2. Recruitment and Patients

The study is multi-centred, with the co-ordinating centre and sponsor of the study being Imperial College London. All participating centres are currently based in the United Kingdom, and are actively involved in the follow-up care of HCV patients post transplantation. Currently seven investigating sites have approval and are actively recruiting into the study. Two further sites are going through ethical and local research approval processes (see Figure 5-2).

All patients transplanted for end-stage liver disease related to HCV infection during the study period are considered for recruitment. The inclusion criteria (see Figure 5-1) are: HCV infection; age >17, and liver transplant within previous 4 months. Patients transplanted for hepatocellular carcinoma may be included. The exclusion criteria (see Figure 5-1) are: patients requiring anticoagulation for existing clinical indications; standard contraindications to anticoagulation (active peptic ulcer disease, past history of haemorrhagic stroke, thrombocytopaenia (platelets count < 60 $\times 10^9$ /L); large oesophageal varices persisting post-transplant; known cerebrovascular abnormalities; HIV antibody positive; and a baseline fibrosis score ≥2. Written informed consent is sought during the post-operative recovery period so that patients' judgement is not influenced by encephalopathy nor by a desire to please the transplant team in order to improve their prioritisation.

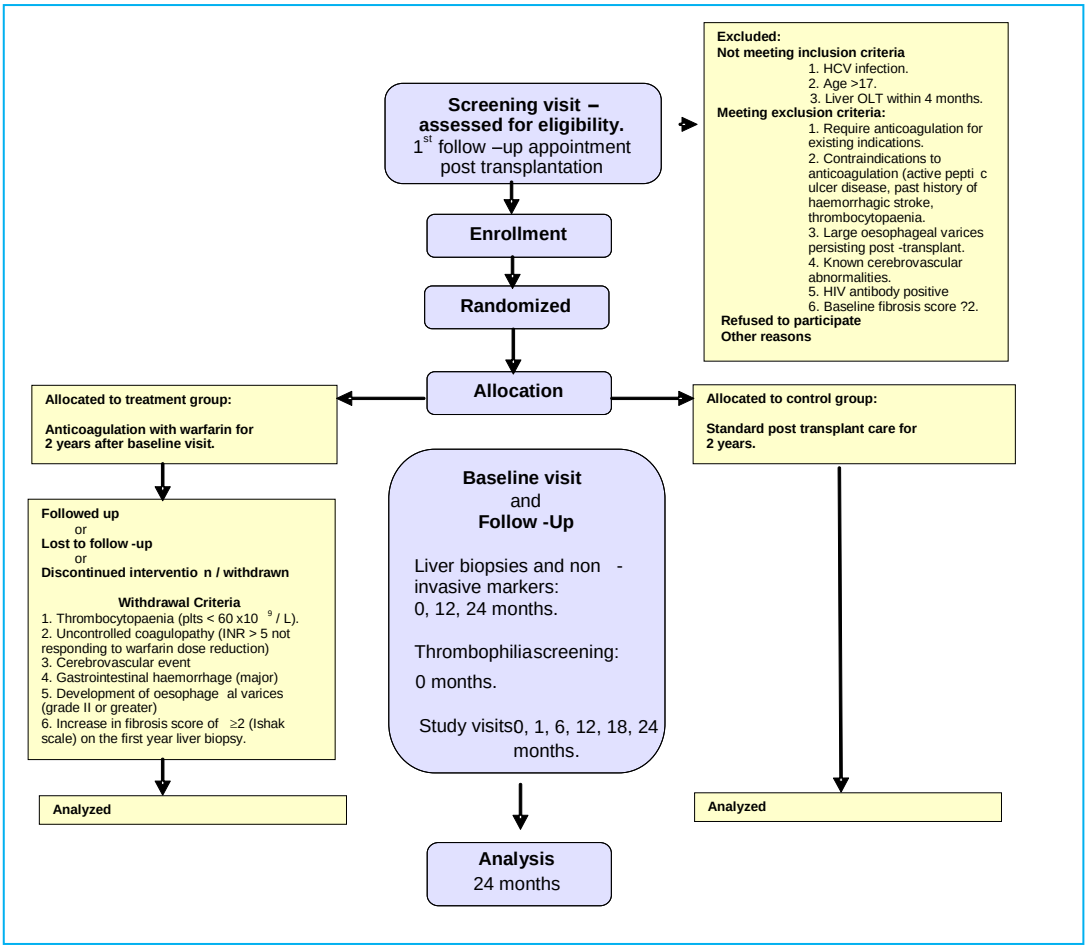


Figure 5-1: Flow chart detailing WAFT-C study design.

Flow chart detailing the study design for the WAFT-C trial. Patients screened for exclusion and inclusion criteria. Recruited patients are allocated to the anticoagulation or control groups. Patients allocated to the treatment arm are given warfarin. Control group patients receive standard post-transplant care. The treatment / observation period is two years. Routine study visits performed on each subject to monitor for withdrawal criteria or adverse events. Final analysis of results will be undertaken when all patients have completed the two year study period

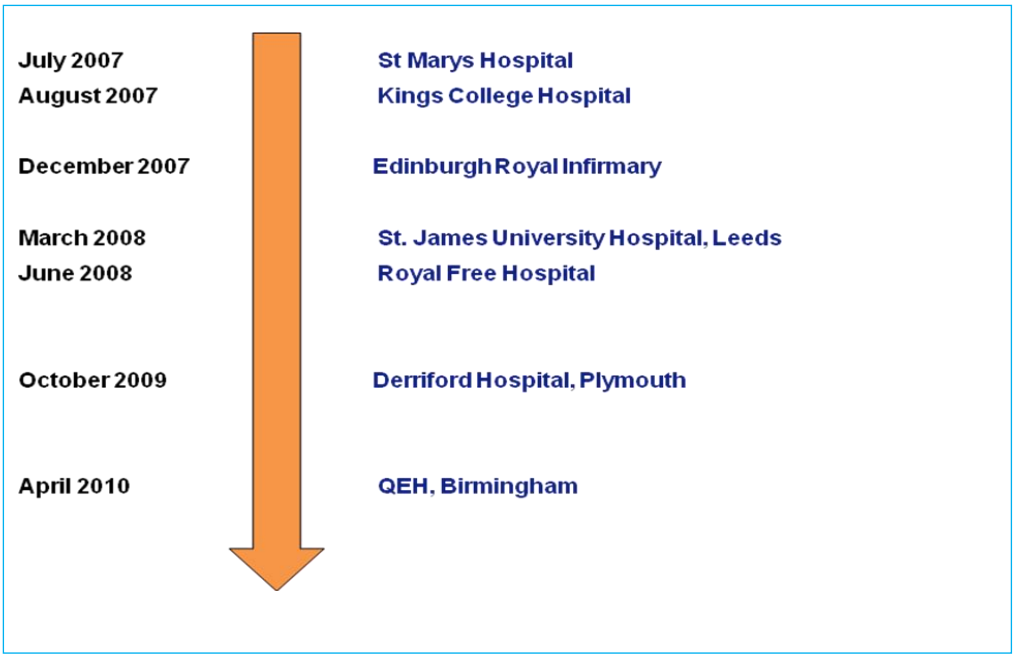


Figure 5-2: WAFT-C study centres and date of entry into study.

Flow chart detailing current participating study centres. Multi-centred investigator led study with a total of 7 sites currently approved for recruitment.

5.3.3. Evaluation of Patients

Routine follow up visits are performed at 6, 12, 18 and 24 months following date of transplant. (see Figure 5-1). Patients are assessed for withdrawal criteria at each visit (see Figure 5-1). The withdrawal criteria are: Thrombocytopaenia (platelets < 60 $x10^9$ / L); uncontrolled coagulopathy (INR > 5 not responding to warfarin dose reduction); cerebrovascular event; significant gastrointestinal haemorrhage; development of oesophageal varices (grade II or greater) and increase in fibrosis score of ≥2 (Ishak scale) on the first year liver biopsy. Adverse events are reported at each study visit in keeping with good clinical practice guidelines.

5.3.3.1. Liver biopsy

Liver biopsies are performed as per routine clinical care, at 12 and 24 months following transplantation. The outcome measures are fibrosis stage measured on liver biopsy after one and two years of treatment. Initial liver biopsies are assessed for fibrosis, steatosis and preservation injury. Annual biopsies are assessed for steatosis and, using the modified histological activity injury scales, for necroinflammatory grade and fibrosis stage. All biopsies will be stained by immunohistochemistry with antibodies specific for α smooth muscle actin to identify activated stellate cells (Carpino *et al.*, 2005) and an independent histopathologist assesses the biopsies, whilst blinded to the anticoagulation status of the patients. Each patient will complete their participation in the trial when the second year post-transplant liver biopsy has been performed.

5.3.3.2. Enhanced Liver Fibrosis (ELF) test

Serum samples are obtained from patients at the baseline visit and 12 and 24 months. Serum samples for the ELF test are collected and processed as described in Section 4.3.3.2.1.

5.3.3.3. Routine blood tests

Routine blood tests as described in section 4.3.3.1 are taken at all study visits. All routine blood tests are processed locally at each participating centre. Additionally serum samples for Cytomegalovirus PCR are taken at each study visit and processed by the virology department of each participating site.

5.3.3.4. Thrombophilic screen

Patients with thrombophilic conditions will be identified at recruitment. A thrombophilia screen is conducted to identify patients with FVL, mutations in thrombin, methylenetetrahydrofolate reductase, protein C, protein S and anti-thrombin III. or with anti-cardiolipin antibodies (Joffe *et al .,* 2002), and the impact of these conditions will be evaluated in terms of rate of fibrosis and response to anticoagulation treatment.

5.3.4. Sample Size and Study Analysis

5.3.4.1. Sample size calculation

In animal studies, anticoagulation reduced the rate of liver fibrosis by 50% and in human trials of anticoagulation in pulmonary fibrosis, mortality was reduced by 50% over a three year period. This is the first trial of anticoagulation for liver fibrosis in humans therefore no previous estimates of the size of effect are available. In a report from Berenguer et al 66% of patients transplanted with HCV infection had stage F1 fibrosis (Metavir scale) or greater one year after their transplant (Berenguer *et al*., 2006). If anticoagulation reduced the proportion achieving F1 or greater fibrosis at the end of the first year of treatment to 33% then a trial with 80% power to detect a difference at the $p<0.05$ level would need 35 patients in each group.

5.3.4.2. Interim analyses

For the purpose of this thesis, an interim analysis was performed comparing biopsy scores 12 months post-transplant between the warfarin and control groups and the number of

adverse events to date. The stage of liver fibrosis at baseline and one year, and the increase in fibrosis scores were compared using an unpaired t-test between the two study groups. Categorical data comparing the number of patients in each group with an increase in fibrosis score were compared using chisquare testing. The sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of serum markers of fibrosis to detect increases in histological fibrosis scores were calculated in this group of patients.

A true study interim analysis will be performed when all patients have had their 12 month post-transplant biopsy. The stage of liver fibrosis and number of activated stellate cells per high powered field will be compared using an unpaired t-test between the two study groups.

5.3.4.3. Final analyses

The final analyses will be undertaken on completion of the study, which is when the final patient has under gone a liver biopsy two years post transplant. The primary endpoint will be the proportion of patients with an increase in their fibrosis score of ≥2 in the treatment group compared to the proportion in the control group using chi squared test for univariate analysis after 2 years of anticoagulation. A multivariant analysis using logistic regression including covariates for gender, age at transplantation, immunosupression regimens, donor and graft characteristics and number of episodes of rejection will also be undertaken. The secondary endpoint will be the safety of anticoagulation as assessed by the number of adverse events noted during the study period.

5.4. RESULTS

The study is still actively recruiting and all investigating centres are contributing to patient recruitment (See Figures 5-4 & 5-5). The results presented below represent an interim analysis based on data collated up to May 2011.

5.4.1. Patient Disposition and Demographics

A total of 96 patients have been screened for study eligibility. Thirty one patients declined to consent to enter into the study, citing reasons relating to perceived inconvenience from monitoring of anticoagulation, concerns about poly-pharmacy and the perceived risk of bleeding.

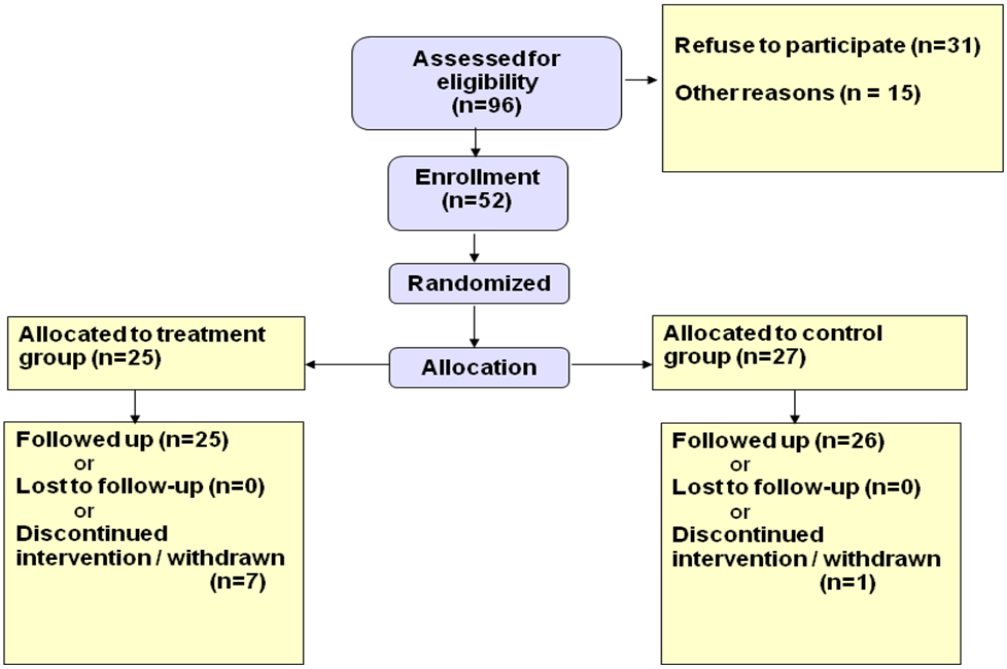
Fifty two patients have been enrolled into the study since July 2007. Twenty-five patients have been randomised and allocated to the treatment group to receive warfarin for a period of two years, and twenty-seven patients have been randomised and allocated to the control group, to receive standard post transplant care (See Figure 5-3).

The mean age of patients is 50.8 years (range 32.1-64.5 years), and the majority of patients are male (M:F, 40:12). There is no significant difference between age, sex, nor racial origin between the treatment and control group patients (See Table 5-1).

Table 5-1: Baseline demographics and select baseline characteristics of subjects enrolled in WAFT-C study.
(n=52)

Characteristic	Control Group	Warfarin Group
Number	27	25
Age, years. mean (range)	50.7 (32-64)	50.8 (33-60)
Sex, n (%)		
Male	22 (75.8)	21 (78.3)
Female	5 (21.7)	4 (24.1)
Race, n (%)		
Caucasian	23 (85.1)	22 (88.0)
Asian	4 (14.9)	3 (12.0)

Figure 5-3: Consort flow chart demonstrating the current disposition of patients through the WAFT-C study.



Consort flow chart detailing the stream of patients through the study as of May2011. Ninety six patients have been screened for study eligibility. Fifty two patients have been enrolled into the study since July 2007. Twenty-five patients have been randomised to the treatment group to receive warfarin and twenty-seven patients randomised to the control group. Seven patients have been withdrawn from the treatment arm and there was one fatality in the control group.

Figure 5-4: Recruitment rates over the course of the WAFT-C study.

Bar graph detailing recruitment rates over the study period. Steady recruitment rates over the course of the study period to date, with a range of one to ten patients over each 6 month period since recruitment commenced.

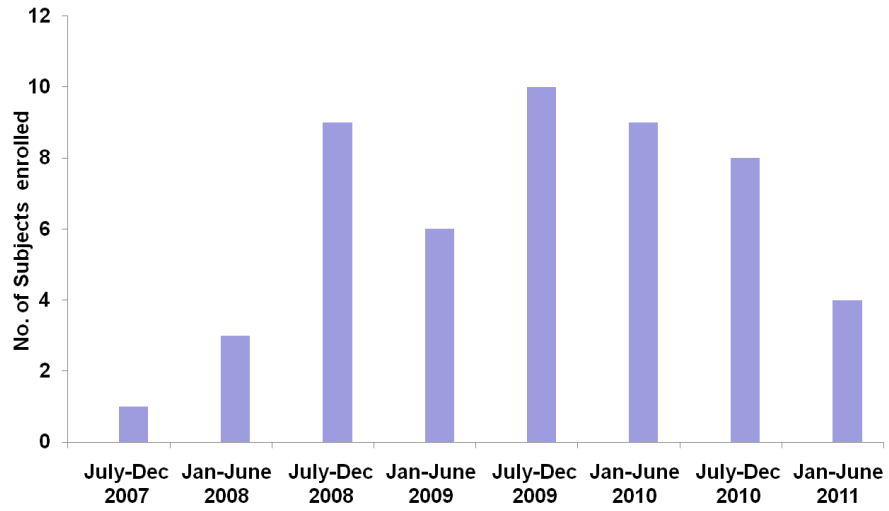
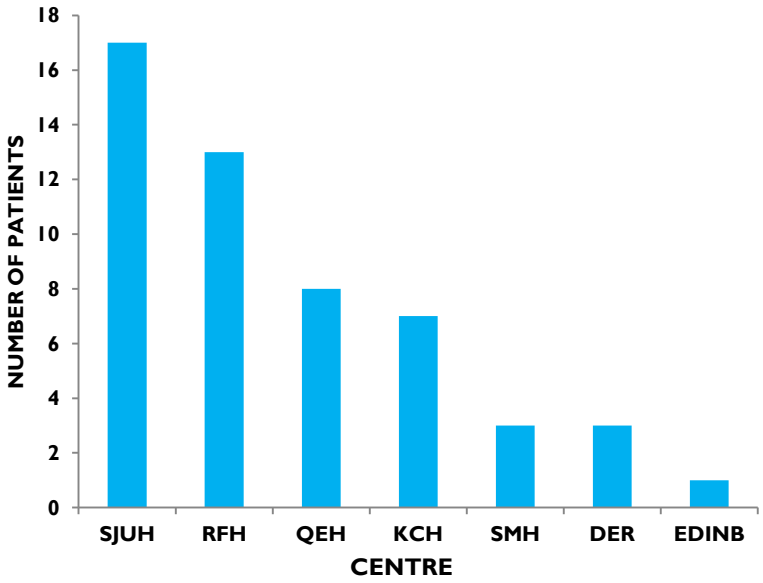


Figure 5-5: Recruitment by centre over the course of the WAFT-C study.

Bar graph detailing recruitment rates by centre. Each site is currently actively recruiting with a range of 1 to 17 patients per centre. Currently 52 patients have been recruited to the study.

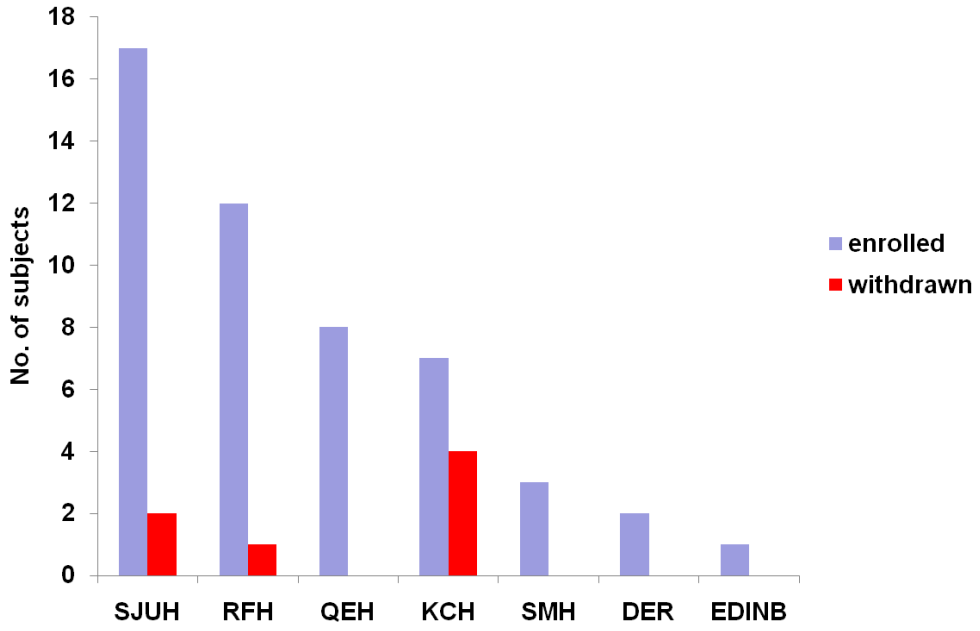


Abbreviations: SJUH, St. James University Hospital; RFH, Royal Free Hospital; QEH, Queen Elizabeth Hospital; KCH, Kings College Hospital; SMH, St. Marys Hospital; DER, Derriford Hospital; EDIN, Edinburgh Royal Infirmary.

5.4.2. Adverse Events and Withdrawals

No serious adverse events have been reported thus far in the treatment group. There has been one fatality in the control group, secondary to graft versus host disease (GVHD) but since no placebo is being used and the control group patients receive standard post transplant care only, no causality was attributed for this fatality to the study. Seven patients have been withdrawn from the treatment group. No patient has been withdrawn secondary to an adverse event relating to warfarin anticoagulation thus far. The majority of withdrawals have been secondary to protocol violations, in particular patients failing to start warfarin within the stipulated period post transplantation as set out in the study protocol (see Table 5-2 and Figure 5-7). Patients withdrawn from the study have been included in the intention to treat analyses, but have been excluded from the per protocol analyses.

Figure 5-6: Withdrawals by centre over the course of the WAFT-C study.
Seven patients from the treatment group and one patient from the control group have withdrawn.



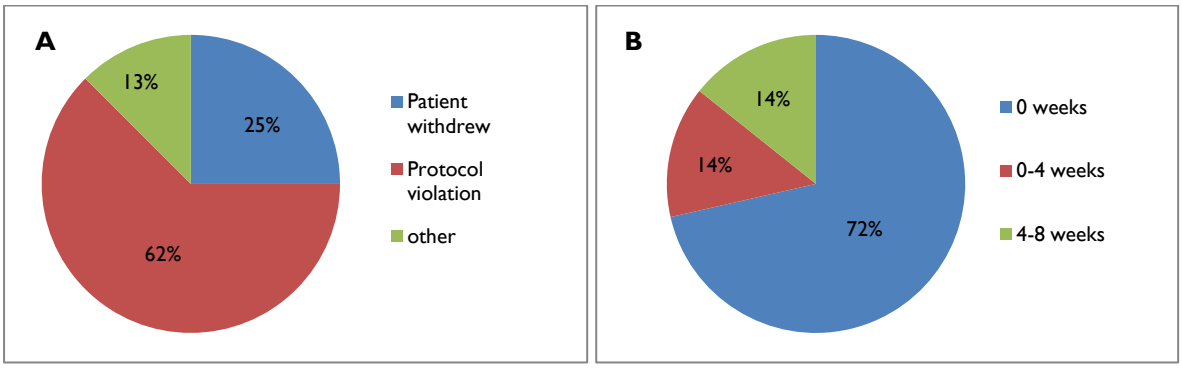
Abbreviations: SJUH, St. James University Hospital; RFH, Royal Free Hospital; QEH, Queen Elizabeth Hospital; KCH, Kings College Hospital; SMH, St. Marys Hospital; DER, Derriford Hospital; EDIN, Edinburgh Royal Infirmary

Table 5-2: Reasons for withdrawal from WAFT-C study to date.

Subject Number	Group	Reason for withdraw	Period of anticoagulation prior to withdrawal in weeks (if applicable)
003	Warfarin	Never started warfarin (protocol violation)	0
009	Warfarin	Patient withdrew from study citing issues with polypharmacy	1
012	Control	Deceased (GVHD)	Not applicable
025	Warfarin	Patient withdrew from study citing issues with polypharmacy	8
029	Warfarin	Never started warfarin (protocol violation)	0
033	Warfarin	Never started warfarin (protocol violation)	0
034	Warfarin	Never started warfarin (protocol violation)	0
036	Warfarin	Never started warfarin (protocol violation)	0

Abbreviations: GVHD, Graft versus host disease.

Figure 5-7: Reasons for withdrawal to date and duration of anticoagulation prior to withdrawal.



Pie charts showing the reasons for withdrawal **(A)** and duration of anticoagulation prior to withdrawal in patients randomised to treatment group **(B).** The majority of patients have been withdrawn due to protocol violations, primarily a failure to start anticoagulation within allotted study period. Two patients withdrew from the study citing issues with compliance and poly pharmacy. One patient in the control group developed graft versus host disease and died 8 months post transplantation. No patient has been withdrawn from the treatment group due to an adverse event as yet. All withdrawn patients in the treatment group had less than 2 months of anticoagulation, with the majority not even starting warfarin.

5.4.3. Fibrosis Scores

Both intention to treat analyses and per protocol analyses have been undertaken. All patients enrolled were included in the intention to treat analyses, irrespective of being withdrawn from the study. Patients who had been withdrawn from the study were not included in the per protocol analyses.

5.4.3.1. Intention to treat analyses

5.4.3.1.1. Baseline fibrosis scores

The baseline fibrosis score for each patient was taken from the most recent biopsy taken prior to enrolment into the study or commencement of warfarin treatment. If a subject did not have a post transplant liver biopsy prior to enrolment into the study, the baseline fibrosis score was determined as the donor fibrosis score at time of transplantation. If no biopsy had been taken of the donor liver at time of transplantation it was presumed that the donor fibrosis score was zero, and hence the presumed baseline fibrosis was zero. Baseline fibrosis scores were compared for 31 patients, whom had a one year biopsy performed to date.

There was no significant difference between baseline fibrosis scores between the treatment group in comparison to the control group (0.06 +/- 0.06 versus 0.07 +/- 0.07, $p>0.05$). Furthermore there was no significant difference between the baseline time point in weeks post transplantation between the treatment and control groups (see Table 5-3 and Figure 5-8).

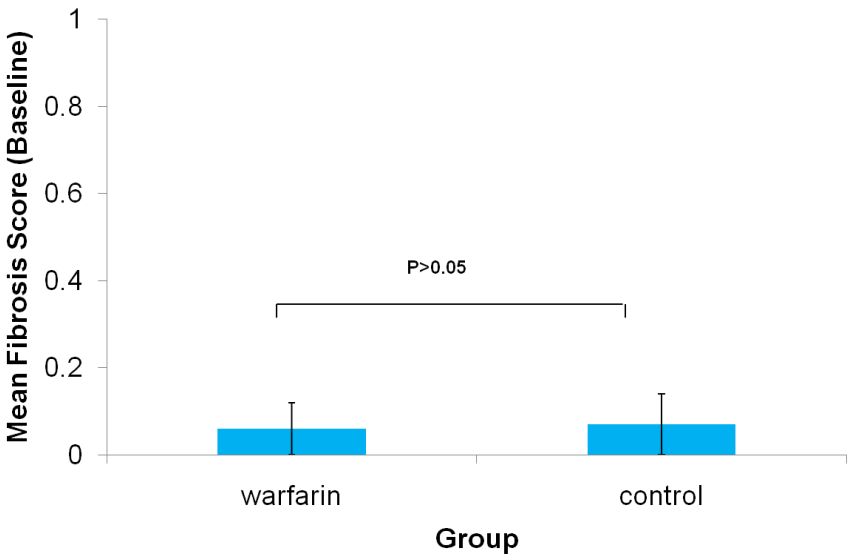
Table 5-3: Intention to treat analysis: Mean Ishak fibrosis score of warfarin and control group patients at baseline.

Values expressed as mean (SEM).

Group	No.	Mean Ishak Fibrosis baseline score	SEM
Warfarin Group	16	0.06	0.06
Control Group	15	0.07	0.07

Figure 5-8: Intention to treat analysis: Mean Ishak fibrosis score of warfarin and control group patients at baseline.

Error bars represent standard error of mean. No significant difference in baseline fibrosis scores were noted between the treatment group patients compared to control group patients.



5.4.3.1.2. One year post transplant fibrosis scores

The one year post transplant timepoint has been reached by 40 of the 52 patients currently enrolled in the study. One year post transplant per protocol liver biopsies have been performed on 31 of these 40 patients to date, and all these patients were included in the analysis. In the treatment group, 16 patients have had one year liver biopsies performed in comparison to 15 patients in the control group.

5.4.3.1.2.1. Mean one year post transplant fibrosis scoring.

A total of 31 patients have undergone one year protocol biopsies to date, patients treated with warfarin anticoagulation had a lower mean fibrosis score (0.68+/-0.21) at one year than patients in the control group (0.94+/-0.28), but this difference did not meet statistical significance ($p > 0.05$) (See Table 5-4 and Figure 5-9). There was no significant difference in the interval in weeks after transplantation at which the biopsies were taken between the two groups (see Table 5-4).

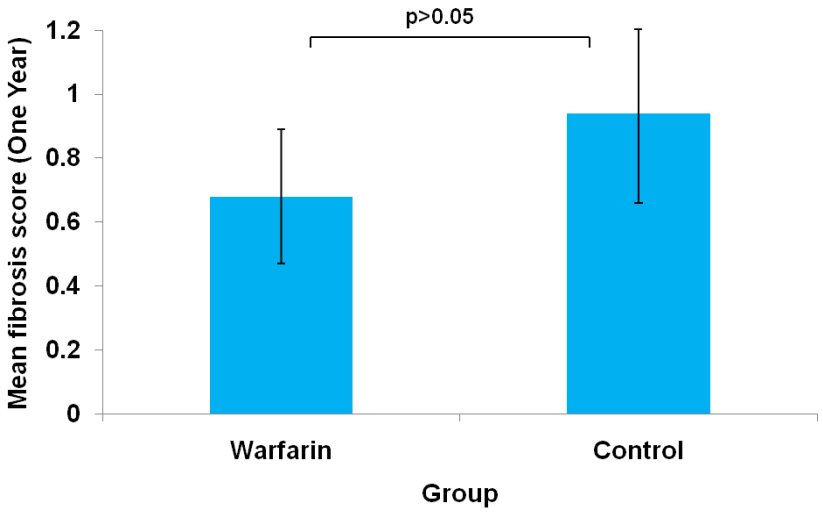
Table 5-4: Intention to treat analysis: Mean Ishak fibrosis score of warfarin and control group patients at one year post transplantation.

Values expressed as mean (SEM).

Group	No.	Mean Ishak Fibrosis score (SEM)	Mean Interval from date of transplantation in weeks (SEM)
Warfarin Group	16	0.68 (0.21)	46.7 (4.92)
Control Group	15	0.94 (0.28)	43.5 (3.65)

Figure 5-9: Intention to treat analysis: Mean Ishak fibrosis scores of warfarin versus control group at one year.

Error bars represent standard error of mean. A non significant reduction in fibrosis scores in treatment group patients compared to control group patients was demonstrated.



5.4.3.1.2.2. Mean increase in fibrosis score at one year post transplant

The mean increase in fibrosis was calculated by subtracting the baseline fibrosis score on entry into the study from the fibrosis score at year one for each patient. Warfarin anticoagulation resulting in a 40 % reduction in mean increase in fibrosis scoring at year one (0.56+/-0.21) compared to the control group (0.93+/-0.28). However in keeping with mean fibrosis scores, this did not meet statistical significance (p >0.05) (See Table 5-5 and Figure 5-10).

Table 5-5: Intention to treat analysis: Mean increase in Ishak fibrosis score from baseline to one year post transplantation in warfarin and control group.

Values expressed as mean (SEM).

Group	No.	Mean increase Ishak Fibrosis score at one year post transplant	Mean Interval length in weeks between biopsies (SEM)
Warfarin Group	16	0.56 (0.21)	46.7 (2.92)
Control Group	15	0. 93 (0.28)	43.3 (3.37)

Figure 5-10: Intention to treat analysis: Mean increase in Ishak fibrosis scores of warfarin versus control group patients at one year.

Error bars represent standard error of mean. A non significant reduction in fibrosis scores in treatment group patients compared to control group patients was demonstrated.

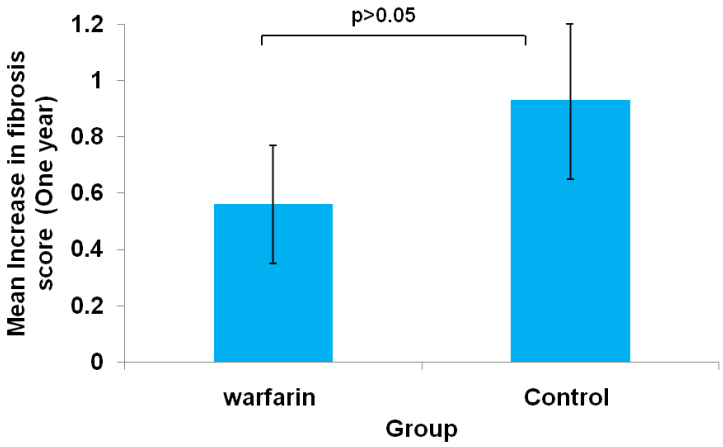
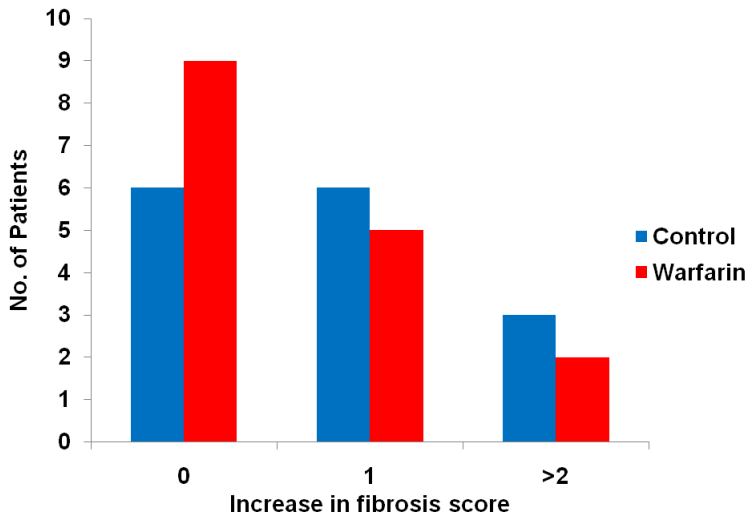


Table 5-6: Intention to treat analysis: Number of patients with an increase in Ishak fibrosis scores at year one post transplantation in warfarin and control group.

Values expressed as numerical value (%). Chi-square = 2.48, p=0.11.

Group	Number of subjects with increase in Ishak Fibrosis score ≥ 1 at one year post transplant	Number of subjects with increase in Ishak Fibrosis score < 1 at one year post transplant
Warfarin Group	7 (43.7)	9 (56.2)
Control Group	9 (60.0)	6 (40.0)

Figure 5-11: Intention to treat analysis: Number of patients with an increase in Ishak fibrosis scores at year one, warfarin versus control group.



5.4.3.2. Per protocol analyses

Patients withdrawn from the study were not included in the per protocol analyses**.** The majority of withdrawals were secondary to protocol violations, in which patients randomised to warfarinisation had failed to start it within the designated time period as set out in the protocol (See Table 5-2, Figure 5-7).

5.4.3.2.1. Baseline fibrosis scores: per protocol analysis

The baseline fibrosis score for each patient was taken as described in section 5.4.3.1.1. There was no significant difference between per protocol baseline fibrosis scores between the treatment group in comparison to the control group (0.08 +/-0.08 versus 0.07+/-0.07, p>0.05)(see Table 5-7, Figure 5-12).

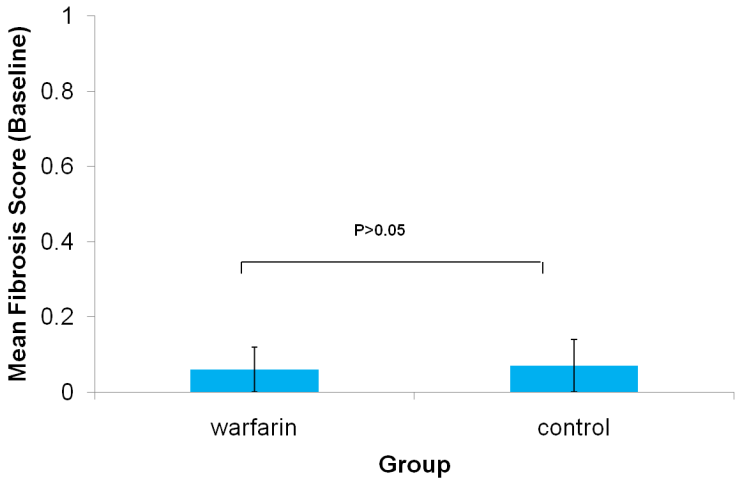
Table 5-7: Per protocol analysis: Mean Ishak fibrosis score of warfarin and control group patients at baseline.

Values expressed as mean (SEM).

Group	No.	Mean Ishak Fibrosis baseline score	SEM
Warfarin Group	10	0.08	0.08
Control Group	15	0.07	0.07

Figure 5-12: Per protocol analysis: Mean Ishak fibrosis score of warfarin versus control group patients at baseline.

Error bars represent standard error of mean.



5.4.3.2.2. One year post transplant fibrosis scores: per protocol analyses

Twenty-five patients fulfilled the criteria for per protocol analysis at the one year time point; 10 patients from the treatment group and 15 patients in the control group.

5.4.3.2.2.1. Mean one year post transplant fibrosis scores: per protocol analysis

A total of 25 patients have undergone one year protocol biopsies to date, and fulfilled criteria to be included in the per protocol analysis. Patients treated with warfarin anticoagulation had a lower mean fibrosis score (0.40+/-0.21) at one year than patients in the control group (0.94+/-0.28), but this difference just failed to meet statistical significance (p =0.06) (See Table 5-7 and Figure 5-12). There was no significant difference in the interval in weeks after transplantation at which the biopsies were taken between the two groups (see Table 5-7).

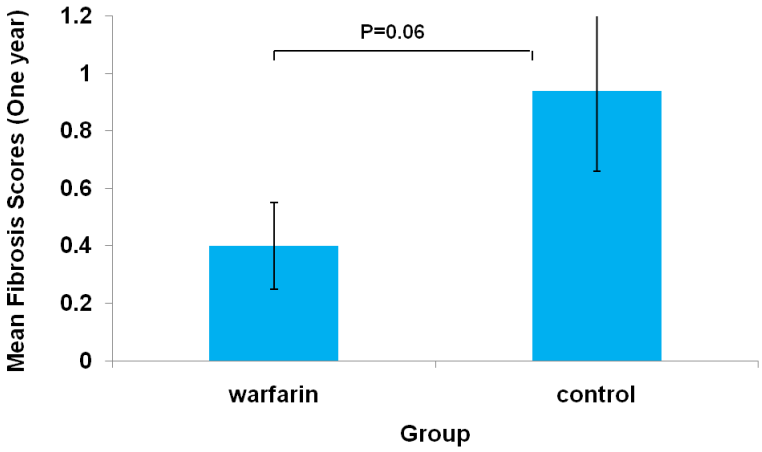
Table 5-8: Per protocol analysis: Mean Ishak fibrosis score of warfarin and control group patients at one year post transplantation.

Values expressed as mean (SEM).

Group	No.	Mean Ishak Fibrosis score (SEM)	Mean Interval from date of transplantation in weeks (SEM)
Warfarin Group	10	0.40 (0.21)	46.7 (3.82)
Control Group	15	0.94 (0.28)	43.5 (3.65)

Figure 5-13: Per protocol analysis: Mean Ishak fibrosis score of warfarin versus control group patients at one year.

Error bars represent standard error of mean. A borderline significant (p=0.06) reduction in fibrosis scores in treatment group patients compared to control group patients was demonstrated one year after transplantation.



5.4.3.2.2.2. Mean increase in fibrosis score at one year: per protocol analysis

The mean increase in fibrosis was calculated for each patient by subtracting the baseline fibrosis score on entry into the study from the fibrosis score at year one for each patient. Warfarin anticoagulation resulted in a greater than 50% reduction in mean increase in fibrosis scoring at year one (0.30+/-0.15) compared to the control group (0.87+/-0.28). In keeping with mean fibrosis scores, this was on the verge of meeting statistical significance (p =0.07) (See Table 5-8 and Figure 5-13).

Table 5-9: Per protocol analysis: Mean increase in Ishak fibrosis score from baseline to one year post transplantation of warfarin and control group patients.

Values expressed as mean (SEM).

Group	No.	Mean increase Ishak Fibrosis score at one year post transplant (SEM)	Mean Interval length between biopsies in weeks (SEM)
Warfarin Group	10	0.30 (0.15)	46.7 (3.82)
Control Group	15	0.87 (0.28)	43.3 (3.39)

Figure 5-14: Per protocol analysis: Mean increase in Ishak fibrosis score of warfarin versus control group patients at one year.

Error bars represent standard error of mean. A borderline significant (p=0.07) reduction in fibrosis scores in warfarin anticoagulation group patients compared to control group patients was demonstrated one year after transplantation.

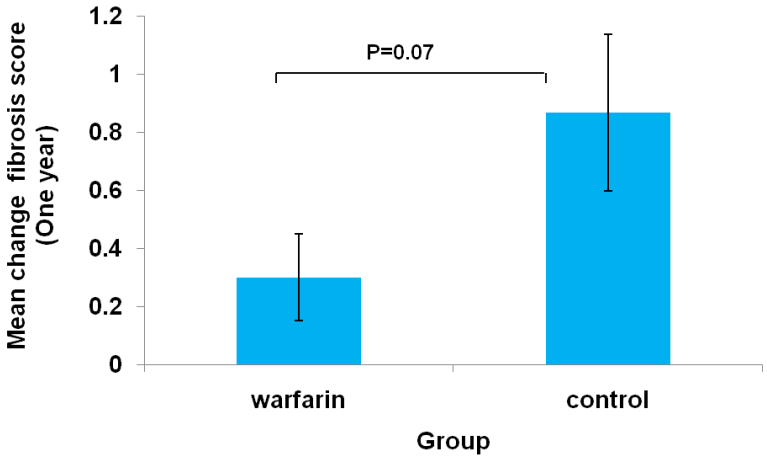


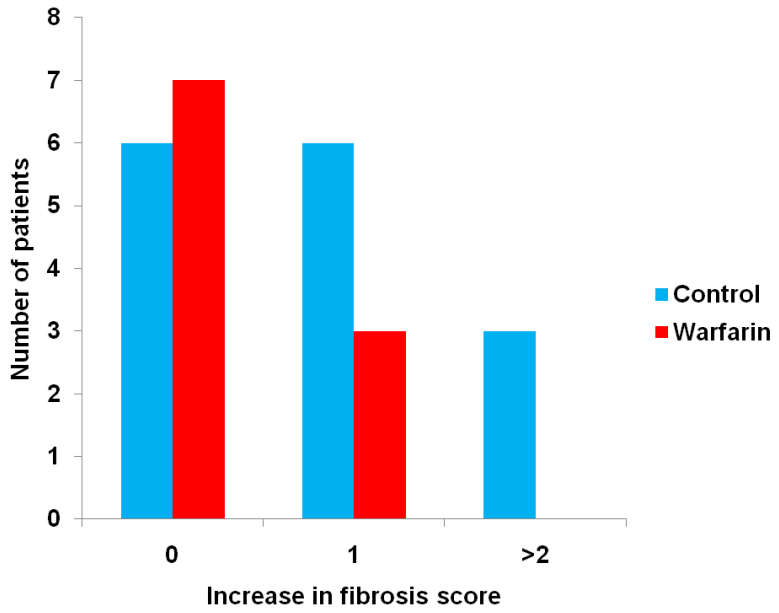
Table 5-10: Per protocol analysis: Number of patients with an increase in Ishak fibrosis scores at year one post transplantation in warfarin and control group patients.

Values expressed as numerical value (%). Chi-square = 2.75, p=0.09.

Group	Number of subjects with increase in Ishak Fibrosis score < 1 at one year post transplant (%)	Number of subjects with increase in Ishak Fibrosis score ≥ 1 at one year post transplant (%)
Warfarin Group	7 (70.0)	3 (30.0)
Control Group	6 (40.0)	9 (60.0)

Figure 5-15: Per protocol analysis: Number of patients with an increase in Ishak fibrosis scores at year one, warfarin versus control group.

In contrast to the control group no patients to date in the warfarin anticoagulation group have demonstrated a 2 or greater increase in their fibrosis score one year post transplantation.



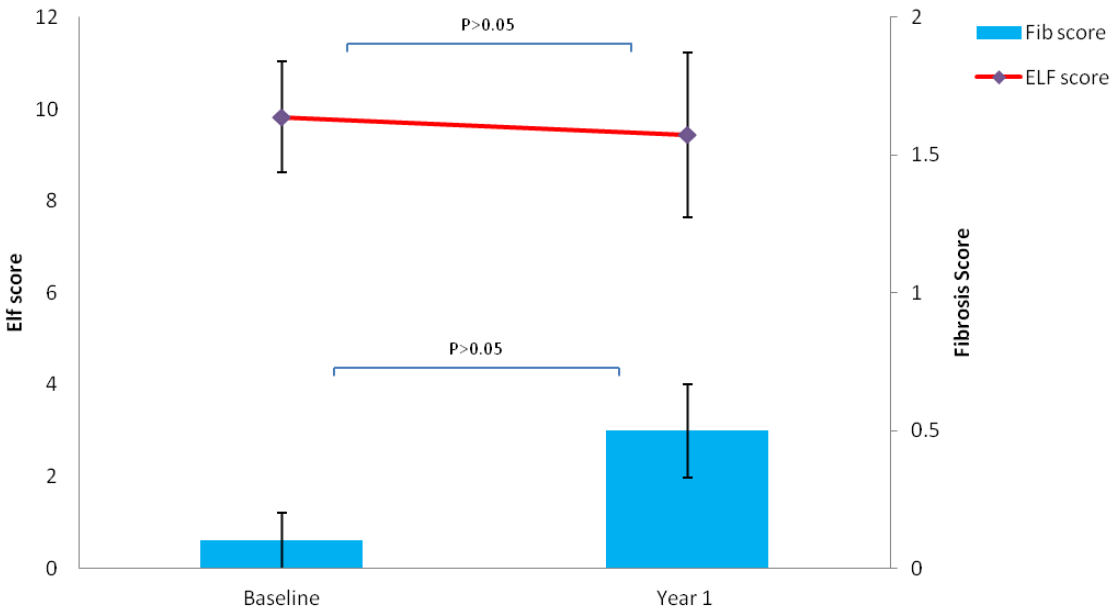
5.4.4. Enhanced Liver Fibrosis (ELF) Test scores:

Only 10 patients have had paired liver biopsies and ELF testing performed at baseline and one year post transplantation. There was a minor non significant improvement in ELF test scores at the one year timepoint compared to the baseline values (9.44 ± 1.8 versus 9.83± 1.2, p>0.05). This was in direct contrast to fibrosis scores in the same patients, which showed a non significant rise (0.1 +/- 0.1 versus 0.5+/- 0.17) (See Table 5-11 and Figure 5-15). Furthermore, the sensitivity of ELF testing in these patients to detect a rise in fibrosis scores at the one year timepoint was only 25%, with a specificity of 50%, and negative and positive predictive values of 25% and 50% respectively.

Table 5-11: Mean ELF test scores and fibrosis scores in patients one year post transplantation.
Values expressed as mean (SEM). n=10.

Timepoint	Mean ELF test score (SEM)	Mean Fibrosis Score (SEM)
Baseline (0 years)	9.83 (1.2)	0.1 (0.1)
1 Year	9.44 (1.8)	0.5 (0.17)

Figure 5-16: Mean ELF test and fibrosis scores in patients at baseline and one year post transplantation.
Error bars represent standard error of mean. The ELF test scores showed a minor non significant improvement at one year post transplantation compared to baseline. In contrast fibrosis scores showed a non significant increase after one year.



5.5. DISCUSSION AND FUTURE WORK

The WAFT-C study is the first clinical trial to evaluate warfarin anticoagulation as an antifibrotic in post transplant patients. Given the body of evidence from in vitro and animal studies, it fulfils the overwhelming demand to further test the potential benefits of anticoagulation as an anti-fibrotic in a group of patients in whom it is ethical justified, and needed, since there is no treatment as yet for accelerated fibrosis and early graft loss in patients transplanted for HCV related liver disease.

5.5.1. The Anti-fibrotic Effect of Warfarin Anticoagulation One Year Post Transplantation

The study is designed to demonstrate a significant effect if anticoagulation prevents liver fibrosis in this group of patients. It is powered such that a minimum of 70 patients are required to complete the study for this effect to be demonstrated. To date we have recruited 52 patients, of which 8 patients have been withdrawn.

The results presented above are an interim analysis of the data thus far, and reflect the results of the study without complete recruitment or hence adequate power. Despite this, interim results of the intention to treat and per protocol analyses both demonstrate a reduction in fibrosis scores in patients treated with anticoagulation compared to patients receiving standard post transplant care alone (see Figures 5-8 to 5-14). As would be expected, the per protocol analyses demonstrated a greater magnitude of beneficial effect with a greater than 50% reduction in both mean fibrosis scores and mean increase in fibrosis score at one year in patients treated with warfarin (see Figures 5-12 to 5-14). At present,

this level of reduction in fibrosis with anticoagulation reaches only borderline statistical significance (see Figures 5-13 & 5-14).

Intention to treat analyses are often valuable in preventing confounding biases and false positive effects, particularly in larger studies (Lachin, 2000). Per protocol analyses only include patients who have fully completed a study or trial without any protocol violations. The borderline statistical reduction in fibrosis scoring with anticoagulation seen in the per protocol analyses is highly encouraging. An argument can be made to suggest that excluding patients in such a manner introduces bias. As the above data demonstrates (see Table 5-2 and Figure 5-7), the majority of the patients withdrawn from the per protocol analysis were from the treatment group and for logistical reasons, did not even commence warfarin anticoagulation. The two patients that received warfarin, did so for only one week and two months respectively. These short periods of anticoagulation are unlikely to have had any substantial effect on year one fibrosis scores. Therefore excluding these patients is justified and the interim per protocol analyses rather than the intention to treat analyses, represent a more accurate representation of the true effect seen to date with anticoagulation (see Figure 5-12).

5.5.2. Safety of Warfarin Anticoagulation in HCV Patients Post Transplantation

To date warfarin anticoagulation in this group of patients has been safe. In keeping with data obtained from the pilot study (see Chapter 4), no serious adverse events have been reported in any of the subjects in the anticoagulation group and no withdrawals have been secondary to treatment related adverse events. These findings are in keeping with unpublished data from the Royal Free Hospital, which demonstrates anticoagulation post

liver transplantation for a variety of indications is safe (Burroughs *et al., unpublished data*). Two patients have self withdrawn from the treatment group citing reasons relating to compliance and issues with poly-pharmacy, which are not unusual concerns in post-transplant patients (Weng *et al.,* 2005). With the added burden of regular monitoring of warfarin anticoagulation, this may affect the potential willingness of some HCV post transplant patients to use warfarin anticoagulation if it is proven to have a significant beneficial effect on preventing fibrosis. The FXa oral inhibitor, Rivaroxaban, which has been shown to have a significant anti-fibrotic effect in thioactemide exposed mice (see Chapter 3), is a practical alternative since it does not require regular monitoring, and hence may appeal to more patients in this setting. Therefore, it is feasible that if this study does show that warfarin anticoagulation is a safe and effective treatment in preventing or reducing fibrosis in this group of patients, further clinical studies using Rivaroxaban should be considered. This however could only take place once long term therapeutic treatment with this drug has been given regulatory approval, and if there are no documented contraindications in post transplant patients.

5.5.3. Non Invasive Markers of Liver Fibrosis

The Enhanced Liver Fibrosis test is a serum panel of biomarkers which combines three direct markers of liver fibrosis HA, TIMP-1and PIIINP, with each individual marker entered into an algorithm to generate a score. The test score was first validated in a mixed cohort of patients with chronic liver disease, and has been shown to correlate with the level of liver fibrosis assessed by liver biopsy (Rosenberg *et al.,* 2004), but has to date not been validated in patients post liver transplantation. Liver biopsy remains the 'gold standard' for assessing hepatic fibrosis post liver transplantation, but is not without disadvantages. It is an invasive

and painful procedure with rare but potential life threatening complications. In addition, the variability of liver biopsies in assessing fibrosis has been questioned due to sampling error and inter-observer variability (Regev *et al*., 2002) which can affect the scoring of HCV related disease (Bedossa *et al.,* 2003). Despite the need to develop non-invasive tests, few studies have attempted to validate currently available serum markers of fibrosis in HCV patients post liver transplantation. One recent study, using a similar panel to the ELF test, has demonstrated in HCV patients within one year after transplantation, a correlation between serum markers and hepatic venous pressure gradients and fibrosis scores (Carrion *et al.,* 2010).

To date only a small number of subjects have had paired biopsy and serum markers of fibrosis performed at baseline and one year in our study. The results from this preliminary data demonstrate a poor sensitivity and specificity for the ELF test in detecting increases in fibrosis scores at one year post transplantation. The small number of patient samples collected however makes it difficult to draw any firm conclusions from this data, but our preliminary results have led to the withdrawal of serum markers from the study, partly due to cost issues and poor compliance with testing across all the sites.

5.5.4. Future Work

We have currently enrolled 52 patients into the study, with a target of 70 subjects required to power the study adequately. Recruitment had initially been slow with a longer than anticipated study set-up time, primarily due to contractual negotiations with several investigating sites. Additionally, a downturn in organ donation had a direct effect on the number of subjects we could screen. We have successfully increased recruitment rates by

the addition of extra investigating sites in the last 12 months. All existing investigating sites remain active and are committed to completing the study, allowing us to maintain an adequate quarterly recruitment rate. The forthcoming addition of new investigating sites allows us to forecast that recruitment should be complete within 8 months. Furthermore both the safety data and interim results to date warrant and support further patient recruitment to allow for adequate powering of the study. Further study numbers are required to achieve adequate power and to demonstrate whether the reduction in fibrosis between the control and warfarin groups reaches statistical significance. If the effect is of a less dramatic magnitude, then the results from this study will provide estimates of the magnitude of antifibrotic effect on which future larger studies may be based.

On completion of the study, final analyses will be conducted as described in section 5.3.4.3. These analyses will include a multivariant analysis using logistic regression including covariates for potential confounding factors. Variables that influence the progression of recurrent HCV following liver transplantation are poorly understood, and potentially include donor or graft characteristics, viral characteristics and recipient characteristics including immunosuppression regimens. Evidence suggests that donor characteristics including age of donor at time of transplantation gender is strongly associated with poorer outcomes (Bereunger *et al.,* 2002). Other factors that may influence outcome but need to be evaluated in a prospective manner are the impact of living or deceased organs including the use of donor livers from non heart beating donors. Viral characteristics, including viral genotype and viral load remain controversial with several studies demonstrating conflicting results despite viraemia being universal post transplantation (Bereunger 2002). Recipient characteristics including racial origin, immunosuppression regimens, total cumulative steroid dose, the presence of diabetes mellitus and CMV infection have been suggested to influence

HCV reoccurrence post transplantation, but again data is conflicting (Berenguer *et al*., 2001; Berenguer *et al.,* 2006; Foxton *et al.,* 2006; Burra *et al*., 2009).

These potential confounding factors therefore need to be considered not only to ensure any effect secondary to anticoagulation is genuine but to evaluate if any these factors are independent predictors of graft function and HCV related fibrosis post transplantation. Accordingly prospective data is being collected on donor, recipient and viral factors for each subject enrolled in the WAFT-C study.

5.5.5. Conclusions

The WAFT-C study is the first clinical trial to evaluate warfarin anticoagulation as an antifibrotic in post transplant patients. Given the body of evidence, including cell line, animal and pilot studies, it fulfills the overwhelming demand to test the potential benefits of anticoagulation as an antifibrotic, in a group of patients in whom it is ethical justified. The study is designed to demonstrate a benefit of anticoagulation in the prevention of liver fibrosis if there is a marked effect. To date we have demonstrated a reduction in fibrosis scores at one year post liver transplantation in HCV patients treated with warfarin anticoagulation compared to patients treated with standard post transplant care, without an increased incidence of adverse events. Further patient recruitment is therefore justified. If anticoagulation safely prevents or slows liver fibrosis in this patient group, then there would be an immediate role for this treatment in liver transplant recipients with HCV infection. The study will also allow for a better understanding of factors that influence fibrosis post transplantation.

6. GENERAL CONCLUSIONS

The aim of this body of work was to further our understanding of the role of the coagulation system in the pathogenesis of liver injury and fibrosis. The studies presented in this thesis have further evaluated both the expression and role of the individual coagulation proteins in the generation of liver injury and the therapeutic potential of their targeted inhibition using murine studies of chemically induced liver fibrosis and clinical trials in humans infected with HCV.

As described in Chapter 2, immunohistochemical techniques have provided evidence to suggest intrahepatic activation of the coagulation cascade occurs in both acute liver injury secondary to paracetamol in mice and humans, and chronic liver injury induced by CCL4 in mice, or related to HCV infection in humans. This activation is not only likely to contribute to the respective haemostatic imbalances in each type of injury, but in conjunction with existing evidence, suggests activation of the coagulation cascade contributes to the pathophysiological mechanisms involved in generating these injuries. In acute liver injury, immunohistochemical staining of both murine and human paracetamol induced acute liver injury demonstrated the concomitant upregulation of both TSP-1, a thrombin induced marker of platelet aggregation, and HIF-1 α, a marker of tissue hypoxia, with fibrin. This suggests that activation of the cascade may contribute to platelet aggregation and tissue hypoxia resulting in microcirculatory dysfunction, the generation of ischaemia and a necrotic injury. In chronic liver injury, we demonstrated hepatic upregulation of TF and fibrin with fibrosis. The upregulation of coagulation proteins support recent evidence that fibrogenesis results in a procoagulant state and is a prerequisite to any hypothesis that individual

coagulation proteins may contribute to the generation of liver injury via mechanisms independent of their procoagulant properties, including PAR activation.

Results presented in Chapter 3 evaluated the role of both FXa and thrombin alone and in combination on HSC contraction and activation. When administered together, the combined effect on HSC contraction was significantly higher than when given alone, suggesting that FXa, in a similar fashion to thrombin is a potent mediator of HSC activity via mechanisms independent of its ability to propagate the activation of the coagulation cascade. These results highlight the importance of both FXa and thrombin in the activation of HSCs, and the need to evaluate their combined inhibition as a potential antifibrotic strategy. Accordingly, direct FXa inhibition significantly reduced the rate of hepatic fibrosis and hepatic stellate cell activity in a thioacetamide murine model of liver fibrosis, and furthermore was a more effective anti-fibrotic than direct thrombin inhibition alone. This added effect was most likely due the inability of direct thrombin inhibitors to block FXa activation of HSCs mediated by PAR and demonstrates the importance of early inhibition of the coagulation cascade when planning therapeutic interventions to prevent hepatic fibrogenesis. In the absence of long term and therapeutic dosing data on oral FXa inhibitors, it would not be feasible to trial these drugs as antifibrotics in liver patients as yet. An alternative would be warfarin in view of its well defined safety profile, including recent safety data in patients with advanced liver fibrosis, and well defined mode of action including early inhibition of the coagulation cascade.

Two clinical trials evaluating the antifibrotic potential of warfarin were undertaken. The results generated from a small pilot study of patients with HCV and moderate fibrosis, following 8 weeks of warfarin anticoagulation, demonstrated a significant reduction in liver stiffness measurements. A possible explanation for this is hepatic stellate cell relaxation and

reduction of intrahepatic resistance, but this needs to be studied further. Importantly no significant adverse events were reported in this patient group while treated with anticoagulation. A non significant prolongation in hepatic transit times and no significant difference in serum markers of fibrosis were observed following anticoagulation, which could result from inherent limitations within the design of the study, including small numbers, a small effect and the chosen methods of assessment. The anti-fibrotic potential of anticoagulation therefore needed to be further evaluated using an alternative patient group for a longer treatment duration to allow for a marked effect with appropriate histological endpoints.

The WAFT-C study is the first clinical trial to evaluate warfarin anticoagulation as an antifibrotic in post transplant patients. Given the body of evidence which suggests a role of for FXa and thrombin in the pathogenesis of liver fibrosis and epidemiological evidence which suggests prothrombic tendencies accelerate liver fibrosis, it fulfils the overwhelming demand to test the potential benefits of anticoagulation as an antifibrotic, in a group of patients in whom it is ethical justified. To date we have demonstrated a reduction in fibrosis scores at one year post liver transplantation in HCV patients treated with warfarin anticoagulation compared to patients treated with standard post transplant care, without an increased incidence of adverse events. Further patient recruitment is being undertaken to power the study adequately and allow for a marked effect. If anticoagulation safely prevents or slows liver fibrosis in this patient group, then there would be an immediate role for this treatment in liver transplant recipients with HCV infection. Furthermore it may lead to the use of anticoagulation as an anti-fibrotic irrespective of the aetiology of liver fibrosis in the future due the postulated mechanism of action. This could lead to substantial healthcare benefits, since it is a relatively inexpensive and readily available treatment.

6.1. Future work and directions

Recruitment for the WAFT-C study continues to increase study numbers and enable adequate power of the study to allow any potential marked effect in reducing fibrosis secondary to warfarin anticoagulation to be exhibited.

The further use of novel murine models of liver injury will provide us a unique opportunity to modulate not only the activity of coagulation proteins, but the target cells by which these mechanisms of injury are generated. The recent development of transgenic mice strains expressing membrane–tethered tissue factor pathway inhibitor (TFPI) or hirudin (anti-thrombin) fusion proteins driven by an both alpha smooth muscle actin and CD31 promoters (Chen *et al*,. 2004; Chen *et al*,. 2006) selectively inhibit TF or thrombin on cells expressing alpha SMA, such as hepatic stellate cells, the primary cell effector in hepatic fibrosis; and cells expressing CD31, which has recently been demonstrated in hepatic sinusoids. The latter is of significance since we have demonstrated in our earlier immunohistochemical study expression of TF in hepatic sinusoids. The use of these transgenic mice therefore not only will provide us with a method to further examine the contribution of TF and thrombin on key effector cells in both acute hepatic injury and liver fibrosis, including the role of hepatic endothelial sinusoidal cells in the activation of the coagulation cascade and liver injury, but answer whether targeting not only individual coagulation proteins but also key cellular targets has added therapeutic potential in preventing acute and chronic liver injury.

6.2. Closing Remarks

This body of work presented in this thesis should make a substantial contribution to your knowledge of the role of the coagulation system in liver injury. The results described have followed the above concept through from initial in vitro work to subsequent in vivo studies using murine models and finally translational human studies. The murine study is the first to evaluate the antifibrotic potential of direct FXa inhibition and highlight the importance of early inhibition of the coagulation cascade in this setting. The pilot study and WAFT-C are the first two prospective studies to evaluate the antifibrotic effects of warfarin in liver patients. These studies have and will hopefully provide us with a rare opportunity to develop a treatment against the growing burden of liver disease with substantial benefits for liver patients.

6.3. Presentations and Publications Related to this Thesis

6.3.1. Orginial Research Papers and Review Articles

Anstee QM; Dhar A; Thursz MR. The role of hypercoagulability in liver fibrogenesis. *Clin Res Hepatol Gastroenterol.* 2011; 35:526-533.

Dhar A, Anstee QM, Sadiq F, Goldin R, Thursz MR. Murine models of liver *fibrosis. Manuscript in preparation 2011*.

Dhar A, Anstee QM, Sadiq F, Levene A, Goldin R, Thursz MR. Factor Xa inhibition suppresses thioacetamide induced liver fibrosis. *Manuscript in preparation 2011*.

Dhar A, Anstee QM, Antoniades CG,Sadiq F, Quaglia A, Wendon J, Goldin R, Thursz MR. Coagulation proteins and platelet aggregation in acute liver injury: A role in initiation and propagation. *Manuscript in preparation 2011*.

6.3.2. Published Abstracts

Dhar A, Kampscheur M, Sadiq F, Levene A, Ladva S, Poskitt B, Goldin R, Thursz MR. Coagulation proteins in liver fibrosis: a role for tissue factor and fibrin. *Gut* 2011;60 (2):A48.

Dhar A, Anstee QM, Sadiq F, Levene A, Goldin R, Thursz MR. Factor Xa inhibition suppresses thioacetamide induced liver fibrosis. *Gut* 2010;59:A33.

Dhar A, Anstee QM, Cobbold J, Stove J, Martinelli A,Taylor-Robinson S, Thursz MR. Anticoagulation for liver fibrosis: a pilot study in hepatitis C infected patients *Gut* 2010;59:A18

Dhar A, Anstee QM, Sadiq F, Levene A, Goldin R, Thursz MR. Factor Xa inhibition suppresses thioacetamide induced liver fibrosis. *Hepatology* 2010;52:1125A

Dhar A, Anstee QM, Cobbold J, Stove J, Martinelli A,Taylor-Robinson S, Thursz MR. Anticoagulation for liver fibrosis: a pilot study in hepatitis C infected patients. *Hepatology* 2010; 52:1133A

Dhar A, Anstee QM, Kudo H, Goldin R, Thursz MR. Coagulation proteins in Acute Liver Injury: A role in initiation and propagation. *Journal of Hepatology* 2010; 52 (1): S322

6.3.3. Presentations at Conferences

Dhar A, Kampscheur M, Sadiq F, Levene A, Ladva S, Poskitt B, Goldin R, Thursz MR. Coagulation proteins in liver fibrosis: a role for tissue factor and fibrin. *Poster presentation*

British Association for the Study of the Liver, London 2011

Dhar A, Anstee QM, Sadiq F, Levene A, Goldin R, Thursz MR. Factor Xa inhibition suppresses thioacetamide induced liver fibrosis. *Poster presentation*

British Association for the Study of the Liver, Edinburgh 2010

Dhar A, Anstee QM, Cobbold J, Stove J, Martinelli A,Taylor-Robinson S, Thursz MR. Anticoagulation for liver fibrosis: a pilot study in hepatitis C infected patients. *Poster presentation*

British Association for the Study of the Liver, Edinburgh 2010

Dhar A, Anstee QM, Sadiq F, Levene A, Goldin R, Thursz MR. Factor Xa inhibition suppresses thioacetamide induced liver fibrosis. *Poster presentation*

American Association for the Study of Liver Disease AGM, Boston 2010

Dhar A, Anstee QM, Cobbold J, Stove J, Martinelli A, Taylor-Robinson S, Thursz MR. Anticoagulation for liver fibrosis: a pilot study in hepatitis C infected patients. *Poster presentation*

American Association for the Study of Liver Disease AGM, Boston 2010

Dhar A, Anstee QM, Kudo H, Goldin R, Thursz MR. Coagulation proteins in Acute Liver Injury: A role in initiation and propagation. *Poster presentation*

European Association for the Study of Liver Disease AGM, Vienna 2010

7. APPENDIX

7.1 APPENDIX A: Dosing Protocols for CCL4

Materials required:

Stock CCl4 (Sigma-Aldrich) and corn oil vehicle; Stock sterile glassware; Externally vented hood with sufficient space for injecting of animals; 1ml disposable syringes with 23 gauge needles (sterile disposable).

Method:

Mix CCL4 with corn oil vehicle in a externally vented hood CCL4 was administered to mice via intraperitoneal injection using a disposable 1ml plastic syringe with a 5/8 x 23 gauge disposable needle.

Week 1:

Target Dose: 0.125ul/g (Ratio 1:31) 3x/week (Monday, Wednesday, Friday)

Weight (gram)	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Volume (ml)	0.06	0.064	0.068	0.072	0.076	0.08	0.084	0.088	0.092

Weight (gram)	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Volume (ml)	0.096	0.1	0.104	0.108	0.112	0.116	0.120	0.124	0.128

Week 2:

Target Dose: 0.25ul/g (1:15) 3x/week (Monday, Wednesday, Friday)

Weight (gram)	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Volume (ml)	0.06	0.064	0.068	0.072	0.076	0.08	0.084	0.088	0.092

Weight (gram)	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Volume (ml)	0.096	0.1	0.104	0.108	0.112	0.116	0.120	0.124	0.128

Week 3:

Target Dose: 0.5ul/g (1:7) 3x/week (Monday; Wednesday; Friday)

Weight (gram)	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Volume (ml)	0.06	0.064	0.068	0.072	0.076	0.08	0.084	0.088	0.092

Weight (gram)	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Volume (ml)	0.096	0.1	0.104	0.108	0.112	0.116	0.120	0.124	0.128

Weeks 4 – 6:

Target Dose: 1ul/g 3x /week (1:3) 3x/week (Monday; Wednesday; Friday)

Weight (gram)	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
Volume (ml)	0.06	0.064	0.068	0.072	0.076	0.08	0.084	0.088	0.092

Weight (gram)	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Volume (ml)	0.096	0.1	0.104	0.108	0.112	0.116	0.120	0.124	0.128

7.2 APPENDIX B: Anticoagulant dosing levels

Group	Mean prothrombin time (secs)	SEM	Prolongation of prothrombin time (versus control animals)
Control animals	10.2	0.07	n/a
Rivaroxaban 10mg	13.6	0.54	1.32
Rivaroxaban 20mg	14.2	0.50	1.38
Rivaroxaban 40mg	18.2	0.23	1.77
Dabigatran 30mg	12.4	1.0	1.20
Dabigatran 50mg	15.7	0.35	1.53
Dabigatran 70mg	15.1	0.68	1.47
Dabigatran 80mg	14.7	0.58	1.42
Dabigatran 100mg	17.4	0.42	1.70

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