

**Portal hypertension and cirrhosis: the role of  
inflammation and nitric oxide**

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**A thesis submitted for the degree of Doctor of Philosophy (Ph.D.) from The  
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As mentioned below, several collaborators have contributed significantly towards this project and include Prof. Werner Muller-Esterl's group (Frankfurt), Dr Neil Dalton (Guy's Hospital), Prof. Patrick Vallance (Rayne Institute, UCL) and Prof. Mick Deutz's Lab (Maastricht University).

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**Rajeshwar Prosad Mookerjee**  
May 2007

## **Role of Various Contributors**

I was intimately involved in all aspects of the studies carried out in London at the Middlesex Hospital, UCLH, and at the Institute of Hepatology, UCL. This included design and conducting of patient studies, sample collection, subsequent laboratory analysis and collation of data in the interventional studies, in addition to performing the clinical interventions. However, the complex nature of these studies required involvement at all levels from all the other members of our research group in order for successful completion of the work. Dr Rajiv Jalan, Dr Sambit Sen, Sister Lisa Cheshire and Dr Debbie Shawcross all helped with the clinical studies requiring patient intervention and monitoring. Dr Sambit Sen, performed the MARS treatments on the patients in whom I performed portal pressure measurements, with our joint interest in their portal vascular dynamics. Dr Nathan Davies and Dr Stephen Hodges provided invaluable scientific support and guidance with all the laboratory analysis, including cytokine and oxidant stress profiling of human and animal material.

The animal experiments were also conducted by a team partly in the animal facilities at The Royal Free Hospital (Bile duct ligated Rat model- Prof Kevin Moore's Group) and the remainder at the animal house facilities at UCL, and involved in the main, Dr Nathan Davies and Dr Gavin Wright. All subsequent analysis of nitric oxide activity and characterization of liver derangement were conducted within the lab at The Institute of Hepatology. The method I finally used to measure nitric oxide synthase activity followed a period of method development, trying to utilize a quantitative cyto-histochemical technique on the animal model tissues for assessing nitric oxide stimulated guanylate cyclase activity. This work was carried out under the supervision of Dr Siroos Mehdizadeh in his lab at Charing Cross Hospital.

The ADMA studies were conducted under the guidance of Prof. Patrick Vallance of the Rayne Institute, UCL, and followed an initial period of assay method development utilizing HPLC techniques based primarily in the Department of Surgery, University of Maastricht, under Prof Mick Deutz and Dr Hans van Eijk. The final assay utilized to generate the data presented was run by Dr Neil Dalton at The Wellchild Laboratories, Guys hospital, London.

Immunohistochemical studies were performed with the assistance of Miss Phillipa Munson in the Department of Histopathology, UCH. The neutrophil studies were

conducted under the supervision of Dr Sukhwinderjit Lidder, and the in-vitro experiments in-conjunction with Dr Vanessa Stadlbauer, at The Institute of Hepatology.

Studies related to NOSTRIN were performed in close collaboration with Professor Werner Muller-Esterl's laboratory in the Wolfgang Goethe University, Frankfurt. The co-precipitation studies and molecular characterization of NOSTRIN  $\beta$  were performed in Frankfurt largely by Miss Anja Wisenthal.

## **Abstract**

### **Portal hypertension and cirrhosis: the role of inflammation and nitric oxide**

Patients with cirrhosis characteristically develop haemodynamic changes which include increased cardiac output, decreased systemic vascular resistance and paradoxically, increased portal pressure. Studies to date largely in animal models, have suggested that a decrease in hepatic nitric oxide may be important. The studies described in this thesis provide evidence for significantly elevated portal pressure in alcoholic hepatitis patients, who were shown to have a marked additional inflammatory response on the background of cirrhosis, and a more severe expression of disease. Studies in patients and a galactosamine rodent model confirmed a decrease in endothelial nitric oxide synthase (NOS) activity in the context of inflammatory liver injury. Following on from these observations, further studies explored the role of potential regulators of NOS which may have accounted for its decrease in activity in liver disease.

Studies of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, showed that it was markedly increased in liver failure in both patients and in an animal model. The data suggested this may result from a decreased metabolism (through reduced expression of its metabolizing enzyme, dimethylarginine-dimethylaminohydrolase) or/and increased synthesis by protein arginine methyltransferases. ADMA was shown to correlate with severity of portal pressure, and with increased organ failure and death in decompensated cirrhosis, suggesting that it may have a potential use as a biomarker of disease severity.

Other novel regulators of endothelial NOS were also explored including the recently described potential NOS inhibitor, NOSTRIN (nitric oxide synthase traffic inducer). It was demonstrated that NOSTRIN was up-regulated at both message and protein levels in liver disease patients and this was most marked in those with additional inflammation. A further novel observation was the identification of a variant of NOSTRIN which was only found in cirrhosis patients and not in normal liver tissue. The findings from these studies provide a better understanding of the importance of inflammation in the context of vascular dysfunction in cirrhosis and highlight some potential novel therapeutic targets.

## Abbreviations

ALD	Alcoholic liver disease
NAD	Nicotinamide adenine dinucleotide
ATP	Adenosine triphosphate
NF $\kappa$ B	Nuclear factor-kappa B
TNF $\alpha$	Tumour necrosis factor alpha
Il-8	Interleukin-8
AH	Alcoholic hepatitis
BH <sub>4</sub>	Tetrahydrobiopterin
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
AH+C+	Patients with histological cirrhosis with superadded alcoholic hepatitis
HVPG	Hepatic venous pressure gradient
HBF	Hepatic blood flow
IHR	Intra-hepatic resistance
SVR	Systemic vascular resistance
MAP	Mean arterial blood pressure
CO	Cardiac output
SIRS	Systemic inflammatory response syndrome (components of this scoring system)
PAH	<i>para</i> -aminohippuric acid
ICG	Indocyanine green
NO	Nitric oxide
NO <sub>x</sub>	Nitrite/nitrate metabolites of Nitric oxide
cGMP	Cyclic guanosine- 3',5-monophosphate
NOS	Nitric oxide synthase
MDA	Malondialdehyde
LPS	Lipopolysaccharide
BDL	Bile duct ligated rat model
Gal	Galactosamine administration
ADMA	Asymmetric dimethylarginine
SDMA	Symmetric dimethylarginine
PRMT	Protein arginine methyltransferase
DDAH	Dimethylarginine dimethylaminohydrolase
ALF	Acute liver failure
AUROC	Area-under-the-curve of a receiver operator curve
DF	Discriminant function
MELD	Model of end-stage Liver Disease score
Pugh (CP)	Child-Pugh score
NOSIP	Nitric oxide synthase interacting protein
NOSTRIN	Nitric oxide synthase traffic inducing protein
HSC	Hepatic stellate cell
CHO cells	Chinese hamster ovary cell line
HUVEC	Human umbilical vein endothelial cell lines
IP	Intra-peritoneal
CRP	C reactive protein
WBC	White blood cell count
HSC	Hepatic stellate cells
ACLF	Acute-on-chronic liver failure

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***“There is no subject so old that something new cannot be said about it.”***

***Dostoevsky; A Diary of a Writer (1876)***

*For Purnima, Tara and Shiv*

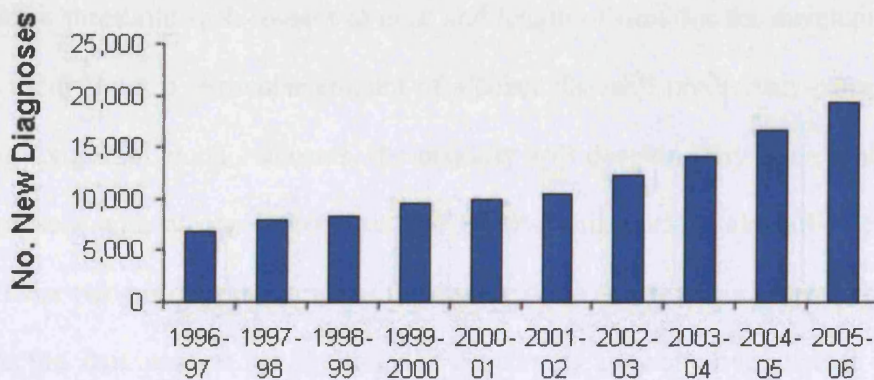


***Chapter 1***  
***General Introduction***

### 1.1 Alcohol and liver disease

References to alcohol and its effects date back to 1543, and the term 'al-kuhl' was used by Arabic chemists to refer to substances such as essences that were obtained by distillation. In a healthy human, ethyl alcohol is metabolized at the rate of about 7 grams (or about 9ml.) per hour, a little less than the amount in an average measure of spirits. Larger quantities or drinking faster leads to an accumulation of alcohol for the liver to process. In heavy drinkers, which comprise 8 % of men and 3 % of women in the UK, there is an increased risk of developing alcoholic liver disease (ALD). According to figures from the Alcohol related Working Party of The British Liver Trust, alcoholic liver disease accounts for greater than 50 % of presentations of UK liver disease and is responsible for 44% of all cirrhosis related mortality, with the resultant economic burden that this entails. [<http://www.britishlivertrust.org.uk/>]

**Number of New Alcohol Related Cirrhosis Cases Per Year**



**Figure 1.1:** Over the last decade, there has been a marked increase in the number of cases of alcohol related cirrhosis. (1)

Within the last 3 decades, figures from the UK Dept. of National Statistics suggest that there has been a four-fold increase in mortality from liver cirrhosis in 45-54 year

old men and an eight-fold cirrhosis mortality increase in 35-44 year old men, largely attributable to ALD. Alcoholic cirrhosis was the primary indication for liver transplantation in the UK between 1996 and 2000, with an estimated £24 million for direct transplant costs. However, the pathophysiology of severe ALD, as seen in alcoholic hepatitis, is poorly understood with treatment modalities being largely supportive and with a lack of consensus on more specific interventions.

There is a significant heterogeneity in the severity of presentation of clinical disease and in the susceptibility of individuals to ALD. There is believed to be a nebulous “threshold” of daily amount and length of time of alcohol intake that has to be exceeded, in order to develop ALD. This has been proposed as being a daily intake of alcohol over 10 to 12 years with doses in excess of 40-80g/day for males (equating to 3-6 cans of 350ml of beer) and of 20-40g/day for females. (2) The lower thresholds are now widely accepted, especially with the increased recognition of high alcohol intake amongst younger drinkers, especially young women. Despite the need for a threshold with respect to dose and length of time for the development of ALD, there is not a particular amount of alcohol that will predictably cause ALD. Among long-term alcohol abusers, the majority will develop fatty liver, whilst 10–35% present with alcoholic hepatitis and 8–20% will develop alcoholic cirrhosis, albeit these two histological forms of the disease often co-exist on a biopsy.

Risk factors that increase the likelihood of developing alcoholic liver disease include quantity of alcohol consumed, (3) female gender, (4) obesity, (5) concomitant viral infection (especially hepatitis C), (6) and genetic factors including polymorphisms in metabolizing enzyme systems and inflammatory mediators.(7) Thus, alcohol probably works as a ‘potential hepatotoxin’, with the development of liver disease depending on the balance of host attributes and co-existing external factors.

## **1.2 Pathophysiology of alcoholic liver disease**

### ***1.2.1 Consequences of alcohol metabolism:***

Ethanol is metabolized by 2 major pathways: (i) oxidation to acetaldehyde by cytosolic alcohol dehydrogenase with subsequent oxidation to acetate by mitochondrial aldehyde dehydrogenase, coupled to the reduction of nicotinamide adenine dinucleotide (NAD) to NADH; (ii) metabolism by the cytochrome P450 system (CYP2E1) which also generates acetaldehyde, in addition to numerous free-radicals. Acetaldehyde is thought to have a direct toxic effect on cells by the formation of protein adducts by binding to cysteine residues. These modified proteins may then evoke an immune response and the production of auto-antibodies perpetuating an inflammatory response. (8) The change in redox state (increased NADH formation) impairs normal carbohydrate and lipid metabolism, whilst also decreasing the supply of adenosine triphosphate (ATP) to cells. Gluconeogenesis is also impaired leading to the diversion of generated Acetyl CoA, to ketogenesis and the synthesis of free fatty acids. The inhibition of mitochondrial  $\beta$ -oxidation coupled with the additional inhibitory effect on lipolysis by acetate, leads to hepatic steatosis and the generation of reactive oxidant species through lipid peroxidation. (9) Loss of normal mitochondrial function (10) together with protein adduct formation (11) contributes to derangements in apoptotic signalling pathways and progression of liver injury.

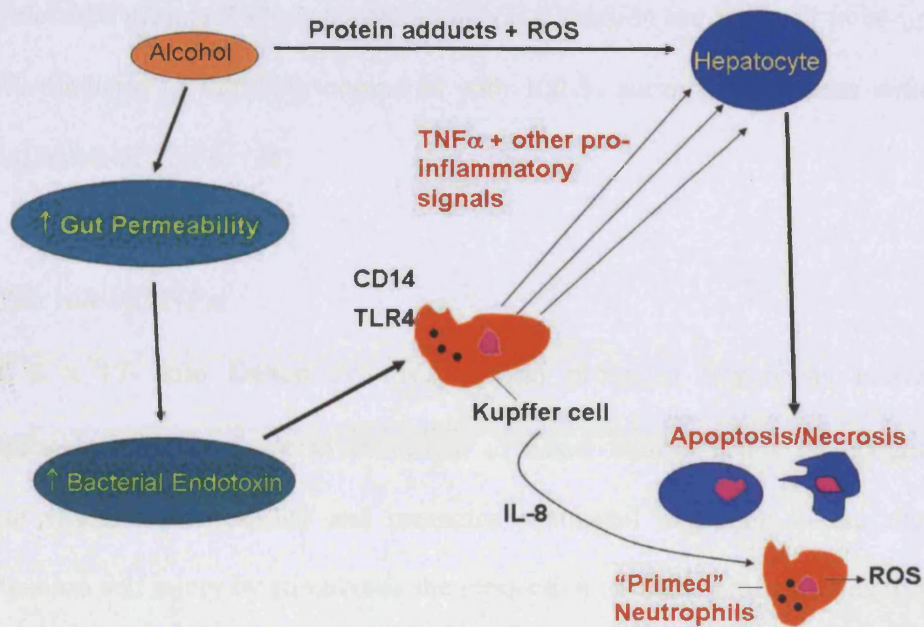
### ***1.2.2 The role of endotoxin and pro-inflammatory cytokines:***

Several lines of evidence suggest that alcohol promotes gut permeability to bacterial translocation and an increase in measured levels of endotoxin. (12, 13) Bacterial lipopolysaccharide (LPS) then binds to Kupffer cells through inducible receptors

such as CD14 and Toll-like receptor-4 causing Kupffer cell activation, (14) transducing signals to the cytoplasm, culminating in activation of nuclear factor-kappa B (NFκB). Activated NFκB promotes an up-regulation of production of pro-inflammatory cytokines such as Tumour necrosis factor alpha (TNFα) and the neutrophil attracting chemokine, interleukin-8 (IL-8). (15) The liver inflammation that ensues is perpetuated by the further generation of neutrophil oxidative burst and protease production, (16) and the continued pro-apoptotic drive through increased mitochondrial permeability transition, promoted by TNFα. (17, 18) Furthermore, alcohol appears to “prime” peripheral blood mononuclear cells to increase cytokine generation in response to LPS stimulation, whilst also sensitising hepatocytes to the inflammatory and pro-apoptotic effects of cytokines. (9) The concept of priming and sensitisation to a “second hit” of injury such as endotoxin implies a series of sequential events in the progression of liver injury following alcohol exposure.

A schematic representation of this concept of a ‘second hit’ is represented in figure 1.2.

## “Second Hit”- Inflammatory Injury



**Figure 1.2** This cartoon illustrates the concept of the primary stimulus for liver injury resulting from the metabolism of alcohol with the generation of reactive oxygen species (ROS) and protein adducts that promote inflammatory injury and apoptosis. However, an increase in gut permeability and consequent translocation of endotoxin, promotes a ‘second hit’ resulting in the activation of pro-inflammatory signals that are perpetuated by neutrophil activation.

### 1.2.3 Contribution of inflammation in alcoholic cirrhosis

As stated above, chronic alcohol ingestion has been suggested to increase intestinal permeability and the resultant translocation of bacterial products from the intestinal lumen to the mesenteric circulation and its lymphatics induces regional and systemic production of pro-inflammatory cytokines. (19) Furthermore, it has been demonstrated that peripheral blood mononuclear cells in alcoholic cirrhotics upon bacterial challenge within regional lymph nodes, undergo a priming activation resulting in significantly increased spontaneous and lipopolysaccharide (LPS)-stimulated release of tumour necrosis factor alpha ( $\text{TNF}\alpha$ ), compared with

monocytes from healthy volunteers. (20) In patients with severe alcoholic hepatitis (AH), elevated plasma TNF $\alpha$  concentrations on admission are believed to be up to an 82% predictor of mortality compared with 100 % survival in patients without elevated levels of TNF $\alpha$ . (21)

#### **1.2.4 The role of TNF $\alpha$**

TNF $\alpha$  is a 17- kilo Dalton cytotoxic protein produced largely by activated macrophages, after exposure to endotoxin or tissue injury. TNF $\alpha$  is thought to increase vascular permeability and promotes neutrophil migration to the site of inflammation and injury by stimulating the production of chemokines such as Il-8 by Kupffer cells and hepatocytes (22), whilst also enhancing neutrophil adhesion to vascular endothelium. (23) TNF $\alpha$  also has a direct role in promoting apoptosis and necrosis, which may involve increased mitochondrial pore transition. (18)

The importance of TNF $\alpha$  in the pathogenesis of alcoholic liver disease is highlighted by 2 studies in intragastric alcohol fed animal models: Yin and colleagues (24) studied alcohol fed TNF receptor knock-out mice and demonstrated significant liver injury and raised alanine aminotransferases only in wild type and TNF receptor-II knock-out mice. This highlights the importance of alcohol induced injury via a TNF receptor-I pathway. A similar model of enteral fed male Wistar rats treated with a neutralizing anti- TNF $\alpha$  antibody demonstrated significant attenuation of hepatic inflammation and necrosis, and a reduction in macrophage inflammatory protein-2, suggesting that TNF $\alpha$  may drive subsequent neutrophil responses to evoke liver injury. (25)

### **1.3 Vascular derangements in cirrhosis**

Patients with decompensated alcoholic liver disease (a precipitation of clinical deterioration of underlying cirrhosis by factors such as infection or bleeding) exhibit a characteristic hyperdynamic circulation which is manifest by increased cardiac output (CO), a dilated and hyporesponsive peripheral circulation with low systemic vascular resistance (SVR), portosystemic shunting, increased portal pressure, and reduced organ blood flow. These factors are responsible for the complications and mortality associated with portal hypertension with which patients often present to hospital, namely: variceal bleeding, hepatorenal failure, hepatic encephalopathy and circulatory dysfunction. (26)

A wealth of evidence suggests that complications of portal hypertension do not appear until the portal pressure gradient (measured between the portal vein and hepatic veins) rises above 10-12 mmHg (normal values: 1-5 mmHg). (27, 28) Conversely, lowering portal pressure below this threshold value is associated with prevention of most portal hypertensive complications. (29)

#### ***1.3.1 Pathophysiology of portal hypertension***

The portal pressure gradient is the result of the interaction between the portal blood flow and vascular resistance that opposes that flow in accordance with Ohm's law, as defined by the relationship:

$$\Delta_p = Q \times R$$

in which  $\Delta_p$  is the portal pressure gradient, Q the total portal venous blood flow and R the vascular resistance of the portal venous system. Thus, an increase in portal pressure can result from changes in portal blood flow, vascular resistance or a combination of both.



### *1.3.1.1 Splanchnic vasodilatation in portal hypertension*

Increased portal venous inflow is the result of marked arteriolar vasodilatation in splanchnic organs draining into the portal vein. This is likely to represent a multifactorial process involving neurogenic, humoral and local vaso-active mechanisms. (30) Amongst the most studied vasodilators include glucagon, (31) and nitric oxide (NO), as first suggested by Vallance and Moncada. (32) More recent studies have shown that patients with cirrhosis have increased plasma levels of nitrates and nitrites (NO<sub>x</sub>), especially noted in the portal vein, (33) suggesting that in these patients there is enhanced release of NO from the splanchnic bed. The mechanisms postulated for this increased NO release relate to changes in endothelial nitric oxide synthase (eNOS) expression following increased shear stress, (34) and TNF $\alpha$  mediated increased availability of the eNOS co-factor, tetrahydrobiopterin. (35) Other proposed mechanisms include effects on NO production following activation of cannabinoid CB1 receptors caused by increased levels of the endogenous cannabinoid anandamide in advanced cirrhosis. (36)

In association with splanchnic vasodilatation, patients with advanced cirrhosis characteristically develop peripheral vasodilatation with reduced peripheral vascular resistance. This leads to activation of endogenous neurohumoral systems resulting in sodium retention, expansion of plasma volume and an increase in cardiac output, all elements known to drive the development of ascites and the hepatorenal syndrome. (37) It is these elements that further contribute to the splanchnic hyperaemia which together with an increase in portal vascular resistance, are believed to contribute to portal hypertension in what has been termed the "forward flow" theory. (38)

### *1.3.1.2 Increased intrahepatic resistance*

Early concepts of increased intrahepatic resistance in cirrhosis related to the belief that liver ultra-structural changes induced by fibrosis altered liver blood flow. However, currently, beyond these mechanical factors there is believed to be an important contribution from a dynamic component provided by hepatic sinusoids and smooth muscle cells of portal venules that are able to constrict in a reversible and graded manner in response to several agonists. (39) Constriction of sinusoids is mediated largely through the contractile properties of activated stellate cells which are located in the peri-sinusoidal space and whose pseudopodia wrap around the sinusoids causing constriction in response to vasoactive factors such as endothelin 1 and angiotensin II which are increased in cirrhosis. (40, 41) By contrast, it has been shown that synthesis of NO is decreased and unable to compensate for this abundance in vasoconstrictors. (42-44) Thus in liver disease, the 'normal' balance in vasoconstrictors and vasodilators are deranged leading to an endothelialopathy manifest as increased vascular tone. This is further compounded by exaggerated stellate cell activity promoted by inflammatory signals such as TNF $\alpha$ , (45) further increasing intrahepatic resistance, resulting in the so-called 'backward flow' theory of portal hypertension. This might suggest a rationale for using vasodilators to reduce portal hypertension. However, when drugs such as isosorbide-5-mononitrate have been used, they also create some degree of systemic and further splanchnic vasodilatation, inducing hypotension, and a further deleterious effect on regional blood flow. (46) There is thus an apparent paradox between systemic vasodilatation and relative increases in hepatic vascular resistance that needs to be reconciled, in spite of factors such as NO contributing to both phenomena- too much in the systemic circulation, and too little within the liver.

#### **1.4 Nitric oxide biology**

NO, an endothelial derived relaxing factor, is synthesized by a family of three nitric oxide synthases (NOS): the constitutively expressed isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), and an inducible form (iNOS). (47) Among these isoforms, eNOS is the major enzymatic source of the vascular NO overproduction in the arterial circulation (35). Endothelium derived NO is synthesized from the amino acid L-arginine by the action of eNOS, yielding L-citrulline as a by-product. (48) NO is labile with a short half life (< 4 seconds in biological solutions) and is rapidly oxidized to nitrite and then nitrate by oxygenated haemoglobin, before being excreted into the urine. Several co-factors are required for NO biosynthesis. These include nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH<sub>4</sub>), and calmodulin. Once synthesized the NO diffuses across the endothelial cell membrane and enters the vascular smooth muscle cells where it activates guanylate cyclase, leading to an increase in intracellular cyclic guanosine- 3',5-monophosphate (cGMP) concentrations. (48) As a second messenger, cGMP mediates many of the biological effects of NO including the control of vascular tone, by regulating intracellular calcium homeostasis and thereby, smooth muscle cell contractility. In addition to vascular regulation, NO has many other physiological functions including as an anti-oxidant, in modulating platelet thrombotic effects and in modulating leukocyte adhesion, hence the central role it plays in the pathology of atherogenesis. (49)

#### ***1.4.1 Nitric oxide an important mediator of vascular disturbances in cirrhosis- too much and yet not enough***

NO related vasodilatation serves as a counterbalance to local vasoconstrictors and sympathetic nervous activity. Investigators have demonstrated that isolated perfused normal rat livers respond to increases in blood flow and subsequent shear stress by developing a flow-dependent increase in NO synthesis to counteract the increased perfusion pressure. (50) The enzymatic origin of this NO production was found to be hepatic endothelial cell derived eNOS.(51) Moreover, preferential inducible nitric oxide synthase inhibition was shown to be ineffective in endotoxaemic conditions of increased shear stress, supporting the importance of eNOS-derived NO production for maintaining intra-hepatic vascular tone. (52)

In cirrhotic rat models with ascites, there is a paradoxical vasoconstriction in response to the eNOS agonist acetylcholine, coupled with reduced hepatic NO production, suggesting that there is a relative NO deficiency in the intrahepatic microcirculation contributing to increased intrahepatic resistance. (42) This deficiency in NO production has been demonstrated in several experimental models, (43, 44) with the suggestion that it is related to the severity of the liver disease as it is more evident in cirrhotic animals with ascites. (42) Human studies in cirrhotic patients are limited, (53) and there is no data to relate the degree of eNOS dysfunction with the degree of disease severity or with underlying pathological factors such as inflammation associated with advanced cirrhosis.

The reduction in NO production in cirrhosis does not appear to be due to decreased eNOS expression as this appears to be the same in control and cirrhotic animals. (43, 44) This finding might implicate posttranslational regulatory mechanisms resulting in defective NO production in cirrhosis. In support of this concept, an increased

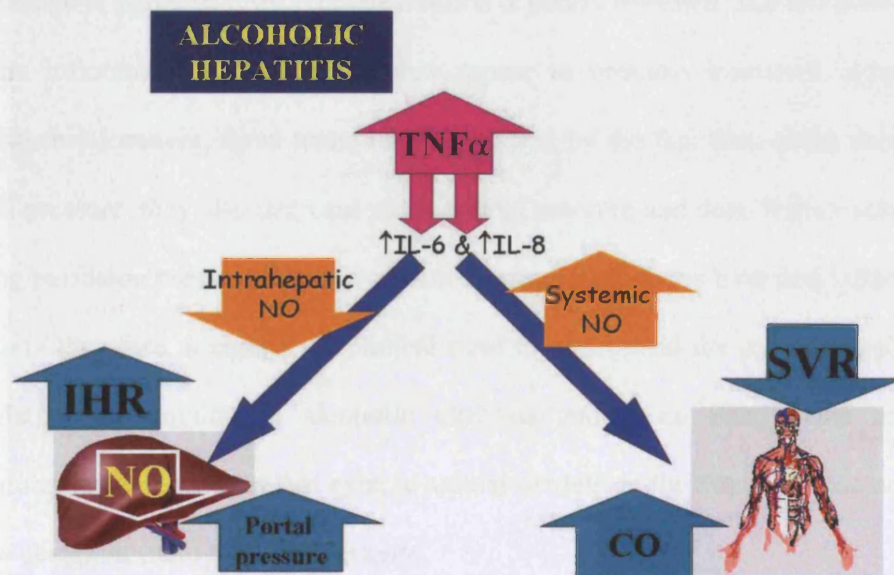
expression of the eNOS regulatory protein caveolin 1 has been described in association with decreased enzyme activity. (54) Several other NOS regulators have been identified and may affect NO-cGMP signalling through modification of eNOS activity, albeit data in liver disease and humans is lacking. (55, 56) Recently, interventions such as eNOS gene transfer (57) and transduction of the liver with activated Akt (a serine protein kinase required for eNOS phosphorylation and subsequent activation) have demonstrated reductions of portal pressure in cirrhotic rats, (58) further emphasizing the importance of hepatic NO generation in the regulation of intra-hepatic resistance, whilst also hinting at a potential target for the management of elevated portal pressure. Indeed some novel compounds to deliver intra-hepatic NO have been tested in animal models to try to ameliorate portal hypertension, (59) but these findings are yet to be substantiated in patients.

#### ***1.4.2 The link between inflammation and vascular derangement in alcoholic liver disease***

Increased pro-inflammatory drive, often as a consequence of increased bacterial translocation in advanced cirrhosis, (60) may interplay with numerous factors that alter vascular tone such as endothelin-1 (61), cysteinyl-leukotrienes (62), and NO (48, 63). Of these mediators, NO is believed to be the most important, and TNF $\alpha$  is thought to promote vasodilatation and a hyperdynamic circulation in mammals by activating NO synthesis. (48) The mechanism reported to be involved is thought to relate to activation of guanosine triphosphate (GTP)-cyclohydrolase I, which generates BH<sub>4</sub> production in mesenteric arteries. This increase in BH<sub>4</sub> is associated with enhanced eNOS activity and eNOS-derived NO overproduction in the mesenteric arterial beds. (35). The resultant hypotension elicited by TNF $\alpha$  can be

reversed via inhibition of NO synthesis, suggesting that L-arginine-derived NO is the principal mediator of this observation.(64)

The importance of  $TNF\alpha$  in the development of portal hypertension is suggested by results from studies on portal vein ligated rats treated with anti-  $TNF\alpha$  therapies, where a blunting of the hyperdynamic circulation and a reduction of portal pressure has been demonstrated albeit, the mechanism by which  $TNF\alpha$  evokes these vascular effects has not been elucidated. (65, 66) These anti-cytokine treatments are believed to act largely through the modulation of intrahepatic resistance, and a regulatory effect on eNOS activity is therefore, an attractive hypothesis (figure 1.3).



**Figure 1.3:** This cartoon depicts the hypothesis that alcoholic hepatitis exists as a state of increased pro-inflammatory drive ( $TNF\alpha$ ), where a paradox exists between high systemic nitric oxide (NO) promoting vasodilatation with low systemic vascular resistance (SVR) and compensatory high cardiac output (CO), whilst low NO in the liver increases hepatic vascular resistance (IHR) and thus increases portal pressure.

### **1.5 The clinical need for studies relating inflammatory mechanisms to the pathogenesis of portal hypertension.**

Despite the high mortality associated with decompensated alcoholic cirrhosis and severe alcoholic hepatitis (up to 40% in some series) (67), there are a limited number of treatment options and a lack of consensus amongst physicians on their use. In general, the management is supportive with the additional use of glucocorticoids in severe alcoholic hepatitis (68) although this treatment, despite encouraging results in selected subgroups of patients, remains controversial. (69) Similarly, treatments to decrease portal pressure such as  $\beta$ -blocker monotherapy achieve a target reduction of  $\geq 20$  % reduction in hepatic venous pressure gradient (HVPG) in only a small percentage of patients. (70) This intervention is poorly tolerated, and has little impact on the inflammatory mechanisms that appear to promote increased intrahepatic resistance. Moreover, these treatments are flawed by the fact that whilst decreasing portal pressure, they also decrease mean arterial pressure and thus, further compound failing perfusion pressure through sensitive organs such as the liver and kidney. (46) There is therefore, a significant clinical need to understand the pathophysiology of circulatory dysfunction in alcoholic cirrhosis and relate this to the apparent inflammatory mechanisms that exist in animal models, in the hope that this will help in the design of more targeted therapies.

### **1.6 Hypothesis:**

*In alcoholic liver disease patients, a marked pro-inflammatory response, for which TNF $\alpha$  is important, regulates vascular resistance by modulating the activity of eNOS in systemic and regional vascular beds.*

### **1.7 Aims:**

1. To define the relationship between inflammatory mediators and the expression of severity of disease and vascular dysfunction in alcoholic cirrhosis, and assess responses to anti-inflammatory intervention
2. To explore the relationship between nitric oxide generation and inflammation in cirrhosis
3. To study novel regulators of nitric oxide synthase and explore their biological significance in alcoholic cirrhosis



## ***Chapter 2***

### ***Methods***

## **2.1 Patient Studies**

### ***2.1.1 General and ethical consideration:***

Patients were recruited from University College London Hospitals (unless otherwise stated for a specific study) with an appropriate alcohol history, and clinical or/and histological evidence of alcoholic cirrhosis. All patients or their relatives gave written informed consent or assent, respectively, in accordance with the Declaration of Helsinki of the World Medical Association, 1951. Several concurrent interventional studies within The Liver Failure group including use of MARS, anti-TNF therapies, Probiotics and L-ornithine-L-aspartate had local ethics (UCLH/UCL) approval for collection and storage of data, plasma and liver biopsy samples obtained during routine clinical acquisition, which were used for the studies described below.

In general, baseline haemodynamic studies (described below) were conducted at the time of a trans-jugular liver biopsy or/and assessment of portal pressure, as clinically indicated. All patients were managed with standard of care in addition to any specific intervention for a defined study, and routine daily observations including blood pressure, heart rate, temperature and urine output. In addition, three times per week if not daily estimation of urea and electrolytes, liver function tests and blood count were conducted.

Any healthy controls studied also gave full informed consent to participate, including the taking of blood samples and subsequent storage for further scientific investigation.

## ***2.1.2 Haemodynamic studies***

### ***2.1.2.1 Hepatic venous pressure gradient assessment***

All haemodynamic studies were performed following an overnight fast and a 1-hour period during which the patient had been resting supine. Patients were sedated for the procedure using midazolam (mean dose of 3 mg; Phoenix Pharma Ltd., Gloucester, UK), as has been shown to be appropriate during HVPG measurements owing to lack of effect on HVPG measures whilst improving patient tolerability to the procedure.<sup>(71)</sup> None of the patients in whom haemodynamic measurements were made were on beta-blockers. A 5Fr Berenstein occlusion balloon catheter (Boston Scientific, Cork, Ireland) was introduced via the right internal jugular route into the right hepatic vein under fluoroscopic screening (Toshiba Spot Film Device Model: SA-900U; Tochigi-ken, Japan). Wedged hepatic venous pressures (WHVP) were assessed in triplicate in at least 2 radicals after inflation of the balloon and injection of 2mls of contrast medium (Iohexol [Omnipaque] Amersham Health, Little Chalfont, UK). Careful attention to fluoroscopic examination ensured wedged positions obtained were without drainage by local venous shunts. Free hepatic venous pressures (FHVP) were measured on deflation of the balloon. Pressure measurements were recorded via pressure transducer sets (Medex Medical, Rossendale, Lancashire, UK) on a Hewlett Packard monitor (Model 86S, HP, Palo Alto, CA, USA). Hepatic venous pressure gradient (HVPG) was calculated as the difference between WHVP and FHVP. In certain studies, where described, 24 hour HVPG measurements were made by keeping the hepatic venous catheter in place for a repeat reading.

### *2.1.2.2 Cardiovascular Haemodynamics*

Heart rate, oxygen saturation and ECG were recorded continuously and the mean arterial pressure (MAP) ( $1/3$  [systolic – diastolic] + diastolic pressure) was measured prior to catheterization and every 5 minutes thereafter (Hewlett-Packard, Model 86S, HP, Palo Alto, CA, USA). The pulmonary artery was catheterized via the internal jugular sheath using a Swan-Ganz catheter (Edward Lifesciences, Irvine, CA) and cardiac output (CO) calculated by thermodilution and displayed electronically (Vigilance monitor, Critical Care Edwards Lifesciences, Irvine, CA). Each measurement was performed in triplicate and an electronic mean calculated. Systemic vascular resistance (SVR) was also calculated when CO, MAP, and central venous pressure (CVP) were known, using the formula:

$$\text{SVR} = 80 \times (\text{MAP} - \text{CVP}) \div \text{CO}.$$

### *2.1.2.3 Hepatic blood flow*

Hepatic blood flow (HBF) was determined using a primed (12 mg) followed by a continuous infusion (1 mg/min) of indocyanine green (ICG) (Akorn Inc., Buffalo Grove, IL, USA). The infusion was started 1 hour prior to sampling to ensure steady state concentrations. HBF was measured by simultaneous sampling of arterial and hepatic venous blood. ICG levels were determined spectrophotometrically at an absorbance wavelength of 805 nanometres, on an Agilent 8453 diode-array spectrophotometer (Agilent UK) with detection capabilities in the ultra-violet range. Plasma flow rate of the liver was calculated on the method of indicator dilution and Fick's principle.(72, 73), and was converted to blood flow using the haematocrit.

### *2.1.2.4 Renal blood flow*

Renal blood flow (RBF) was determined using a primed infusion (bolus of 0.5g), and a continuous infusion (0.5g/hour) of *para*-aminohippuric acid [PAH] (Apotheek

Academisch Ziekenhuis, Maastricht, NL). As with HBF, sampling commenced after steady state was reached. RBF was determined by measurement of PAH in the arterial and renal venous blood by spectrophotometric analysis (as above) and the flow rate calculated by the method of indicator dilution and Fick's principle.

## **2.2 Neutrophil oxidative burst and phagocytosis determination**

Neutrophils were either investigated in a whole blood assay (as described below) or after isolation by a one-step gradient centrifugation.

### ***2.2.1 Neutrophil Isolation***

Whole blood (4ml) was layered over 5 ml of Polymorphprep (Axis-Shield, Oslo, Norway) and spun for 30 min at 400g at room temperature. Neutrophils were harvested from the second interface and washed with phosphate buffered saline (Sigma Aldrich, St. Louis, MO, USA). Neutrophils were counted in a Thoma-haemocytometer and re-suspended in phosphate buffered saline at a density of  $5 \times 10^5$  in 50  $\mu$ l. 50  $\mu$ l of cell suspension and 50  $\mu$ l of plasma were used for one assay. Viability was tested by Trypan-Blue exclusion and was over 98%.

### ***2.2.2 Neutrophil activation (oxidative burst)***

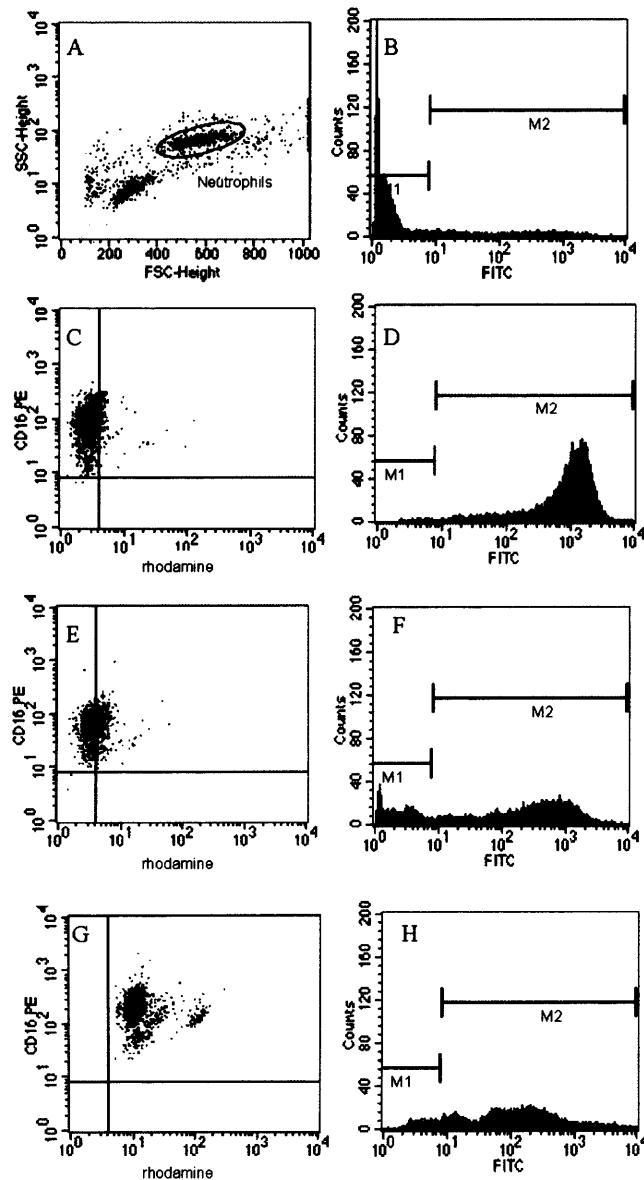
The Phagoburst<sup>®</sup> kit (Orpegen Pharma, Heidelberg, Germany) was used to determine the percentage of neutrophils that produce reactive oxidants with or without stimulation according to the manufacturer's instructions. In brief, 100  $\mu$ l of heparinized whole blood or 50  $\mu$ l of isolated neutrophils and 50  $\mu$ l of plasma (as indicated) were incubated for 20 minutes with 20  $\mu$ l of opsonized *E.coli* ( $2 \times 10^7$  bacteria), N-formylmethionyl-leucyl-phenylalanine (fMLP; 5 $\mu$ M), phorbol-12-

myristate-13-acetate (8.1  $\mu\text{M}$ ) or without stimulus at 37°C. Formation of reactive oxidants was monitored by oxidation of dihydrorhodamine 123 to rhodamine which gives green fluorescence. To identify neutrophils, cells were stained with anti-CD16-PE antibody (Immunttools, Friesoythe, Germany) and analysed by fluorescence activated cell sorting (FACS; Becton Dickinson FACScan, San Jose, USA), using Cellquest™ software. Neutrophils were gated on forward and side scatter characteristics (Figure 2.1A) and subsequently the percentage of CD16-positive cells producing reactive oxygen metabolites (green fluorescence) was calculated (Figure 2.1 C, E, and G). Samples were analyzed in duplicate or triplicate. The inter-assay coefficient of variation (CV) for resting burst was 5.4% and for stimulated burst 4.2%. The intra-assay CV for resting burst was 4.7% and 2.4% for stimulated burst.

### ***2.2.3 Neutrophil phagocytic capacity***

The Phagotest® (Orpegen Pharma, Heidelberg, Germany) was used to measure phagocytosis by using FITC-labelled opsonized *E. coli* bacteria. 100  $\mu\text{l}$  of whole blood or 50  $\mu\text{l}$  of isolated neutrophils and 50  $\mu\text{l}$  of plasma (as indicated), were incubated with 20  $\mu\text{l}$  of bacteria ( $2 \times 10^7$ ) at 37°C for 20 min, while a negative control sample remained on ice. Phagocytosis was stopped by placing the samples on ice and adding quenching solution that allowed discrimination between attachment and internalization. Erythrocytes were lysed and DNA staining solution allowed exclusion of aggregation artefacts in the form of bacteria or cells. The cells were spun at 250g for 5 minutes and further re-suspended in 50 $\mu\text{l}$  of washing solution, and incubated with 10 $\mu\text{l}$  anti-CD-16 antibody (IOTest®, Beckman Coulter) for 30-40mins at room temperature and analyzed by flow cytometry. Neutrophils were gated on forward and side scatter (Figure 2.1A) characteristics and subsequently the

percentage of CD16 positive cells– FITC positive cells, corresponding to the percentage of neutrophils undergoing phagocytosis and the geometric mean of fluorescence intensity (GMFI), corresponding to the number of bacteria engulfed by one cell, was analyzed. Figure 2.1 B, D, F, and H. To avoid misinterpretation of results due to batch-to-batch variability of bacteria, results were normalized to the mean of at least 3 healthy control samples for each new batch of bacteria used. Samples were analyzed in duplicate or triplicate. The inter-assay CV was 10.1% (reflecting the potential differences between batches of bacteria used as stated above), the intra-assay CV was 1.6%.



**Figure 2.1:** (A) Neutrophils gated according to their forward and side scatter (B) Analysis of phagocytosis: On a sample without bacteria, markers are set such that 99% of the gated neutrophils appear within the first marker (C) FACS-plot from a healthy control subject; percentage of double positive cells (CD16 positive cells producing reactive oxygen metabolites determined by green fluorescence in FL-1) are measured. (D) Corresponding FACS-plot of a healthy control with normal GMFI (E) FACS-plot from a patient with low resting burst, (F) Corresponding FACS-plot of a patient with low GMFI, (G) FACS-plot from a patient with high resting oxidative burst (H) Corresponding FACS-plot of a patient with very low GMFI



## **2.3 Animal model studies**

All animal experiments were performed under a Home Office directed animal project license and adhered to the Animals Scientific Procedures Act 1986.

### ***2.3.1 Bile duct ligation in rats***

The bile duct ligated (BDL) rat is a model of rapid liver fibrosis that translates as a chronic model of cirrhosis. (74)

Surgery was performed under anaesthesia (diazepam 1mg/kg intra-peritoneal (I.P), followed by Hypnorm® 150µl/kg intramuscular (I.M), Janssen Pharmaceutica, Belgium). All rats underwent bile duct ligation to induce biliary cirrhosis, or a Sham operation. A midline abdominal incision was made under anaesthesia. The common bile duct (CBD) was isolated, triply ligated (to ensure definitive ligation) using 3-0 silk sutures and sectioned between the 2 distal ligatures. Sham-operated rats underwent laparotomy and isolation of the CBD without ligature or section, leaving the biliary system intact.

Twenty-eight days after operation, the rats were randomized into groups, with BDL or Sham-operated rats as the respective controls and test animals for counterparts that were injected with bacterial lipopolysaccharide (LPS – from *Salmonella typhimurium*, 0.5 mg/kg I.P made up to 0.5mls in saline) or saline. As per protocol, the rats were allowed free access to food and water for a period of 3 hours post-intervention in a temperature controlled environment and then sacrificed by exsanguination under terminal anaesthesia (Hypnorm 200µL/kg I.M), 20 minutes after diazepam (1mg/kg I.P). Blood was withdrawn from the descending aorta and immediately put into heparin/EDTA containing tubes until full exsanguination,

centrifuged, and the plasma collected and stored at -80°C until assayed. Liver tissue was snap frozen for further analysis.

### **2.3.2 Galactosamine treated rats**

Sprague Dawley rats (male, 250-300g) were administered a single IP injection of galactosamine (1g/kg, Sigma, Poole UK) in saline, to induce acute liver failure, or saline only as a control. After 24 hours animals were given a further IP injection of either saline or bacterial lipopolysaccharide (LPS 1mg/kg, from *Klebsiella pneumoniae*, Sigma, Poole UK) in saline, or saline only control, and returned to their cages. After three hours, 3 groups of five animals (sham, galactosamine, galactosamine plus LPS) were anaesthetised (Hypnorm, 250µl IP, Janssen Pharmaceutica, Belgium) and blood was withdrawn from the descending aorta into heparin containing tubes until full exsanguination, centrifuged, and the plasma collected and stored at - 80°C until the point of assay. Tissue samples were collected and flash-frozen in liquid nitrogen and stored at -80°C until use.

## **2.4 Laboratory analytical methods**

### **2.4.1 Cytokine/chemokine analysis**

Capture antibodies (BioSource AS, Belgium) for TNF $\alpha$ , IL-6, IL-8 and soluble TNF receptors I and II (TNF-R1, TNF-R2) were coated at 1 µg/ml onto 96 well Maxisorb (NUNC, Denmark) ELISA plates overnight at 4°C. The plates were washed 3x before being blocked with bovine serum albumin (300µl; 5g/L) (Fraction V, Sigma-Aldrich, Poole, UK) for 2hours at room temperature. Standard (7.5 – 1000pg/ml) or sample in 100µl together with biotin-conjugated detection antibody (100 µl; 0.4 µg/ml) (BioSource AS, Belgium) were co-incubated at room temperature for a

further 2hours. In order to accurately quantify TNF-R1, TNF-R2 serial dilution (from 1/32 and down) of sera samples was necessary. After washing, streptavidin (100µl; 125ng/ml) (BioSource AS, Belgium) was added to each well for 20min at room temperature. The plates were again washed before incubation with substrate (100µl, 300µg/ml o-phenylenediamine HCl; Sigma-Aldrich, Poole UK). The reaction was stopped with 50µl 1.8M sulphuric acid and the optical density measured at 450nm referenced against 630nm. Individual standard curves were established and a lower limit for TNFα detection set at 5pg/ml. Each standard and sample was measured in duplicate. After a single batch analysis, the intra-assay coefficient of variation was 5.4%.

#### **2.4.2 Measures of oxidant stress**

**2.4.2.1 Malondialdehyde (MDA) measurement:** The use of a thiobarbituric acid (TBA) test to define the presence of lipid peroxidation aldehydes such as MDA, is an accepted test of oxidative stress in disease.(75) Patient plasma (0.5 ml ) was added to a reaction mixture (1.0 ml) formed by equal parts of 15% trichloroacetic acid, 0.25 N HCl, and 0.375% TBA, plus 2.5 mM butylated hydroxytoluene (BHT) and 0.1 ml of 8.1% sodium dodecyl sulfate (SDS), followed by 30 min heating at 95°C; pH value of the analytical reaction mixture was about 0.9. BHT was used to prevent lipid peroxidation during heating. After cooling, the chromogen was extracted with *n*-butanol and read spectrophotometrically at 532 against a “blank” reaction mixture lacking plasma but subjected to the entire procedure and extracted with *n*-butanol. To correct for background absorption, absorbance values at 572 nm were subtracted from those at 532 nm, the latter representing the absorption maximum of the 2:1 TBA:MDA adduct.

*2.4.2.2 Measurement of F2-isoprostanes in plasma:* Free 8-Isoprostane F2alpha was assayed with a commercial enzyme immuno-absorbance (EIA) kit (Cayman Chemical, Ann Arbor, MI) according to manufacturers instructions.(76, 77) 200µl plasma was deproteinised with 600µl ethanol containing 8KBq <sup>3</sup>H-PGE2 as an internal standard to account for losses. After centrifugation the supernatant was reduced to near dryness, 2ml acetic acid was added and the mixture applied to a preconditioned (acetic acid washed) C18 SPE cartridge (Waters Corp, Milford, MA). The column was washed with 5ml water, dried with oxygen-free nitrogen and eluted with 5ml HPLC grade hexane, before the prostanoid fraction was eluted with 5ml ethylacetate containing 1% methanol. The eluant was reduced to dryness, reconstituted in 450ul of EIA buffer, 100µl being used to determine recovery of <sup>3</sup>H-PGE2 and 50µl added to the EIA plate with 50µl isoprostane tracer and 50µl antibody and incubated for 18h at room temperature. The plate was washed before the addition of 200µl of Ellman's reagent. The optical density of each well was measured at 420nm and isoprostane levels were determined by reference to authentic standards and corrected for losses calculated from radioactive prostaglandin recovery.

### **2.4.3 Nitric oxide measurement**

#### *2.4.3.1 NOx: (nitrate/nitrite) production*

A method for simultaneous detection of nitrate and nitrite in plasma with a modified Greiss reaction, as described by Miranda et al (78) was used. All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) and were of the highest laboratory grade. A saturated solution of vanadium (III) chloride (400mg) was prepared in 1M hydrochloric acid (50 mls). Greiss Reagent A was prepared from N-(1-Naphthyl)

ethylenediamine dihydrochloride (0.1% w/v) in ddH<sub>2</sub>O. Greiss Reagent B was prepared from sulfanilamide (2% w/v) in 5% hydrochloric acid. All three solutions were stored in the dark.

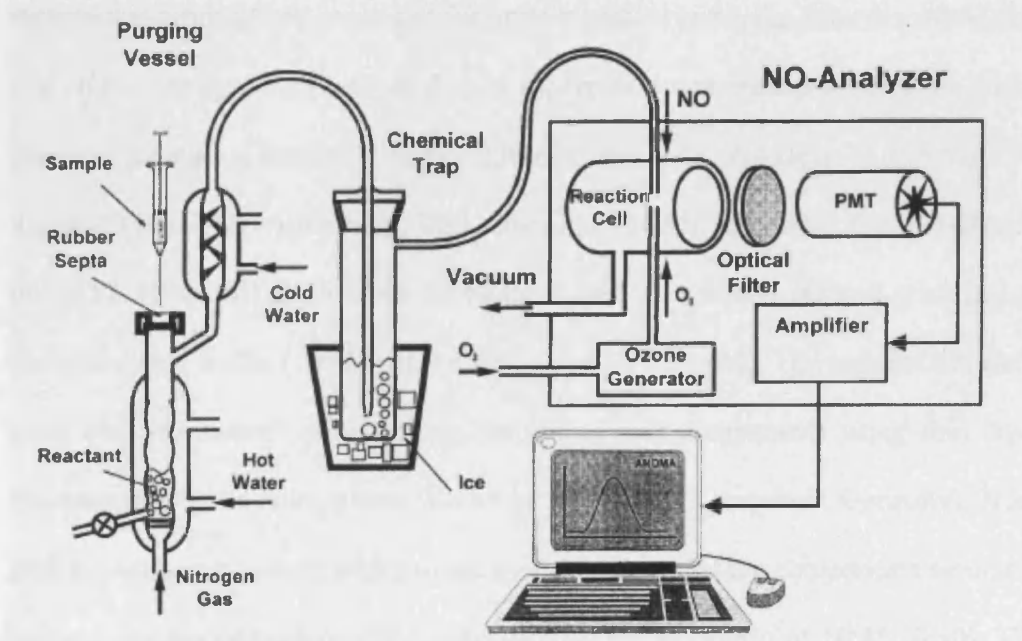
100 µL of plasma was diluted 1 in 4 with phosphate buffered saline. This was placed in a Whatman 12 kDa molecular weight cut off filter (Vectaspin, Whatman, Maidstone UK) and spun at 13,000 rpm for 30 minutes at 4 °C to centrifugally remove proteins as described by Giovannoni et al.(79) For the measurement of nitrate, 50 µL of the filtrate was pipetted out into a 96 well plate (Maxisorb, NUNC, Denmark). To this were added 50 µL of vanadium (III) chloride solution, 50 µL of Greiss Reagent A, *N*-(1-Naphthyl)ethylenediamine dihydrochloride (NEDD), and 50 µL of Greiss Reagent B, sulfanilamide (SULF), whereby nitrate was catalytically converted to nitrite by vanadium (III) chloride. Nitrite was measured in a similar manner except that samples were only exposed to Griess reagents A and B. The nitrite and nitrate in the filtrate were determined against a standard curve measured at 550nm on a Sunrise 96 well plate reader with accompanying Magellan 3 software (Tecan Sunrise, Salzburg Austria). Linear regression of the mean values of the absorbance at 540 nm for each standard set minus the blank values was utilized to determine the nitrite or total NO<sub>x</sub> concentrations in samples. These values were then subtracted to give the nitrate concentration. Each sample was analysed in duplicate.

#### *2.4.3.2 Measurement of nitrite in plasma*

A method for sensitive and specific measurement of nitrite in plasma using a chemiluminescence assay (80) was used. I<sub>3</sub><sup>-</sup>, synthesised by the addition of potassium iodide and iodide to acetic acid and water, will reduce nitrite (but not nitrate) to NO gas. The released NO gas is carried from the solution in a stream of

nitrogen gas into the chemiluminescent NO analyser which can detect from 0.3 to 1 pM NO gas. The instrumental setup is illustrated in Figure 2.2.

All chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK) and were of the highest laboratory grade. A stock solution of 180 ml of  $I_3^-$  reagent was prepared from 2g potassium iodide and 1.3g of iodine dissolved in 40 ml ddH<sub>2</sub>O. 140 ml of acetic acid was then added and mixed thoroughly for 30 minutes. Nitrogen gas was bubbled through the 9ml  $I_3^-$  reagent in a glass purge vessel. The vessel is linked to a trap containing 15 ml of 1 M sodium hydroxide and is then connected to the chemiluminescent NO analyser (Sievers, Model 280, Boulder, Colorado, USA) as shown in Figure 2.2. The system was then allowed to reach a steady baseline.



**Figure 2.2** A schematic diagram of the chemiluminescence instrumental setup

100  $\mu$ L of plasma was diluted 1 in 4 with phosphate buffered saline. This was placed in a Whatman 12 kD molecular weight cut off filter (Vectaspin, Whatman, Maidstone UK) and spun at 13,000 rpm for 30 minutes at 4 °C to centrifugally remove proteins as described by Giovanni et al.(79) 10  $\mu$ L of the filtered plasma was

then injected via a syringe into the purge vessel. The NO released then passes into the NO analyser to produce a mV peak which was then measured and recorded. The mV peaks were allowed to return to baseline prior to subsequent injections.

#### *2.4.3.3 Tissue eNOS activity determination*

The method used was a variation on the widely reported  $^3\text{H}$ -arginine to  $^3\text{H}$ -citrulline assay,(81, 82) modified for use with liver tissue. Briefly, snap frozen liver tissue was homogenised in ice-cold HEPES buffer (20mM, pH 7.4) containing EDTA (1mM); sucrose (250mM); valine (60mM); protease inhibitor cocktail (at 1ml/20g tissue); and PMSF (3.4mg/ml). This was centrifuged (1000g, 10min, 4°C) and the supernatant retained and measured for protein content using the Biuret method.(83) For NOS activity determination, 5µl of supernatant was incubated with 40µl of a reaction medium [Tris-HCL buffer (30mM, pH 7.4); NADPH (1.25mM);  $^3\text{H}$ -arginine (10µCi/ml, Amersham, UK); norvaline (5mM); and either  $\text{CaCl}_2$  (400µM) or EGTA (600µM)] at 30°C for 30 minutes, and the reaction stopped with 1ml of ice-cold citrate buffer (50mM pH5) containing EDTA (1mM). The arginine:citrulline ratio was determined by separating the amino acid components using thin layer chromatography on silica plates (Kieselgel 60, Merck, Darmstadt, Germany). Non-tritiated amino acids were added to aid spot detection, and the components separated using a running mixture of  $\text{CHCl}_3$ :MeOH: $\text{NH}_4\text{OH}$ : $\text{H}_2\text{O}$  (ratio of 10:45:20:10). The individual spots were removed and the scintillation activity measured (Ultima Gold scintillant, Perkin Elmer, Boston, MA), using a Tri-Carb 2100TR, liquid scintillation analyser (Packard Biosciences, Berks, UK.). Activity was expressed as µmoles citrulline made/ mg protein/ hr. All chemicals and reagents were supplied by the Sigma Chemical Company (Poole, UK) unless otherwise stated.

As previous studies on hepatic tissue from decompensated alcoholic cirrhotic liver patients had suggested that eNOS is the only significant calcium dependent, constitutive, isoform of NOS expressed in these patients' livers,(84) the difference in the measured NOS activities between the calcium present / calcium chelated (EGTA present) assays described above, was interpreted as representing eNOS activity.

#### **2.4.4 eNOS protein expression**

Immunohistochemistry was carried out using standard antigen-retrieval techniques.- Immunohistochemical staining was performed on 4µm thick formaldehyde-fixed/paraffin-embedded, mounted hepatic tissue sections, counter-stained with haematoxylin. After 10 minutes heating at 70°C, slides were de-parafinized with xylene, then re-hydrated with decreasing grades of ethanol and finally with water. Optimum antigen retrieval was achieved by microwaving slides in citrate buffer for 20 minutes. Specific mouse anti-human monoclonal antibodies for eNOS (Transduction Laboratories/Pharmingen, San Jose, CA) were applied and normal mouse serum used as a negative control.

#### **2.4.5 ADMA measurement**

Plasma ADMA was measured at The Wellchild Laboratory, Guys Hospital, Kings College London by Dr RN Dalton, using fragmentation specific stable isotope dilution electrospray mass spectrometry-mass spectrometry. Samples (50µl) were deproteinized with acetonitrile containing <sup>2</sup>H<sub>6</sub>-ADMA, chromatographed (acetonitrile:water, 1:1, with 0.025% formic acid) on a Teicoplanin guard column 10mm x 2.1mm ID (Chirobiotic T, ASTEC Ltd, Congleton, UK), and analysed using a SCIEX API4000 (Applied Biosystems, Warrington, UK) in positive ion multiple



reaction monitoring mode. The acquisition time was 2.5min with an inject-to-inject time of <3min. Intra-assay imprecision for ADMA was 2.1% at a concentration of 0.37  $\mu\text{mol/l}$ . Inter-assay imprecision for ADMA was 7.4%, 5.8%, and 5.1% at concentrations of 0.39, 1.15, and 3.96  $\mu\text{mol/l}$ , respectively.

Tissue homogenates were prepared as for the NOS activity assay described in section 2.4.3.3. The supernatants from these preparations were extracted and assayed for ADMA using the same methodology as for plasma samples, outlined above, but using 10 $\mu\text{l}$  of each homogenate sample.

#### **2.4.6 DDAH and PRMT Western blot analysis**

Human liver biopsy specimens were assessed for quantification of DDAH and PRMT. Homogenized frozen liver tissue was re-suspended in 100 $\mu\text{L}$  of lysis buffer (PBS, pH 6.6, containing 0.1% SDS, 0.5% sodium deoxycholate and 1% Nonidet). After protein concentration determination, 30 $\mu\text{g}$  of protein was separated by SDS polyacrylamide gel (12%) and transferred to a nitrocellulose membrane, which was blocked with 5% non-fat dry milk in PBS containing 0.1% Tween for one hour. Samples were incubated overnight at 4°C with the primary antibody (mouse monoclonal DDAH II or rabbit polyclonal PRMT-1 antibody, 1:1000), before washing and further incubation with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (DDAH II and PRMT-1, respectively) at a dilution of 1:3000. The membranes were developed with enhanced chemiluminescence substrate plus (ECL plus, Amersham, UK). Blots were imaged and quantified using Genesnap/Gene tools (Syngene, SLS, UK). Membranes were then stripped at 55°C and re-probed using an  $\alpha$ -tubulin mouse monoclonal primary antibody followed by secondary anti-mouse horseradish peroxidase-conjugated

secondary antibody and developed as above, in order to quantify the expression of DDAH and PRMT by correcting it for the  $\alpha$ -tubulin content.

## **2.4.7 NOSTRIN study methods**

### *2.4.7.1 Quantitative real-time PCR*

*mRNA estimation of NOSTRIN, NOSIP, Caveolin 1 and eNOS:* This work was done in collaboration with Prof Werner Muller-Esterl's group in The Institute Biochemie II laboratories, Goethe University, Frankfurt.

mRNA and protein was isolated from liver tissue using the TRI-Reagent (Sigma, Taufkirchen, Germany) following the manufacturer's instructions. From the mRNA, cDNA was synthesised using the iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, CA, USA) according to manufacturer's recommendations. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays were developed using the TaqMan technology on an iCycler detection system (Bio-Rad). Assay mix of primers and TaqMan MGB probes (FAM™ dye-labelled) for eNOS, caveolin, NOSTRIN $\beta$  and control ubiquitin C (UbC) spanning exon-exon junctions to exclude detection of genomic DNA were from Applied Biosystems (Foster City, CA, USA). To detect NOSTRIN $\alpha$ , an "assay-on-design" (Applied Biosystems) with the probe spanning the junction of exon-2 to exon-3 of the NOSTRIN sequence was used. The whole cDNA of each liver sample was split up into six equal aliquots and analysed for target genes and endogenous control. Duplicate cycle threshold ( $C_T$ ) values were normalised against the internal control UbC and analysed using the  $\Delta C_T$  method. The relative expression levels of NOSTRIN $\alpha$  and NOSTRIN $\beta$  RNAs were calculated from the normalised mean  $C_T$  values.

#### *2.4.7.2 NOSTRIN Immunohistochemistry*

Paraffin-embedded sections were pre-treated with xylene and then micro-waved in citrate buffer for 20 minutes, to optimize antigen retrieval. Specific murine monoclonal antibodies to human eNOS, caveolin-1 (Transduction Laboratories, Lexington, USA) and human NOSTRIN (raised against GST-NOSTRIN<sub>242-506</sub> by Nanotools, Teningen, Germany) were applied. As a control, the antibodies were blocked with GST-NOSTRIN prior to the staining.

#### *2.4.7.3 Western blots*

Protein fractions of whole liver samples were isolated with TRI-Reagent according to the manufacturer's instructions. These fractions as well as whole cells were dissolved in Laemmli buffer (63 mM Tris•HCl, pH 6.8, 2.5% SDS, 5% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue), subjected to SDS-PAGE and transferred to PVDF membranes, which were then probed with mouse monoclonal anti human Lamp-1 (Transduction Laboratories, Lexington, USA) and human NOSTRIN.

### **2.5 Statistical Analysis**

Data are represented as median and range or mean ± standard error of the mean (SEM). Unpaired t-tests have been used to define differences between means of normally distributed, equal variance data, using a commercially available package (Graph Pad Prism, version 3.0; Graph Pad Software, Inc., SanDiego, CA, USA). For statistical evaluation of data that was not normally distributed, an appropriate non-parametric test such as a Mann Whitney test, has been used. Results were considered significant if  $p < 0.05$ .

## ***Chapter 3***

### ***The role of inflammation in alcoholic cirrhosis***

### **3.1 The effects of inflammation on structure and function**

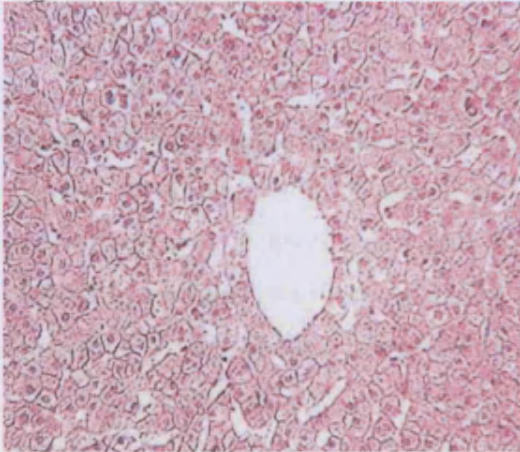
The initial elements of study were to determine the fundamental differences that inflammation imparts onto patients with pre-existing alcoholic liver disease and animal models of liver injury, with reference to liver structure, systemic measurable indicators of biochemical function and inflammatory parameters. Markers of oxidative stress were also studied and an assessment of function made with reference to disease presentation and outcome.

#### ***3.1.1 Structural changes in cirrhosis with inflammation***

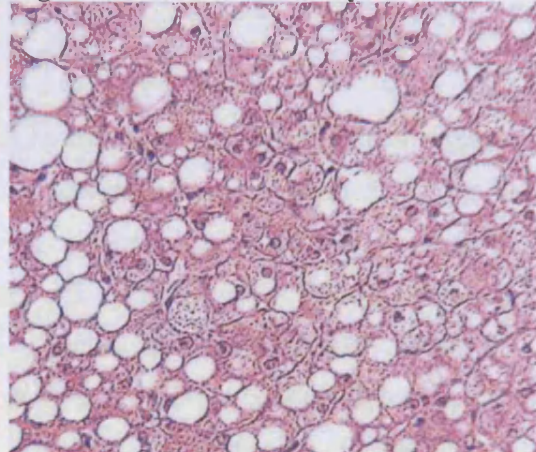
##### ***3.1.1.1 Histological presentation with human alcoholic liver disease***

As can be seen within the haematoxylin and eosin preparations at low magnification during light microscopy, shown in Figure 3.1, in normal liver there are plates of hepatocytes radiating out from a central vein forming the basic unit of a hepatic lobule (Figure 3.1 a). With the development of alcoholic liver disease, there is often an increasing deposition of fat initially within the cytoplasm of hepatocytes as seen in Figure 3.1b. Further progression in about 15% patients leads to the development of cirrhosis as denoted by fibrous bands and loss of the normal architectural pattern as seen in Figure 3.1c. This pattern of injury can be readily distinguished from the effects of a superimposed alcoholic hepatitis where the additional inflammatory insult leads to the deposition of Mallory's hyaline (a collection of cytoplasmic microfilament inclusions) within swollen "ballooned" degenerating hepatocytes (Figure 3.1d). Other characteristic features of alcoholic hepatitis include the ingress of polymorphonuclear cells, necrosis of hepatocytes predominantly around the central vein and perivenular and pericellular fibrosis. A number of these features are shown in Figure 3.1e.

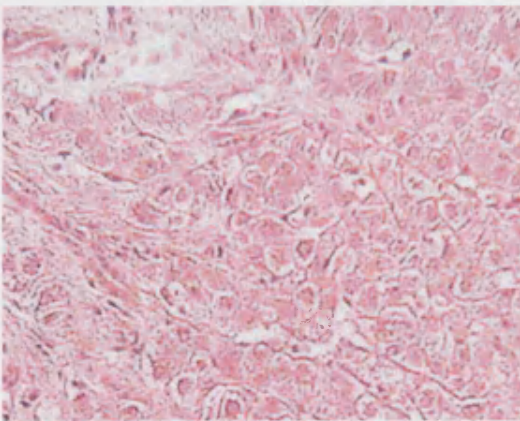
**Figure 3.1a: Normal liver**



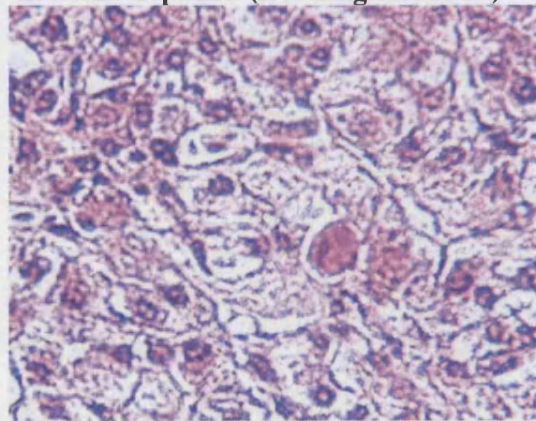
**Figure 3.1b: Alcoholic fatty liver**



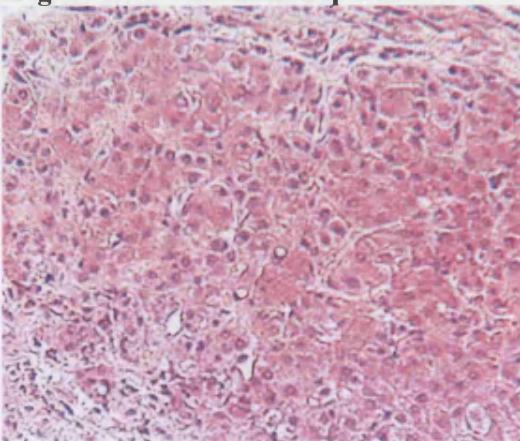
**Figure 3.1c: Alcoholic cirrhosis**



**Figure 3.1d: Mallory's hyaline in alcoholic hepatitis (x40 magnification)**



**Figure 3.1e: Alcoholic hepatitis**

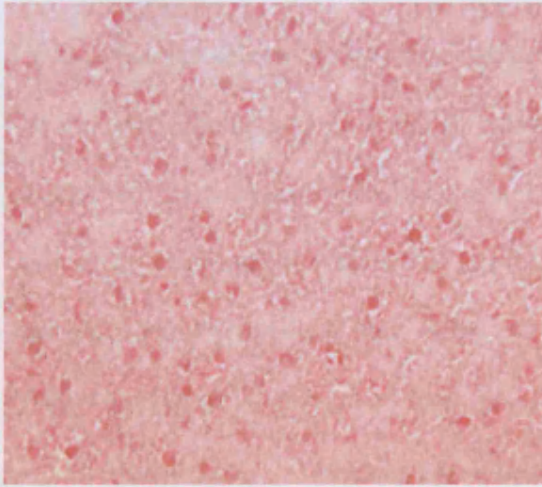


**Figure 3.1** Histological examples of normal human liver and alcoholic liver disease patients of varying severity: **a)** normal liver architecture; **b)** liver section from a patient with early alcoholic liver disease with evidence of fatty infiltration, no peri-cellular fibrosis and relatively normal hepatocytes; **c)** an example of a section from a cirrhotic liver biopsy with clear distortion of the architecture by fibrous bands; **d)** high magnification of ‘Mallory’s hyaline’ in a biopsy from a patient with alcoholic hepatitis demonstrating this phenomenon consisting of collection of cytoplasmic inclusion; **e)** biopsy from a patient with acute alcoholic hepatitis with the key features of balloon degeneration of hepatocytes, peri-cellular fibrosis and an acute inflammatory infiltrate of polymorphonuclear cells.

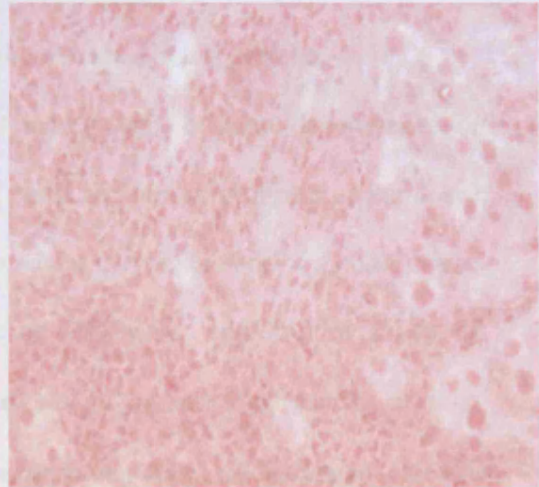
#### *3.1.1.2 Histological presentation in the bile duct ligated rat*

As seen in patients with alcoholic cirrhosis, BDL rats undergo significant loss of the normal cellular architecture (Figure 3.2a) seen in sham animals, with increasing fat deposition and loss of defined portal tracts, as seen in Figure 3.2b. This distortion and loss of liver architecture is further pronounced by the addition of LPS, as an inflammatory stimulus to BDL animals, with greater parenchymal loss. Figure 3.2c.

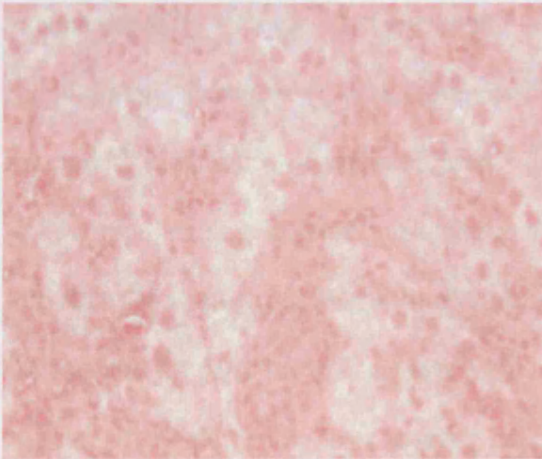
**3.2a: Sham Rat**



**3.2b: BDL Rat**



**3.2c: BDL+LPS Rat**



**Figure 3.2 a-c:** Histological specimens from rat liver showing: **a)** normal architecture of liver sinusoids and normal hepatocytes; **b)** following bile duct ligation with considerable loss of architecture, deposition of fat and with varying morphology of parenchymal cells; **c)** the effects of BDL with superadded LPS resulting in even greater architectural disruption.



### ***3.1.2 Serological changes***

#### ***3.1.2.1: Routine laboratory measures in Alcoholic liver disease patients***

For this study, patients were included if they had evidence of acute decompensation of alcoholic liver disease (increasing ascites, pedal oedema or progressive jaundice), clinical and radiological evidence of cirrhosis, and a current history of alcohol abuse. Patients were excluded if they were <18 or >75 years and had evidence of: additional/another aetiology of liver disease; severe cardiac dysfunction or renal failure (creatinine >150 $\mu$ mol/L); hepatic/extra-hepatic malignancy; hepatic encephalopathy  $\geq$ Grade 2 (precluding informed consent), and if there was microbiological evidence (serial cultures of blood, urine, stool and sputum dependent on clinical indication) of infection after 72 hours of broad-spectrum antimicrobials, or abnormal chest radiography. Alcoholic hepatitis was defined histologically taking into account steatosis, neutrophil infiltration, ballooning of hepatocytes, peri-cellular fibrosis and the formation of Mallory's hyaline.

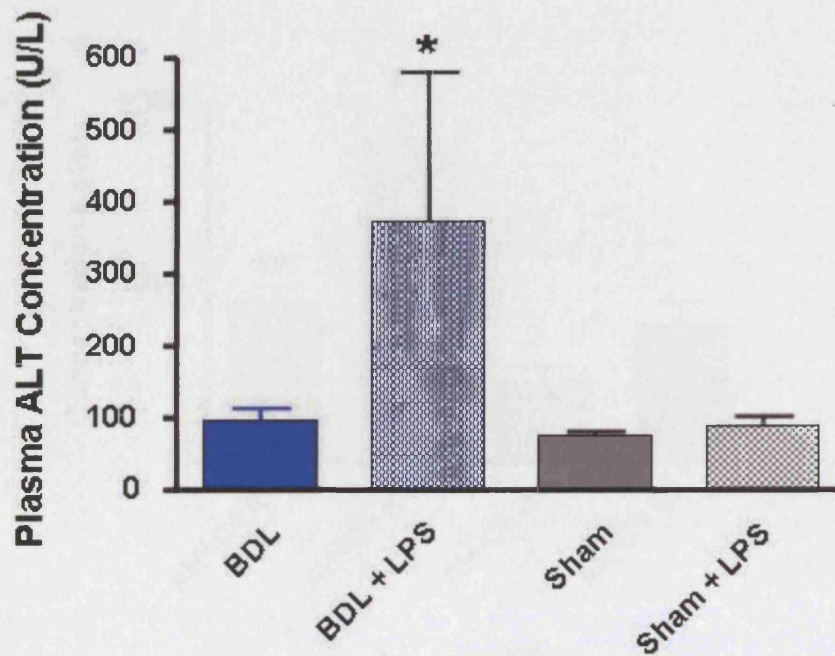
A total of 52 alcoholic liver disease patients were studied sequentially, 27 with alcoholic hepatitis (denoted as AH+C+) and 25 with alcoholic cirrhosis without any histological features of alcoholic hepatitis (denoted AH-C+). The definition of alcoholic hepatitis utilised a grading system which in addition to identification of Mallory's hyaline assessed the degree of hepatocyte ballooning and the numbers of acute inflammatory cells according to:- Grade 0-1: ballooned hepatocytes comprising <5% of parenchyma and no neutrophils, ranging up to grade 5 where ballooned hepatocytes comprised >50% of parenchyma and there were many inflammatory cells. Applying this system, patients with grade 2 and above were deemed to have histological AH+C+ superimposed on their cirrhosis. Indeed most patients were grade 3-5.

The white blood cell count (WBC) was significantly elevated in AH+C+ patients as compared to patients with cirrhosis alone:  $13.7 \pm 1.5$  versus  $6.4 \pm 0.5$ ,  $p < 0.00001$ . Similarly, the C reactive protein (CRP) was significantly greater in AH+C+ patients being  $50 \pm 7.7$  mg/L versus  $16.6 \pm 3.2$  mg/L in AH-C+ patients. Measures of liver function demonstrated significantly elevated plasma bilirubin levels in AH+C+ patients compared with AH-C+ patients:  $249 \pm 38.5$   $\mu\text{mol/L}$  vs.  $56.4 \pm 13.1$   $\mu\text{mol/L}$ ,  $p < 0.001$ . The serum aminotransferases did not differ significantly between the patient groups; serum ALT  $60 \pm 15$  U/L in AH+C+ patients, vs.  $47 \pm 9$  U/L AH-C+ patients,  $p = 0.46$ . Similarly, plasma albumin was not statistically significantly different between the groups albeit AH+C+ patients did have lower mean levels ( $28.4 \pm 1.7$  vs.  $31.7 \pm 1.1$  g/L;  $p = 0.14$ ). The prothrombin time, however, as a further marker of liver synthetic function was significantly higher in AH+C+ compared with AH-C+ patients ( $18 \pm 1$  vs.  $13.4 \pm 0.6$  seconds,  $p < 0.001$ ). In addition to these findings pertaining to the liver, the mean plasma creatinine as a marker of renal function, was also significantly elevated in AH+C+ patients compared to those with AH-C+ ( $92.5 \pm 11$  vs.  $59.8 \pm 5$   $\mu\text{mol/L}$ ,  $p = 0.01$ ), albeit this remained within the normal hospital reference range of 66-112  $\mu\text{mol/L}$ . It is important to note, however, a mass spectrometry method was used to measure creatinine, whilst normal hospital laboratory measures are via a modified Jaffe assay, which is open to misinterpretation in the context of hyper-bilirubinaemia. (85)

#### *3.1.2.2: Routine laboratory measures in BDL and Sham rats*

Four groups of animals were studied namely Sham operated (Sham), Sham+LPS, BDL and BDL+LPS. Bilirubin, creatinine, albumin and plasma aminotransferases were measured using standard kits on a Cobas Integra analyser (Roche Diagnostics

Ltd, West Sussex, UK). The serum aminotransferases were not significantly different between Sham animals with and without LPS, or the BDL animals. However, BDL animals also given LPS did have a higher plasma ALT compared with BDL alone animals ( $168 \pm 54$  vs.  $98 \pm 15$  U/L,  $p < 0.05$ ; Figure 3.3). Furthermore, the plasma bilirubin was significantly elevated to the same degree in both BDL and BDL+ LPS animals ( $180 \pm 18$   $\mu\text{mol/L}$ ) compared to Sham and Sham +LPS animals,  $p < 0.01$ . However no differences were noted between any of the 4 groups in relation to plasma creatinine, which remained within the normal range.

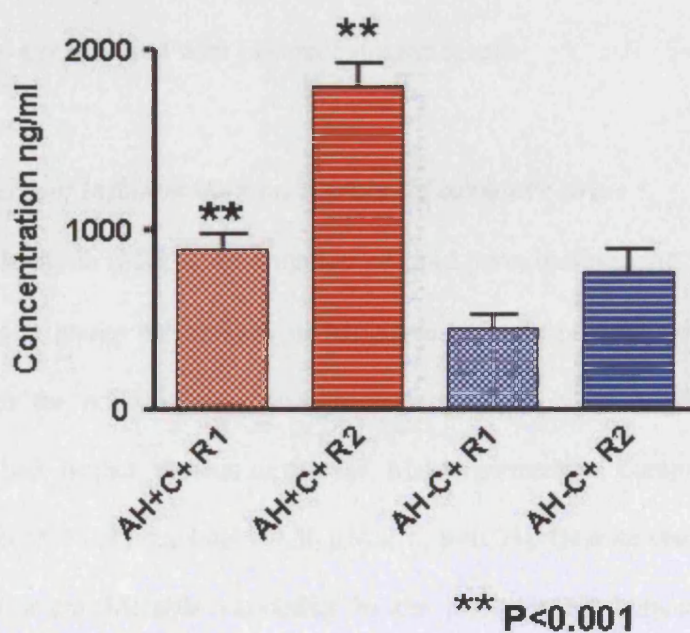


**Figure 3.3** shows the marked increase in plasma ALT (\*  $p < 0.05$ ) in BDL animals treated with LPS, which is not seen following LPS injection in sham animals without liver disease.

### ***3.1.3 Plasma Cytokines in patients with alcoholic liver disease***

Amongst the alcoholic patients studied, as anticipated from the increased white cell count and CRP, AH+C+ patients also had significantly elevated plasma cytokines.

The plasma TNF $\alpha$  was markedly higher in AH+C+ patients than was observed in AH-C+ patients ( $46 \pm 20$  versus  $4 \pm 0.5$  pg/ml,  $p < 0.01$ ). Similarly, IL-6 levels were much higher in AH+C+ patients ( $55.7 \pm 19$  versus  $9.3 \pm 2$ ,  $p < 0.01$ ) as shown in table 3.1, suggesting a potential to evoke further inflammatory signals and promote neutrophil recruitment. Plasma levels of TNF R 1 and TNF R 2 were found to be almost double the plasma level of that found in AH-C+ patients (TNFR1:  $885 \pm 93$  vs.  $455 \pm 74$  ng/ml,  $p < 0.001$ ; TNFR2:  $1798 \pm 128$  vs.  $759 \pm 136$  ng/ml,  $p < 0.001$ ), as shown in Figure 3.4. No consistent and significant differences were noted between AH+C+ patients and AH-C+ patients in relation to IL-8 and IL-10 plasma levels.



**Figure 3.4.** Plasma concentrations of TNF receptor 1 (R1) and TNF receptor 2 (R2) in patients with alcoholic cirrhosis with (AH+C+) and without additional alcoholic hepatitis (AH-C+).

### ***3.1.4 Markers of Systemic Inflammatory Response Syndrome (SIRS)***

The SIRS score(86) has been defined to reflect the effects of inflammatory events resulting in injury to numerous organs. In these studies SIRS was graded according to the criteria: temperature  $>38^{\circ}\text{C}$ ; a heart rate  $> 90$  beats/minute; tachypnoea  $>20$  breaths/minute; white blood cell count of  $>12 \times 10^9/\text{L}$  or  $< 4 \times 10^9/\text{L}$  or the presence of more than 10% immature neutrophils; with each component assigned 1 point. In AH+C+ patients, the SIRS score was significantly elevated with a mean of  $1.6 \pm 0.4$ , compared with AH-C+ patients in whom the mean was  $0.3 \pm 0.1$ ,  $p<0.0001$ . The severity of the SIRS components was in keeping with the high levels of serum cytokines and systemic markers of inflammation such as CRP, and had less variability as compared with plasma cytokine levels.

### ***3.1.5 Effects of inflammation on markers of oxidative stress***

Malondialdehyde (MDA), as a marker of lipid peroxidation, and Prostaglandin F $2\alpha$  were used to gauge the severity of oxidative stress in patients with cirrhosis and in those with the additional inflammatory state of alcoholic hepatitis. Patients with AH+C+ had higher plasma levels of MDA generation compared with AH-C+ patients ( $6.65 \pm 0.83$  vs.  $4.48 \pm 0.56$   $\mu\text{Mol/L}$ ;  $p=0.04$ ). Despite statistical significance there was a considerable variability in the MDA levels between patients. MDA values from healthy volunteers were noted to be  $3.3 \pm 0.21$   $\mu\text{Mol/L}$ .

By contrast, there was a highly significant difference between elevated Prostaglandin F $2\alpha$  levels in patients with AH+C+ compared to cirrhosis alone ( $404 \pm 38$  vs.  $220 \pm 17$  pg/ml;  $p<0.001$ ), although all liver disease subjects exceeded the 'normal range'. Normal range for Prostaglandin F $2\alpha$  using this assay as supplied by the manufacturers' data sheet (Cayman Chemical, Ann Arbor, MI), was (40-100 pg/ml).

### ***3.1.6 Functional effects of inflammation super-imposed on cirrhosis:***

#### ***3.1.6.1 Severity of presentation of liver disease***

AH+C+ patients had more marked expression of their liver disease at clinical presentation compared to patients with cirrhosis alone, as evidenced by increased incidence of hepatic encephalopathy and more resistant ascites reflected in higher Child-Pugh scores(87) ( $11.5 \pm 0.3$  vs.  $8.4 \pm 0.4$ ;  $p < 0.001$ ), and higher MELD scores, (88) ( $15 \pm 2.3$  vs.  $0.26 \pm 1.4$ ;  $p < 0.001$ ). These patients also had a severe presentation of AH as defined by a discriminant function (89) of greater than 32, as reflected by the higher bilirubin and prothrombin times, with the mean discriminant function score of  $46 \pm 6$ . Moreover, AH+C+ patients had a greater propensity to develop culture positive infections during the in-patient episode (65% versus 28%,  $p = 0.004$ ).

#### ***3.1.6.2 Patient outcome***

There were thirteen in-patient deaths in the fifty-two studied patients, twelve with AH+C+ and one with only cirrhosis. Of the AH+C+ deaths, eight developed renal failure, four of whom who also had concomitant sepsis. Patients who developed culture positive infections were also more likely to develop organ failure ( $p = 0.001$ ) and had a higher incidence of mortality ( $p = 0.002$ ). However, there was no direct association between development of organ failure and subsequent mortality and SIRS score or plasma level of pro-inflammatory cytokines.

In the cirrhotic only group, one in-patient died from renal failure despite supportive therapy, whilst the remaining 24 patients were successfully discharged from hospital.

## ***Chapter 4***

### ***Neutrophil dysfunction and contribution to the effects of inflammation in cirrhosis***

#### **4.1 Introduction**

In patients with severe alcoholic hepatitis, infection complicates the course of illness and is associated with significant morbidity and mortality. (90-92) Neutrophils are an essential component of the innate immune response and key players in the pathogenesis of alcoholic hepatitis. (93) Data on neutrophil function in alcoholic hepatitis is paradoxical with some studies suggesting neutrophil “priming”, indicating a readiness to respond to bacterial challenge as measured by hydrogen peroxide overproduction, decreased L-selectin expression (94) and high levels of neutrophil elastase (95). In contrast, other studies show decreased neutrophil phagocytic capacity correlating with disease severity. (96) Endotoxin is known to be elevated in patients with alcoholic hepatitis (97) and has the capability to both prime (98, 99) and also activate neutrophils. (100, 101)

Clarification of this controversy has important clinical implications.- Current strategies to treat alcoholic hepatitis include administration of immunosuppressive agents such as corticosteroids and more recently, anti-TNF strategies are being evaluated, that may further potentiate susceptibility to infection. Results of studies using these drugs in alcoholic hepatitis patients are conflicting, (102, 103) and whilst there is some data showing benefit with anti-TNF strategies,(104, 105) one trial has shown increased risk of infection and mortality resulting in early trial termination. (106)

In order to reconcile the apparent paradox, this study was conducted to assess whether neutrophils exist in either a ‘primed’ or a fully ‘activated state in different alcoholic cirrhosis patients. The study determined the effect that activation status had on susceptibility to infection and clinical outcome, using the hypothesis that full



activation would impair neutrophil responses to on-going bacterial challenge, rendering patients more susceptible to infection.

#### **4.2 Patient selection**

Patients admitted with alcoholic cirrhosis were enrolled into the study at the time of portal pressure assessment and liver biopsy, performed to evaluate the severity of disease and presence or absence of alcoholic hepatitis. Included patients had been admitted with acute decompensation of alcoholic cirrhosis manifest by increasing jaundice, with no clinical or microbiological evidence (chest X-ray, routine cultures of urine, blood, sputum and ascites) of infection. Prophylactic antibiotics (cefotaxime) were prescribed following initial cultures if there was a suspicion of infection, and stopped if subsequent cultures proved negative. Patients were excluded if they were <18 or >75 years, and had evidence of: organ failure (inotrope requirement, creatinine >150µmol/L, hepatic encephalopathy >grade 2, requirement for mechanical ventilation), hyponatraemia, hepatic/extra-hepatic malignancy, <3 days post gastrointestinal bleeding or if they received any immunomodulatory therapy prior to entry in the study. Blood from 20 age and sex matched healthy volunteers with no history of liver disease was studied to serve as controls for comparison of neutrophil function. Patients were classified histologically into those with cirrhosis and superimposed inflammatory alcoholic hepatitis (cirrhosis+AH), as described in Chapter 3, section 3.1.2.1.

#### **4.3 Study design**

Following correction of any electrolyte disturbances or hypovolaemia, peripheral venous blood was aseptically collected into pyrogen-free tubes (BD Vacutainer Lithium-Heparin, 60U per tube, BD, Plymouth, UK) and used for routine

biochemistry and neutrophil function. For experiments with cells, blood was kept at room temperature (maximum 1 hour); for harvesting plasma, blood was placed immediately on ice. After centrifugation at 4°C, plasma was aliquoted under pyrogen-free conditions into non-pyrogenic cryotubes (Corning Inc., Corning, USA) and stored at -80°C until further analysis. Whole blood or isolated neutrophils mixed with plasma were used to perform the Phagoburst® or the Phagotest® as described in the methods in chapter 2, section 2.2.2 and 2.2.3. For all experiments strict precautions were taken to avoid endotoxin contamination by working aseptically and using endotoxin-free equipment. In addition to routine laboratory data collection, discriminant function (89) and MELD (Model of End Stage Liver Disease) (88) and Pugh score (87) were calculated. The patients were followed prospectively over a period of 90 days. The occurrence of organ dysfunction and mortality were recorded

#### **4.4 Results**

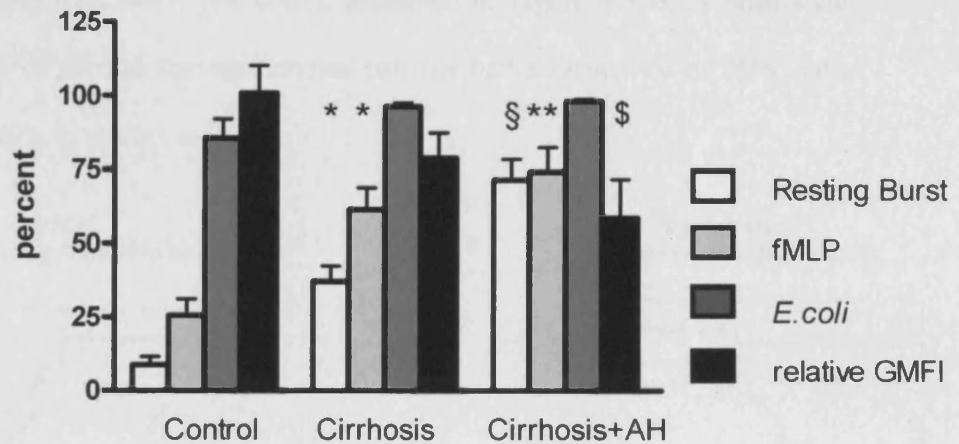
##### ***4.4.1 Oxidative burst and phagocytosis in patients with alcoholic cirrhosis***

A total of 63 patients with alcoholic cirrhosis (41 of whom have been described above in chapter 3, section 3.1.2.1) were screened for neutrophil activation status, and the patients were subsequently classified according to histological criteria into those with features of alcoholic hepatitis (graded through a modified NASH scoring system) (107) and those with cirrhosis alone. Twenty-three patients were found to have alcoholic hepatitis and as previously noted, they had features of a pro-inflammatory state with elevated CRP, white blood cell count and pro-inflammatory cytokines.

The effects on neutrophil function were determined by the capacity to undergo phagocytosis and from the quantification of neutrophil oxidative burst. In circulating

un-stimulated patient neutrophils, oxidative burst was increased significantly compared with healthy controls. Neutrophils from all studied patients had a 5.6 times higher resting oxidative burst ( $p < 0.001$ ) compared to healthy controls (mean control oxidative burst  $8.9\% \pm 2.7$ ). Neutrophils from patients with alcoholic hepatitis had significantly higher resting oxidative burst compared to patients with cirrhosis alone ( $72 \pm 7$  vs.  $37 \pm 5$ ,  $p < 0.001$ ) or controls ( $p < 0.001$ ), as shown in Figure 4.1.

Stimulation with N-formylmethionyl-leucyl-phenylalanine (fMLP), a synthetic peptide that triggers minimal oxidative burst in unstimulated neutrophils, continued to show a significantly greater oxidative burst in alcoholic hepatitis patients compared with cirrhosis ( $75 \pm 8$  vs.  $61 \pm 7$ ,  $p = 0.01$ ) and also compared to controls ( $75 \pm 8$  vs.  $19 \pm 3.5$ ,  $p = 0.001$ ), suggesting prior priming of these patients' neutrophils. However, following stimulation with *E. coli*, the absolute increase in oxidative burst from resting levels (bacterial stimulated burst minus resting burst) was significantly reduced in alcoholic hepatitis patients compared with cirrhosis alone ( $26 \pm 7$  vs.  $60 \pm 5$ ,  $p < 0.0001$ ) or controls (control increase in burst:  $81 \pm 4$ ,  $p < 0.001$ ), as shown in the dark grey bars in Figure 4.1. Phagocytic capacity was measured by the geometric mean of fluorescence intensity (GMFI), which indicates the number of bacteria engulfed by one cell. Neutrophils from patients with cirrhosis+AH engulfed significantly less bacteria than those from control subjects ( $59 \pm 13$  vs.  $99 \pm 6$ ,  $p < 0.05$ , as depicted in Figure 4.1).



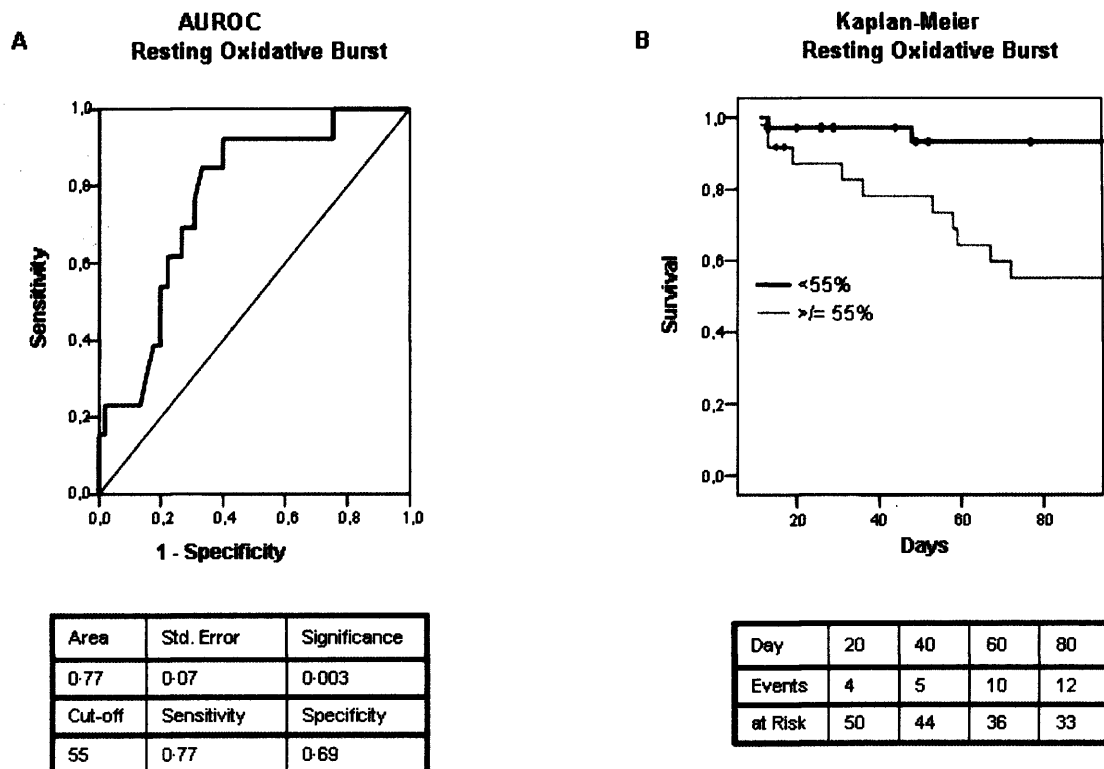
\* p<0.01 versus control  
 \*\* p<0.001 versus control  
 § p<0.001 versus control and versus cirrhosis  
 \$ p<0.05 versus control

**Figure 4.1** The bars represent the percentage of cells undergoing resting oxidative burst in untreated neutrophils (white) and after stimulation with fMLP (light grey) or challenge with *E.coli* (dark grey) in samples from control subjects (n=13), patients with alcoholic cirrhosis (n=40) and alcoholic hepatitis superadded on cirrhosis (cirrhosis+AH) (n=23). The last column bar (black) depicts the phagocytic capacity (relative GMFI) in the study groups.

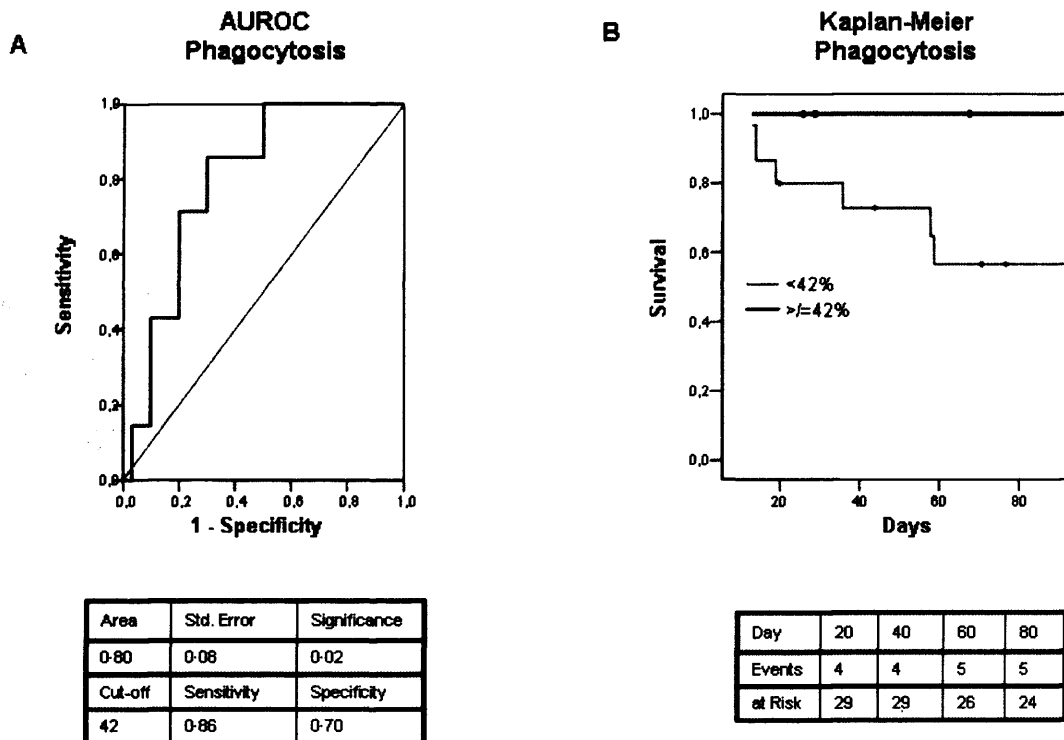
#### 4.4.2 Association of resting oxidative burst and phagocytosis with organ failure and survival

Seventeen patients (26%) developed organ failure, whilst 14 (22%) died within 90 days (30-day mortality 13%) of admission. Renal failure occurred in 15 (88%) and was associated with ventilation and circulatory failure in 4. Resting oxidative burst was found to be predictive of 90-day mortality (AUROC 0.77, p<0.005,) and organ failure (AUROC 0.76, p<0.001), as illustrated in Figure 4.2 A. A cut-off of resting burst >55% from the ROC curve (almost entirely consisting of patients with alcoholic hepatitis) had a sensitivity of 77% and a specificity of 69% for predicting death. Phagocytic function was also predictive of death (AUROC 0.80, p<0.05) and

organ failure (AUROC 0.91,  $p < 0.0001$ ), as shown in Figure 4.3 B. A phagocytic capacity  $< 42\%$  of normal amongst studied patients had a sensitivity of 86% and a specificity of 70% to predict mortality.



**Figure 4.2 (A)** Area under the receiver operator curve (AUROC) shows the predictive utility of measurement of oxidative burst in determining survival. **(B):** A resting burst of greater than 55% had the most optimal values of sensitivity and specificity derived as the best average from the AUROC analysis to group the patients, and was used to construct a Kaplan-Meier survival curve; log-rank analysis showed a significantly higher mortality ( $p < 0.01$ ) in the group of patients with high burst ( $\geq 55\%$ )



**Figure 4.3 (A)** AUROC analysis also revealed that the phagocytic capacity was predictive in determining survival. **(B)** A phagocytic capacity of less than 42%, had the optimal sensitivity and specificity on AUROC analysis and was used to construct a Kaplan Meier survival curve; log-rank analysis showed a significantly higher mortality ( $p < 0.001$ ) in the group of patients with decreased phagocytic capacity (<42%).

#### ***4.4.3 Association of resting oxidative burst and phagocytosis with development of infection***

Although none of the included patients had proven infection at the time of assay for neutrophil function, in 38 (60%) patients, infection was clinically suspected during the course of the hospital admission. In 19 of 38 patients (50%), culture positive infection was verified within 30 days of admission, although our hospital management protocol necessitated the use of broad spectrum antibiotics as soon as

an infection was suspected. In 8, more than one organism was found. The documented culture positive infections are summarized in Table 4.1

As shown in the table, the following species were cultured: *Enterococcus* (n=9), coagulase-negative *Staphylococcus* (n=8), *Candida sp.* (n=3), *E. coli* (n=2), methicillin-resistant *Staphylococcus aureus* (n=2), *Staphylococcus aureus* (n=2), *Propionibacterium sp.* (n=1). It is evident from the table that after stratifying patients by resting burst, those with a high resting burst ( $\geq 55\%$ ), which tended to be patients with alcoholic hepatitis, developed more culture positive infections when compared with low resting burst patients (48% versus 15%,  $p < 0.005$ ). Moreover, only high burst patients developed infections with more than one organism and also developed these infections earlier during their hospital stay as compared to low burst patients (7 versus 16 days,  $p = 0.005$ ). Additionally, 53% of patients with a phagocytic capacity  $< 42\%$  developed culture positive infection as compared to 13% of patients with a phagocytic capacity  $\geq 42\%$  ( $p < 0.05$ ). Those patients who developed culture positive infections were more likely to develop organ failure ( $p = 0.001$ ), and to die ( $p < 0.0001$ , Figure 4.4).

**Table 4.1** Documented culture positive infections in patients studied during 30 days

High Burst					Low Burst		
First Positive Culture			Second Positive Culture		First Positive Culture		
Patient Number	Day	Organism	Day	Organism	Patient Number	Day	Organism
1	4	<i>EC</i>	29	<i>MRSA</i>	2	16	<i>EC</i>
4	14	<i>EC</i>	19	<i>CNS</i>	14	23	<i>E. coli</i>
5	9	<i>CNS</i>	34	<i>EC</i>	17	9	<i>MRSA</i>
7	4	<i>EC</i>	10	<i>CNS</i>	23	15	<i>Staph. aureus</i>
12	2	<i>C. albicans</i>			33	15	<i>Propionibacterium</i>
13	10	<i>Staph. aureus</i>					
15	8	<i>CNS</i>					
20	5	<i>EC</i>	5	<i>CNS</i>			
35	6	<i>EC</i>					
36	4	<i>CNS</i>	4	<i>EC</i>			
45	2	<i>CNS</i>	3	<i>CNS</i>			
48	20	<i>E. coli</i>					
61	6	<i>C. albicans</i>					
62	7	<i>C. albicans</i>	9	<i>EC</i>			

*EC: Enterococcus*

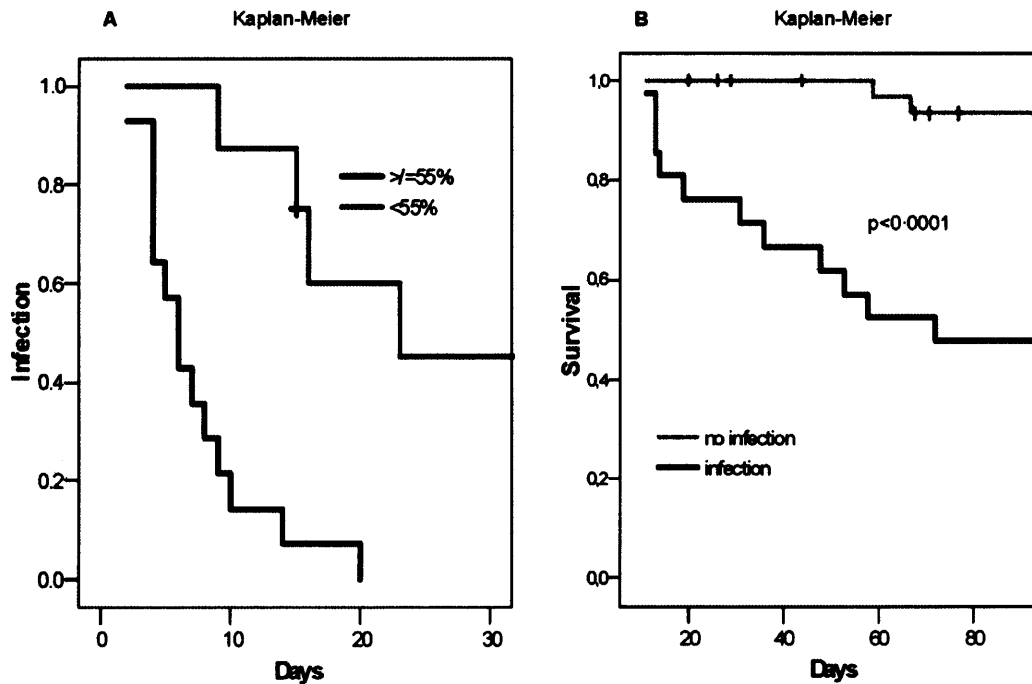
*MRSA: methicillin-resistant Staphylococcus aureus*

*CNS: coagulase-negative Staphylococcus*

*Staph: Staphylococcus*

*C: Candida*

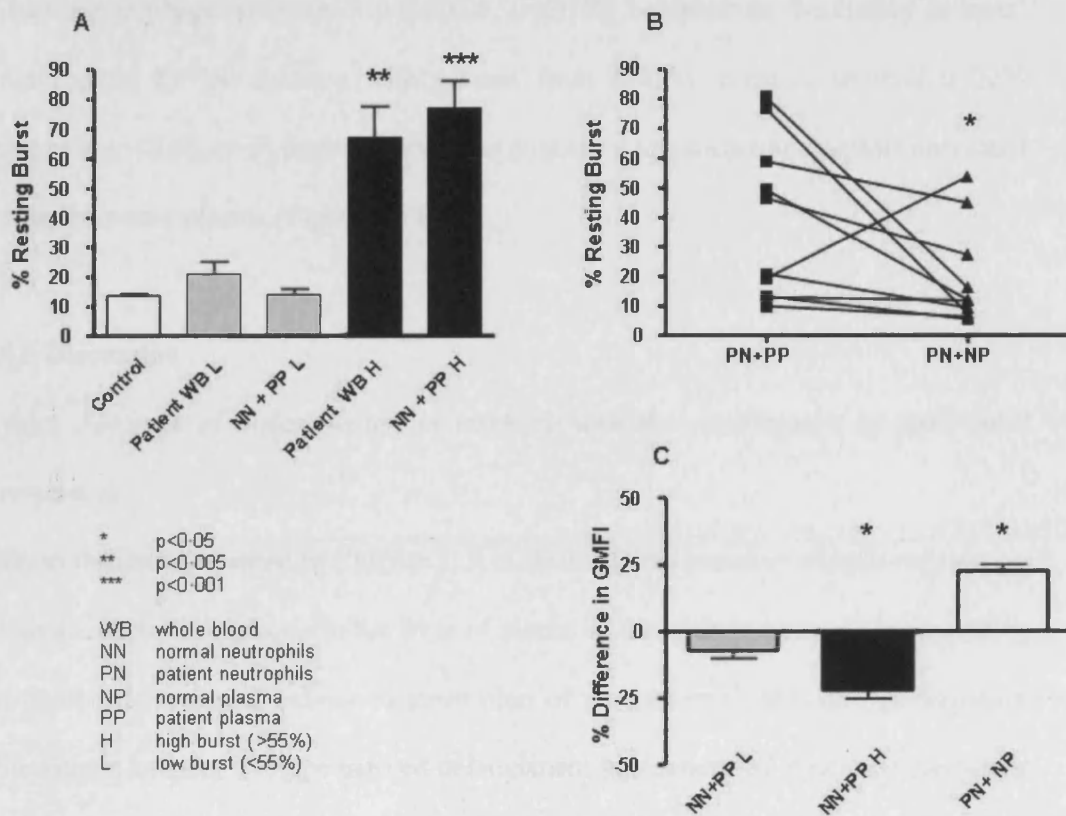




**Figure 4.4.** (A) Analysis for time to infection (Kaplan-Meier and log-rank) shows patients with high resting burst ( $\geq 55\%$ ) have a significantly higher risk ( $p < 0.005$ ) to developing culture positive infections within 30 days. (B) Using Kaplan-Meier and log-rank analysis to determine differences in survival between patients with and without culture positive infections, those with infections have a significantly higher 90-day mortality ( $p < 0.001$ ).

#### 4.4.4 Effect of patients' plasma and normal plasma on neutrophil oxidative burst

Plasma from patients with a high resting burst ( $\geq 55\%$ ;  $n=6$ ) induced a high resting burst in normal neutrophils ( $p=0.005$ ) whereas plasma of patients with a low resting burst ( $< 55\%$ ;  $n=6$ ) failed to do so, as shown in Figure 4.5 A. This result suggested presence of a transmissible factor in patient's plasma promoting neutrophil activation. When isolated neutrophils from patients with known high resting burst were incubated with normal plasma, the resting burst decreased significantly as compared to isolated neutrophils incubated with the patients' own plasma ( $p=0.02$ ) as shown in Figure 4.5 B.



**Figure 4.5 (A)** Resting oxidative burst in whole blood (WB) of controls, in whole blood of patients, and in normal neutrophils (NN) incubated with patients' plasma (PP). Plasma from patients with high resting burst (H) induced a high burst in normal neutrophils, whereas plasma from patients with low resting burst (L) failed to do so, **(B)** Elevated resting oxidative burst in patients' neutrophils (PN) incubated with their own plasma is reversed after incubation with normal plasma (NP), **(C)** Incubation of normal neutrophils with plasma from patients with low resting burst does not change phagocytosis, whereas plasma from patients with high burst decreases phagocytosis. Incubation of patients' neutrophils with normal plasma restores phagocytic function.

#### 4.4.5 Effect of patients' plasma and normal plasma on phagocytosis

Neutrophils from healthy controls incubated with plasma from patients with a low neutrophil resting burst did not differ from control, whereas neutrophils from healthy controls incubated with plasma from patients with high resting burst showed a 22%

decrease in phagocytic capacity ( $p < 0.05$ ,  $n = 6$ ). By comparison, incubating patients' neutrophils for 60 minutes with plasma from healthy controls showed a 22% increase ( $p < 0.05$ ,  $n = 6$ ) in phagocytosis as compared to patients neutrophils incubated with their own plasma (Figure 4.5 C).

## **4.5 Discussion**

### ***4.5.1 The role of inflammation in cirrhosis and the contribution by neutrophil responses***

From the data presented in Chapter 3, it is clear that the presence of inflammation in human alcoholic cirrhosis in the form of alcoholic hepatitis is distinct histologically, with the presence of balloon degeneration of parenchymal cells and peri-cellular fibrosis in keeping with the marked derangement in biochemical function (increasing bilirubin and prothrombin time) evident in these patients. Furthermore, increasing plasma creatinine, despite the highly catabolic state of these patients, would suggest an increasing propensity to the development of renal failure compared to patients with cirrhosis alone, suggesting that the inflammatory activity in the liver, may impact on function of other organs. The inflammatory process was also noted to be evident in systemic parameters, with elevation of the pro-inflammatory cytokines, in addition to SIRS components and CRP. Of interest, soluble TNF receptors are known to be up-regulated in severe alcoholic hepatitis and their levels associated with an increased risk of mortality, (108, 109) suggesting that signalling through the TNF $\alpha$  pathway may be important in the evolution of the disease. By contrast, plasma TNF $\alpha$  levels in patients with alcoholic hepatitis are widely variable in the literature, ranging from values as low as 5pg/ml to as high as 400 pg/ml (13, 94, 110, 111), suggesting that other factors such as concomitant infection, degree of endotoxaemia, genetic factors and soluble receptor expression may be important in determining the

measured levels of TNF. Of interest, in the studies described in this thesis, there was also considerable variation in plasma TNF $\alpha$  levels with numerous patients having values beneath the detection threshold.

In addition to elevation of these inflammatory parameters, there was also evidence for a marked increase in indicators of oxidative stress. The metabolism of ethanol per se increases the generation of oxidant stress and has been implicated in the pathology of alcoholic liver disease,(112) and this is further compounded by the effects of endotoxin.(113) Oxidative stress has also been linked with the pathophysiology of hepatic encephalopathy, through effects upon mitochondria, oxidation of membrane phospholipids and various enzymes involved in energy metabolism. (114-117) In this group of studied patients, those with alcoholic hepatitis had a higher incidence of hepatic encephalopathy compared to those with cirrhosis alone and this was reflected in their higher Child-Pugh scores. Moreover, oxidative stress also impacts on the levels of numerous vascular modulators including asymmetric dimethylarginine (118, 119) (as discussed later in this thesis in chapters 8 and 9), which may aggravate the state of portal hypertension and the development of organ failure (120)and thus, the clinical course of disease in these patients. The consequence of the inflammation noted via histological and plasma factors is evident in the more severe nature of decompensation at presentation, and by the course of the disease. Patients with alcoholic hepatitis had a much higher mortality and expressed a higher incidence of organ failure in addition to an increased burden of sepsis.

The BDL animal model was chosen to parallel the nature of injury with cirrhosis, on which additional inflammatory injury can be superimposed. The results from the BDL studies complimented the findings in patients, with animals in which an inflammatory LPS stimulus had been given in addition to the primary injury (BDL), manifesting a more advanced histological severity of disease, and higher levels of

biochemical liver injury. Although this was at slight variance with the results from the AH+C+ patients in whom there was no significant increment in aminotransferase levels, the BDL +LPS model was thought to support the concept that the effects of inflammation on pre-existent liver injury were not just confined to the effects of alcohol induced disease but are likely to be applicable to other forms of inflammatory liver injury.

Given that an acute inflammatory infiltrate was a characteristic of acute alcoholic hepatitis, and that neutrophils are known to generate superoxide through oxidative burst, the rationale for the study of neutrophil responses was to determine the contribution of neutrophil activation to the perpetuation of injury and the overall outcome in the patient, when inflammation is superimposed on cirrhosis. Circulating neutrophils have been shown to be primed in alcoholic hepatitis, (94, 95) a process involving functional and structural changes, resulting in enhanced responses to a microbial challenge. (98) The results presented confirm that neutrophils in patients with alcoholic cirrhosis and alcoholic hepatitis are primed as shown by increased oxidative burst upon stimulation with fMLP as compared to controls. The presence of primed neutrophils would suggest good bactericidal function but with the potential for promoting local damage if further activated.

Paradoxically, despite data suggesting neutrophil priming, it is also established that patients with alcoholic cirrhosis and especially those with alcoholic hepatitis, are prone to infections, (121), have a higher prevalence of sepsis (122) and are more likely to die from sepsis related complications. (102) This clinical finding is supported by several studies showing defects in phagocytosis, bactericidal capacity, chemotaxis and neutrophil locomotion. (96) It was therefore considered important to be able to distinguish between primed (but not activated) neutrophils and those that are fully activated. Activation of neutrophils was defined in this study as neutrophils

that have a high resting burst. Additionally, presentation of a bacterial challenge to activated neutrophils was associated with an inability of these cells to generate a further oxidative burst, as compared with primed cells. The neutrophils in this study population of alcoholic hepatitis patients showed both neutrophil activation and impairment of phagocytosis. One can only speculate on the mechanism by which an increased resting oxidative burst might be related to a decreased phagocytic capacity. Since both oxidative burst and phagocytosis are energy dependent processes, the induction of oxidative burst may lead to an energy depletion and reduction in phagocytosis. This is supported by data showing that activation of AMP-kinases in a state of low cellular energy contributes to a reduction in host defence mechanisms. (123)

The clinical importance of these neutrophil abnormalities identified by a high resting oxidative burst of  $\geq 55\%$  and reduced phagocytic capacity (relative GMFI of  $< 42\%$ ), are highlighted by the observation of increased risk of infection and the association with organ failure and mortality in these patients. In general, these observations were limited to patients with alcoholic hepatitis, as opposed to those with low burst who were in the main, patients with alcoholic cirrhosis alone. It is notable that the group with high-burst neutrophil activity as described in this study had infection with multiple organisms despite antibiotic therapy, within a relatively short time frame. Although every possible attempt was made to exclude patients with pre-existing infection at study entry, one cannot fully exclude the possibility of an underlying sub-clinical infection that remained un-detected by current routine microbiology techniques, which may have precipitated high resting oxidative burst in some patients. It has been shown that 32% of cirrhotic patients with culture negative ascites have detectable bacterial DNA in the blood, which may indicate systemic bacterial seeding. (124)

An important observation made through *ex vivo* experiments in this study was the demonstration that the neutrophil functional defect could be transmitted to normal neutrophils incubated with patient's serum. It was also possible to show that the neutrophil dysfunction in the patient was reversible following incubation with normal serum, suggesting removal of this transmissible factor from patients' plasma by placing the neutrophils in a more 'healthy' environment. An attractive candidate to study further for consideration as such a transferable serum factor might be endotoxin, as endotoxin is known to be elevated in patients with alcoholic liver disease. Endotoxin is not only a priming agent (98) but can also fully activate neutrophils (100, 101), possibly through up-regulating of the NADPH oxidase assembly. (125) The highest levels of endotoxin reported in patients with liver disease are found in portal venous blood, (126, 127) highlighting the importance of the bowel and altered gut permeability as the possible source of endotoxin.(128) However, the difficulties with measurement of endotoxin make its identification in clinical samples a challenge, as is reflected in the wide range of values in the literature. (99, 126, 127, 129, 130)

This data on neutrophil responses in cirrhosis can thus be summarized as in part explaining the apparent paradox in neutrophil function with heightened responses in the context of inflammation on the one hand, which may further contribute to inflammatory injury, and increased susceptibility to infection on the other, which in turn impacts on patient survival.

***Chapter 5***

***Does inflammation impact on vascular function in  
cirrhosis?***



## **5.1 Circulatory differences between patients with decompensated alcoholic cirrhosis and patients with alcoholic hepatitis**

### ***5.1.1 Introduction:***

Portal hypertension is a frequent clinical syndrome in patients with advanced liver disease defined by an increase in the pressure gradient between the portal vein and the hepatic veins. The importance of this syndrome is recognized by the frequency and severity of its complications which include bleeding from gastro-oesophageal varices, renal dysfunction, increasing ascites, bacteraemia and hepatic encephalopathy. (26) There is some data that suggests that in decompensated alcoholic cirrhosis, major changes in portal blood flow including reversal of flow is associated with a lower 1 year survival in those patients who have proven alcoholic hepatitis. (131) Moreover, Poynard and co-workers suggested that the degree of liver cell necrosis and the extent of the neutrophilic infiltrate may relate to the level of portal pressure gradient. (132) These studies may intimate that in the context of the inflammatory state of alcoholic hepatitis, that there is a change in regulation of either portal blood flow or/and intra-hepatic resistance, though the specific role of inflammation and the potential mechanisms involved have not been explored. An understanding of such mechanisms is clearly paramount in treating the severe complications of portal hypertension in these patients, which contribute to their recognized poor survival in many series. (67)

Given the findings of earlier studies presented in Chapters 3 and 4 demonstrating significant differences in liver architecture, biochemistry and immune function in cirrhosis with superadded inflammation, it was considered important at the outset, to establish the contribution of inflammation to systemic and hepatic vascular function,

before considering how this impacts on patient outcome and progression to organ failure.

### ***5.1.2 Methods:***

#### ***5.1.2.1 Patients***

Twenty-three patients with alcoholic liver disease who were undergoing routine portal pressure evaluation were studied. These patients were a subset of those previously described in Chapter 4, section 4.2. The study was conducted to facilitate an assessment of the vascular effects of additional inflammation on the background of cirrhosis.

#### ***5.1.2.2 Measurements***

Systemic and hepatic haemodynamic evaluation was performed in accordance with the methods described in detail in Chapter 2: HVPG pressure measurement (section 2.1.2.1), cardiovascular haemodynamics (section 2.1.2.2) and hepatic blood flow (section 2.1.2.3). All the haemodynamic studies were conducted after an overnight fast, after the patients had been supine for at least 1 hour.

HVPG was calculated as the difference between WHVP and FHVP. The coefficient of variation of the measurements of HVPG within a given patient was 3% (0.42) for the study group.

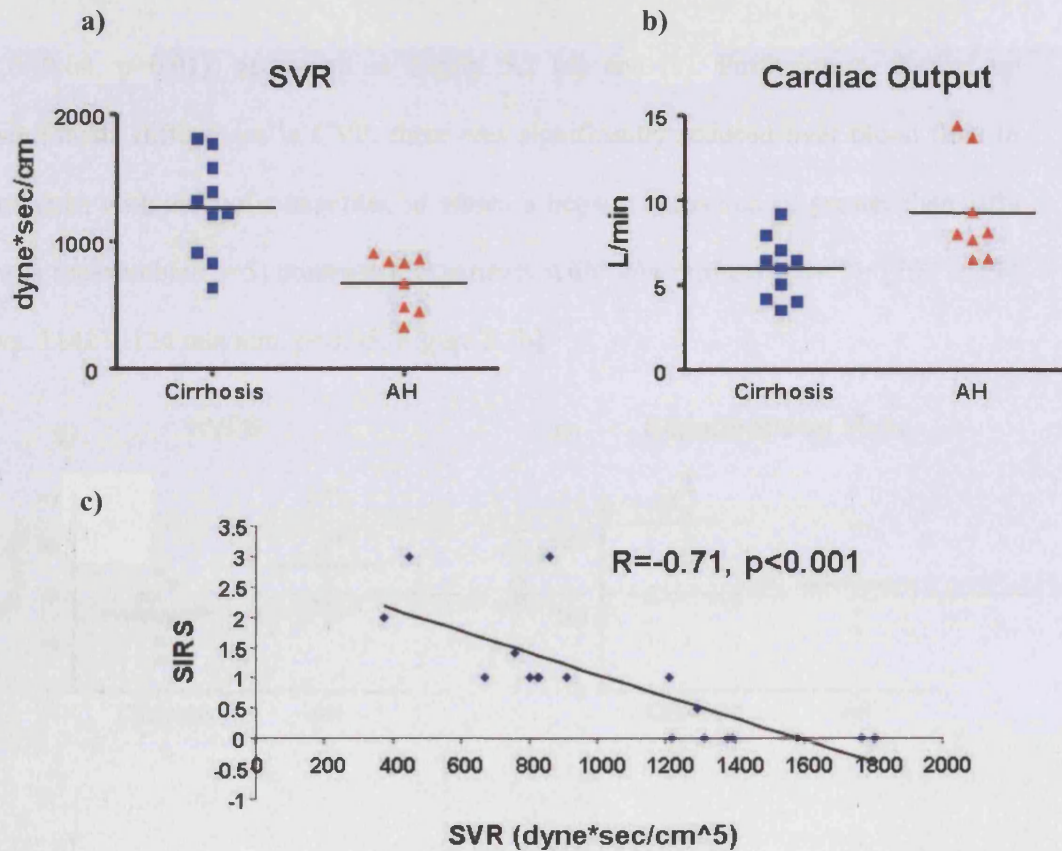
### ***5.1.3 Results***

Of the 23 patients with alcoholic liver disease studied, 10 patients were subsequently found to have histological features of alcoholic hepatitis and the remaining 13 had decompensated alcoholic cirrhosis.

### 5.1.3.1 The systemic circulation

Patients with alcoholic hepatitis had significantly lower systemic vascular resistance (SVR) compared to alcoholic cirrhosis without inflammation ( $671 \pm 79$  vs.  $1266 \pm 124$  dyne\*sec/cm<sup>5</sup>,  $p < 0.01$ ) as shown in the Figure 5.1a. In keeping with this observation, mean arterial blood pressure (MAP) was also significantly lower in alcoholic hepatitis patients ( $74 \pm 2$  vs.  $86 \pm 3$  mmHg,  $p = 0.01$ ). It should be noted that in spite of the differences in SVR and MAP, there were no significant differences in central filling pressure between the patient groups, as gauged through central venous pressure measurement (CVP alcoholic hepatitis vs. alcoholic cirrhosis:  $13.2 \pm 1.1$  vs.  $11.7 \pm 0.9$  mmHg). Cardiac output was, however, significantly greater in alcoholic hepatitis patients. This finding is symptomatic of a more deranged hyperdynamic state than was observed in decompensated alcoholic cirrhosis alone ( $9.3 \pm 1$  vs.  $6 \pm 0.6$  L/min,  $p < 0.01$ ; (Figure 5.1b). The heart rate was significantly greater amongst the inflammatory alcoholic hepatitis patients compared to cirrhosis alone ( $92 \pm 3$  vs.  $82 \pm 3$  beats/min,  $p < 0.05$ ), as anticipated from the SIRS component evaluation.

A significant inverse correlation was found for SVR ( $r = -0.71$ ,  $p < 0.001$ ; Figure 5.1c) and MAP ( $r = -0.47$ ,  $p = 0.02$ ) in relation to SIRS components. Similarly, there was a strong inverse association between TNF receptors 1 and 2 and SVR and MAP ( $r = -0.5$ ,  $p < 0.01$  and  $r = -0.68$ ,  $p < 0.001$ ), respectively. These findings supported an association of inflammation with the observed systemic haemodynamic disturbances. There was, however, no significant association between elevated SIRS components or cytokine levels and cardiac output ( $r = 0.31$ ,  $p = 0.1$ ), which may in part reflect the wide range of measured cardiac output readings amongst this patient group.

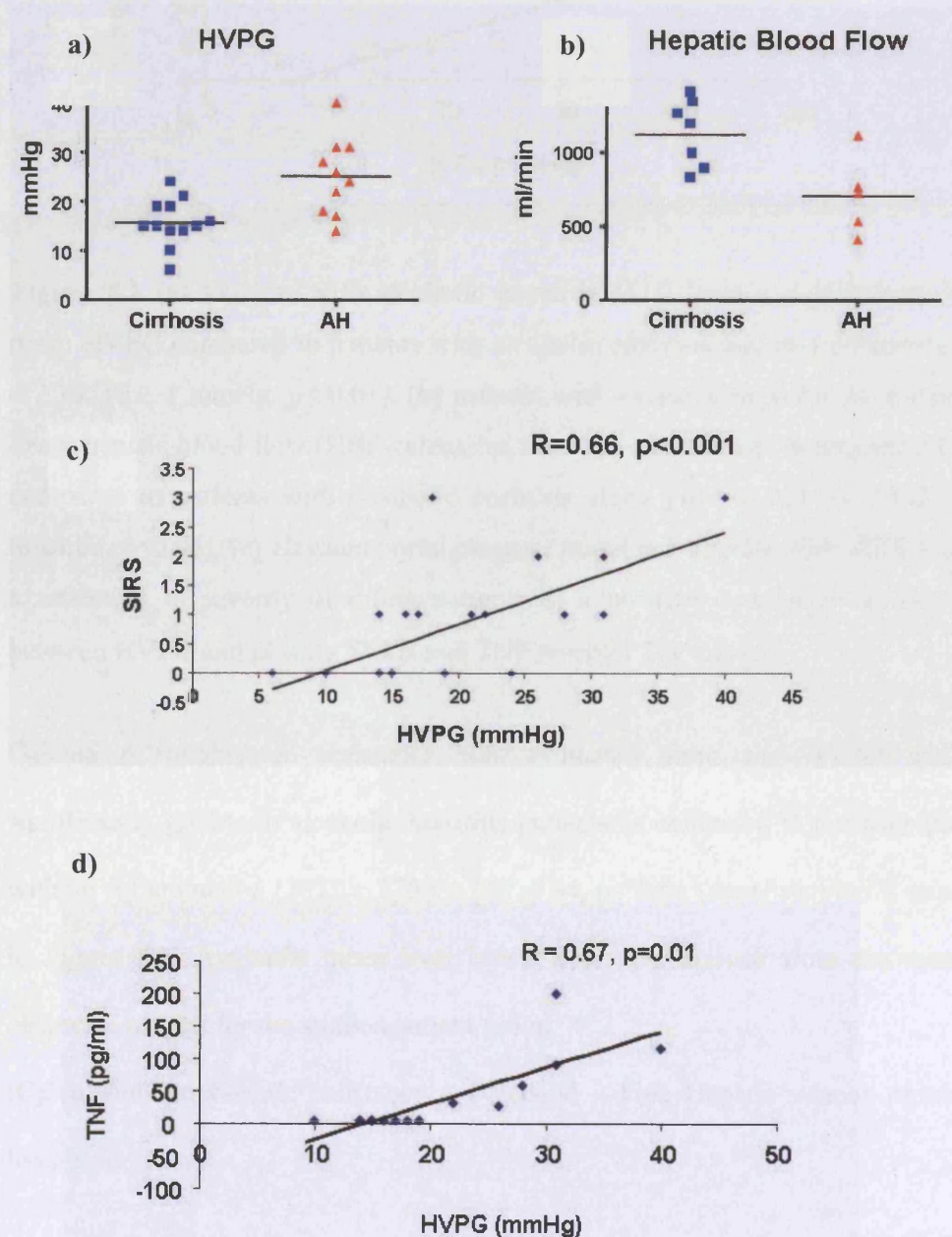


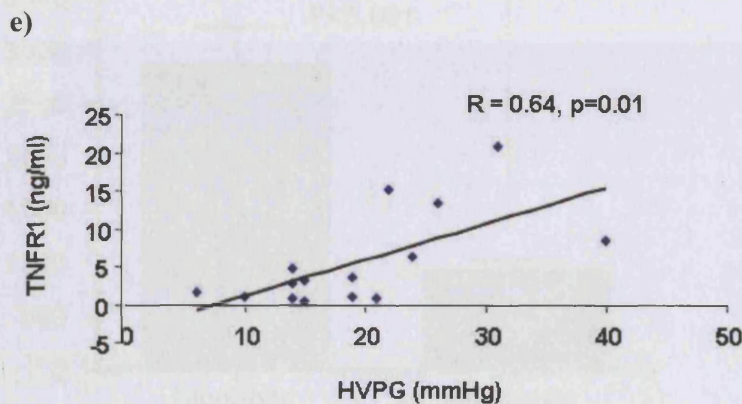
**Figure 5.1 (a)** demonstrates that there is a significant reduction in systemic vascular resistance (SVR) in patients with alcoholic cirrhosis in which there is the additional inflammatory injury of alcoholic hepatitis ( $p < 0.01$ ), compared to patients with cirrhosis alone, **(b)** shows a markedly higher mean cardiac output in patients with alcoholic hepatitis compared to those patients without inflammation, in whom the mean value is still greater than 5 litres/min, panel **(c)** shows the inverse relationship between a low SVR and a high SIRS score.

### 5.1.3.2 The hepatic circulation in alcoholic hepatitis

Patients with alcoholic hepatitis had significantly higher hepatic venous pressure gradients (HVPG) as shown in Figure 5.2a compared to those with alcoholic cirrhosis alone ( $25 \pm 2$  vs.  $16 \pm 1$  mmHg,  $p < 0.01$ ). Elevated HVPG was also shown to have a positive correlation with higher SIRS score components ( $r = 0.66, p < 0.001$ ), as shown in Figure 5.2c. A significant association was also noted between HVPG and plasma TNF $\alpha$  concentrations ( $r = 0.67, p = 0.01$ ) and TNF receptor 1 concentrations

( $r=0.64$ ,  $p=0.01$ ), as shown in Figure 5.2 (d) and (e). Furthermore, despite no significant differences in CVP, there was significantly reduced liver blood flow in patients with alcoholic hepatitis, in whom a hepatic extraction of greater than 10% was measurable ( $n=5$ ) compared to patients with only cirrhosis ( $n=7$ ): [ $707 \pm 154$  vs.  $1142 \pm 124$  mls/min,  $p<0.05$ ; Figure 5.2b].



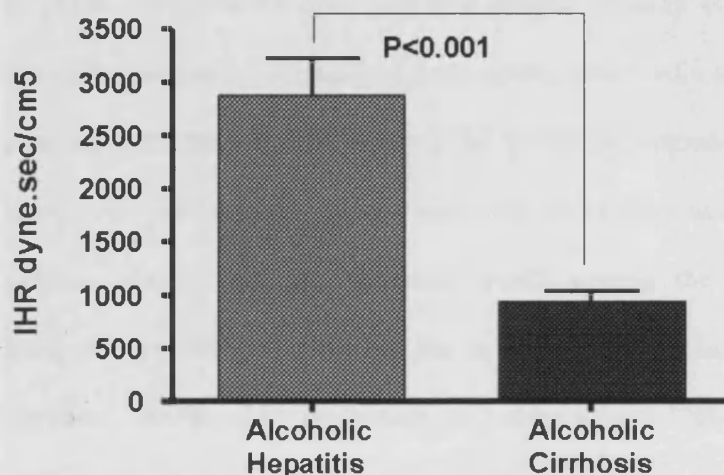


**Figure 5.2 (a)** Patients with alcoholic hepatitis (AH) have a significantly higher mean HVPG compared to patients with alcoholic cirrhosis and no inflammation [ $25 \pm 2$  vs.  $16 \pm 1$  mmHg,  $p < 0.01$ ], **(b)** patients with alcoholic hepatitis have markedly lower hepatic blood flow (HBF-calculated from the clearance of Indocyanine Green) compared to patients with alcoholic cirrhosis alone [ $707 \pm 154$  vs.  $1142 \pm 124$  mls/min,  $p < 0.05$ ], **(c)** elevated portal pressure correlates directly with SIRS score, as a reflection of severity of inflammation, **(d)** a positive correlation is also noted between HVPG and plasma  $\text{TNF}\alpha$  and TNF receptor 1 levels **(e)**.

Calculated intrahepatic resistance was estimated from the HVPG and was significantly greater in alcoholic hepatitis patients as compared to cirrhosis patients without inflammation ( $2871 \pm 375$  vs.  $877 \pm 84$ ,  $p < 0.01$  (dyne\*sec)/ $\text{cm}^5$ ), as shown in Figure 5.3. Available mean liver blood flow was derived from the measured clearance of ICG for the studied patient group.

[Calculated Intrahepatic resistance = (Wedged – Free Hepatic venous pressure) / liver blood flow]

### Effect of inflammation on IHR



**Figure 5.3** Markedly increased intrahepatic resistance (IHR) is noted in patients who have additional inflammatory alcoholic hepatitis on the background of alcoholic cirrhosis.

#### 5.1.4 Discussion

The findings of this study do support and extend the observations by Poynard (132) and Duvoux (131) confirming clear haemodynamic differences in decompensated alcoholic cirrhosis patients who have the additional inflammatory component of alcoholic hepatitis. In this study, these differences have been more formally characterized demonstrating a markedly lower systemic vascular resistance and lower mean arterial pressure in patients with alcoholic hepatitis, confirming an exaggerated hyperdynamic circulation in these patients. Furthermore, the patients with alcoholic hepatitis also have significantly higher portal pressures as defined by hepatic venous pressure gradients. A clear association is observed between elevated portal pressure gradient and SIRS components, in addition to plasma  $\text{TNF}\alpha$  and TNF receptor 1 concentrations. Conversely, there is an inverse relationship for both lower mean arterial pressure and systemic vascular resistance with these systemic inflammatory indices. However, it should be noted that the measurable  $\text{TNF}\alpha$  levels

were at the level of the detection threshold for most patients, and similarly, TNF receptor 1 levels were low for many patients with an HVPG <20 mmHg. Thus, although statistically correlations were noted, these really are most applicable to the patients with the greatest severity of portal hypertension. Notwithstanding the limitations with these cytokine assays and the relative underpowered nature of the patients studied, the data observed would support the assertion that increased inflammatory drive aggravates the systemic hyperdynamic circulatory state in cirrhosis, whilst also promoting a compromised hepatic circulation. This is evidenced by the markedly decreased hepatic blood flow in the inflammatory patients, with a consequent elevation of intra-hepatic resistance.

It is interesting that patients with alcoholic hepatitis are often described as having an audible hepatic bruit, believed to be the consequence of intrahepatic arterial dilatation and regions of hypervascularity. This may be the consequence of a hepatic arterial buffer response to diversion of portal venous blood early in the course of rising portal pressure. However, the relationships defined in this study would suggest that the severity of portal hypertension in severe alcoholic hepatitis is more complex than simply a greater distortion of liver architecture re-routing hepatic blood flow through shunts, or any direct contribution by an increased number of inflammatory cells. (132) It is more likely that the increased pro-inflammatory cytokines previously described in these patients (133) to which activated peripheral neutrophils may contribute, drive key vascular regulators that are involved with local hepatic vascular tonic control at the level of the resistance vessels. This may cause regional or a more global hepatic hypoperfusion, in the context of splanchnic vasodilatation.

It was of interest that no direct association was found in this study between systemic inflammatory components and cardiac output, since the increase in cardiac output is a recognized response in cirrhosis to arterial underfilling as a consequence of



increased splanchnic vasodilatation. (30) A possible explanation may be that an increase in cardiac output serves to maintain mean arterial pressure in compensated cirrhosis and during early decompensation. However, in the context of sepsis or severe inflammation, as noted in alcoholic hepatitis, loss of cardiac reserve and unmasking of an underlying dilated cardiomyopathy (related to alcohol or/and cirrhosis (134) results in a steady decrease in cardiac output, and a consequent disassociation from the relationship with reduced systemic vascular resistance. (135) These changes may themselves precipitate a progression to multi-organ failure.

## ***Chapter 6***

***Can anti-inflammatory intervention modulate  
vascular dysfunction in alcoholic cirrhosis?***

## **6.1 Introduction:**

Given that patients with inflammation superimposed on cirrhosis have a more deranged hyperdynamic circulation and yet also a reduced hepatic perfusion and elevated portal pressure, it was considered important to determine whether the blockade of inflammatory pathways or their modulation resulted in a resolution of these haemodynamic differences. This would help to confirm the direct contribution of inflammation to these circulatory abnormalities in cirrhosis and suggest potential avenues for novel therapy. Moreover, such study would provide an insight into what factors alter the course and progression to organ failure, which might also contribute to the design of further studies to address mechanisms to account for any changes in vascular function observed.

## **6.2 Treatment with an anti-TNF $\alpha$ monoclonal antibody (Infliximab)**

### ***6.2.1 Background***

TNF $\alpha$ , a pro-inflammatory cytokine, is believed to exert vascular effects by increasing vascular permeability and causing vasodilatation, thought to be mediated through nitric oxide dependant pathways. (48) TNF $\alpha$  has been reported to be elevated in alcoholic liver disease, and in particular, high levels of TNF $\alpha$  are found in alcoholic hepatitis (AH). (133) In a pilot study, the administration of the chimeric anti-TNF $\alpha$  antibody Infliximab (Schering-Plough Corporation, Kenilworth, NJ, USA) to patients with severe AH was shown to be safe and resulted in an improvement in liver function and a reduction in inflammatory mediators. (105)

An important role for TNF $\alpha$  in the pathogenesis of portal hypertension has been suggested by studies on portal vein ligated rats treated using anti-TNF $\alpha$  therapies, which demonstrated a blunting of the hyperdynamic circulation and a reduction of

portal pressure. (65, 66) We hypothesized that in view of the reported role of TNF $\alpha$  in the pathogenesis of AH, administration of an anti-TNF $\alpha$  antibody to AH patients would result in a reduction in portal pressure and an improvement in the systemic haemodynamic derangement. This would also confirm the role of TNF $\alpha$  in the inflammatory mechanisms that might be related to the development of portal hypertension.

This study evaluated the acute and short-term effect of a single infusion of Infliximab, on the portal, cardiovascular and renal haemodynamics in patients with severe AH.

### **6.2.2 Study Design**

**Patients:** Ten patients (8 males, mean age  $53 \pm 8$  years) with severe AH were recruited into this study, following approval by the local ethics committee. Following admission, patients were observed for 72 hours prior to inclusion. Patients were included if they had severe AH defined by a compatible clinical history (alcohol consumption of  $>80$ g alcohol/day for men and  $>60$ g/day for women up until the time of admission), a discriminant function of  $>32$  (89), histological evidence of AH (balloon degeneration of hepatocytes; Mallory bodies; neutrophilic infiltrates and apoptotic acidophilic bodies), and a deterioration in liver function tests over the observation period, despite supportive medical management. Patients were excluded if they were  $<18$  or  $>75$  years and had any of the following: renal dysfunction (plasma creatinine  $>130$   $\mu\text{mol/L}$ ), severe cardiovascular or cerebrovascular disease, active or latent tuberculosis, hepatic or extra-hepatic malignancy and treatment with vasoactive agents or corticosteroids therapy.

Haemodynamic assessment was performed prior to entry into the study, 24 hours after administration of Infliximab (Schering-Plough Corporation, Kenilworth, NJ,

USA) and on the day prior to discharge. The study was terminated either on day 28 or the day of discharge, whichever occurred earlier. Day -2: Baseline haemodynamic assessment was performed in nine patients at the time of undergoing a transjugular liver biopsy. In the tenth patient, a biopsy had been performed prior to hospital transfer and it was therefore, difficult to justify a baseline haemodynamic assessment. Day 0: 48 hours following the biopsy and histological confirmation of AH, Infliximab was administered intravenously (Schering-Plough Corporation, Kenilworth, NJ, USA) over a 2-hour period, at a dose of 5mg/Kg body weight. Day 1: Repeat haemodynamic assessment was performed the next morning, 24 hours after the Infliximab infusion, in 8 patients through an indwelling 9 French internal jugular sheath (William Cook Europe, Bjaeverskov, Denmark), negating further venepuncture. 1 patient declined a repeat haemodynamic study at this time. Prior to the day of discharge or day 28: A further haemodynamic assessment was performed at a mean of  $19\pm 4$  days in 5 of the patients who consented to the third phase of the study.

*Sampling*: Blood was collected before the study began and then daily for one week after infusion; thereafter, two times per week until day 28 for routine haematology, biochemistry and CRP. During each haemodynamic assessment, blood was collected from an artery, hepatic and renal veins in pre-cooled heparinised tubes. Plasma was separated by centrifugation (3000rpm, 10minutes at 4°C) and stored at -80° C for subsequent analysis. A 24-hour urine collection enabled determination of sodium excretion.

### **6.2.3 Measurements**

The methods applied for haemodynamic evaluation of hepatic venous pressure gradient, systemic haemodynamics and regional blood flow have been described in Chapter 2, sections 2.1.2.1-2.1.2.4.

Plasma cytokines were assayed by standard ELISA techniques (Chapter 2, section 2.4.1) and levels of NOx (nitrate/nitrite) through a modified Greiss reaction as described in Chapter 2, section 2.4.3.1.

Standard biochemical parameters (bilirubin, ALT etc) were obtained from hospital records. The study was terminated either on day 28 or the day of discharge, whichever occurred earlier.

### **6.2.4 Results**

The 10 patients were deeply jaundiced at the time of entry into the study, with a mean bilirubin of 335.9 ( $\pm 41.6$ )  $\mu\text{mol/L}$  and an alanine aminotransferase (ALT) of 46 ( $\pm 3.5$ ) U/L. All had ascites and 6 had mild hepatic encephalopathy. The mean discriminant function was 68.9 ( $\pm 8.9$ ). Treatment with Infliximab resulted in beneficial biochemical effects on liver function as manifest by a significant reduction in prothrombin time ( $p < 0.05$ ) after 1 week and also a significant improvement in the serum bilirubin levels ( $p < 0.05$ ) by the time of discharge, as shown in Table 6.1. There was a corresponding reduction in discriminant function score at 1 week ( $p < 0.01$ ), which was sustained at 28 days ( $62.9 \pm 9$  at day 0;  $53.6 \pm 9$  at day 7;  $42.3 \pm 5$  at day 28;  $p < 0.05$ ).

**Table 6.1** Changes in liver function and SIRS components prior to and after treatment with Infliximab

	Baseline (Day-2)	Day 1	Day 7	Day 14	Day 28
Prothrombin Time [10-13] (seconds)	20.7 ± 1.7	20.1 ± 1.8	18.8 ± 1.6 *	18.3 ± 1.2 *	17.8 ± 0.9**
Bilirubin [3-17] (µmol/L)	335.9 ± 41.6	285.5 ± 48.2	267.6 ± 43.4	219.2 ± 40.4 *	141.1 ± 17.7 *
C-reactive protein [0-5] (mg/L)	86.8 ± 13.7	53.1 ± 9.2 **	48.7 ± 10.3 **	39.2 ± 5.6 ***	32.7 ± 4.6 ***
White blood cell count [3-10] x 10 <sup>9</sup> /L	17.5 ± 2.7	13.5 ± 1.6 **	13.8 ± 1.6 **	13.5 ± 1.2 **	12.3 ± 1.8 ***
Temperature °C	37.7 ± 0.3	37 ± 0.2 *	37 ± 0.3 *	36.8 ± 0.2 *	36.8 ± 0.2 *
Heart Rate (beats/min)	93.8 ± 3.7	85.3 ± 3.1 *	85.7 ± 2.9 *	79 ± 3.2 **	73.5 ± 1.6 ***
Respiratory Rate /min	22.3 ± 1.5	18.7 ± 1.1	18.5 ± 2.1	17.1 ± 0.8	17.7 ± 1.1

\* p<0.05, \*\* p<0.01, \*\*\* p<0.001 compared with baseline values, [normal range]

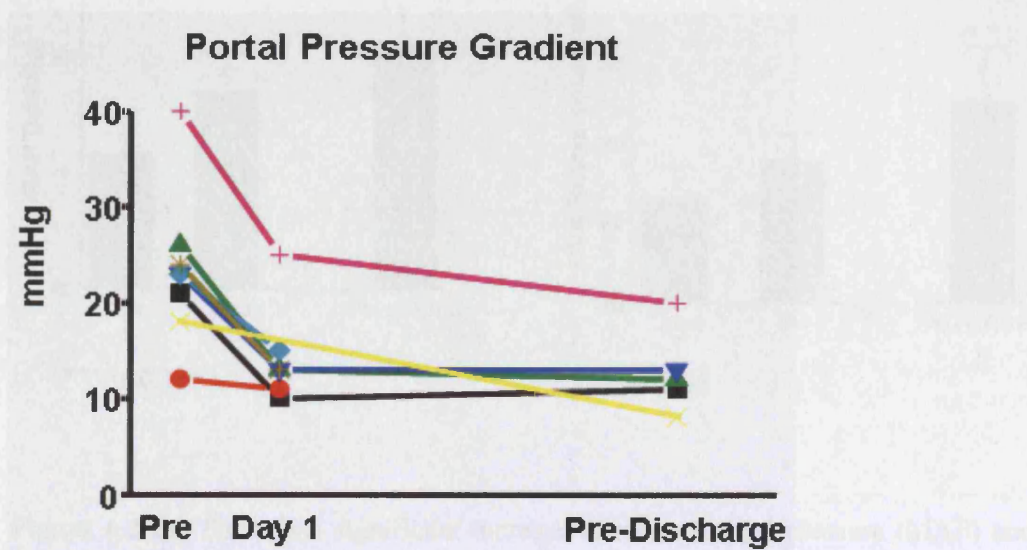
The biochemical changes observed occurred in the light of a reduction in inflammation, as evidenced by a reduction and sustained improvement in the SIRS score ( $2.5 \pm 0.4$  at day 0 to  $0.5 \pm 0.27$  at day 28;  $p<0.001$ ). There was also a substantial reduction in CRP 24 hours after treatment with Infliximab ( $86.8 \pm 14.4$  to  $53.1 \pm 9.2$  mg/L;  $p<0.001$ , Table.6.1).

Clinical evidence of improvement was sustained during the study period in all but one patient who died at 28 days from an aspiration pneumonia and subsequent

septicaemia. Aside from this particular patient, no other individual required any vasoactive agents, admission to the intensive care unit or antibiotic therapy or developed any signs of progressive hepatic encephalopathy. In 3 patients, the occurrence of ascites required paracentesis.

#### 6.2.4.1 Haemodynamic changes

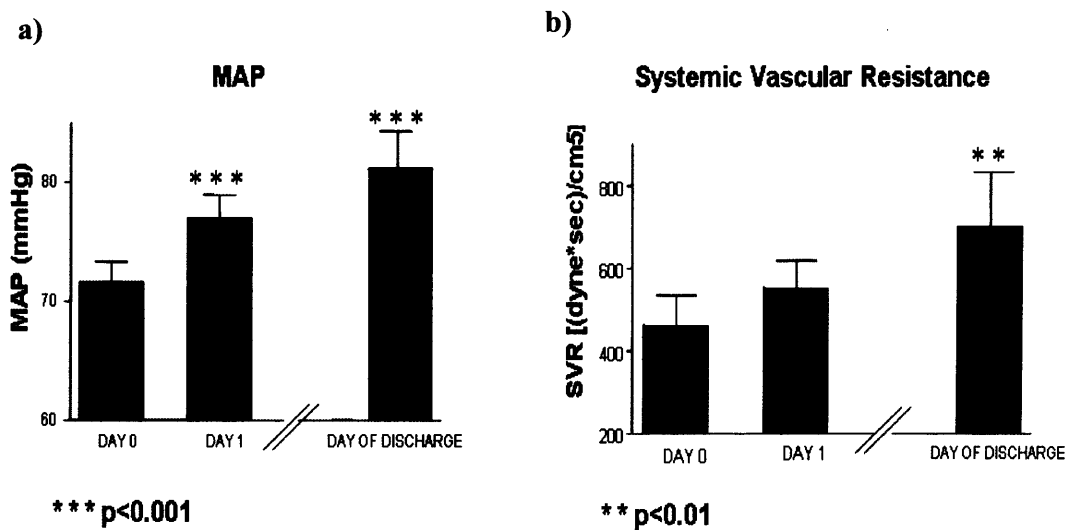
6.2.4.1.1 Portal haemodynamics: HVPG was reduced by more than 30% of the baseline value in 7 of the 8 patients who had a repeat assessment within 24 hours of the treatment with Infliximab. Overall, the mean HVPG was reduced by 38.9 % from a mean of  $23.4 \pm 2.8$  mmHg at baseline to  $14.3 \pm 1.9$  mmHg on day 1 ( $p < 0.001$ ). Moreover, the mean HVPG on the day prior to discharge (a mean time interval of  $19 \pm 4$  days) was  $12.8 \pm 1.9$  mmHg ( $p < 0.001$ ). Figure 6.1.



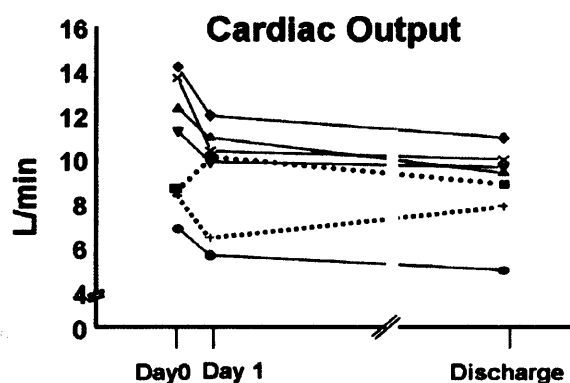
**Figure 6.1.** Infliximab causes a significant reduction (>30%) in portal pressure following a single i.v. administration (5mg/Kg) in 7 out of 8 patients who had a repeat pressure measurement within 24hours. This effect on portal pressure was sustained throughout the patients' admission, a mean interval of 19 days.



6.2.4.1.2 Cardiovascular Haemodynamics: In all 10 patients, MAP was increased significantly from its baseline value of  $71.6 \pm 1.7$  mmHg to  $77 \pm 1.9$  mmHg on Day 1 ( $p < 0.001$ ), and remained elevated on the day prior to discharge (mean  $81.1 \pm 3.2$  mmHg,  $p < 0.001$ ), as shown in Figure 6.2a. This was associated with a significant increase in SVR from  $477.7 \pm 72$  (baseline) to  $786.2 \pm 124$  dyne\*sec/cm<sup>5</sup> prior to discharge,  $p < 0.01$ , [Figure 6.2b]. There was a corresponding 22% reduction in CO ( $p < 0.05$ ) by the end of the study ( $10.9 \pm 1$  to  $8.4 \pm 0.8$  L/min), [Figure 6.2c], in addition to a significant reduction in heart rate ( $93.8 \pm 3.7$  to  $85.3 \pm 3.1$  at day 1:  $p < 0.05$ ; and  $73.5 \pm 1.6$  on the day prior to discharge:  $p < 0.001$ ). No significant changes in central venous pressure were observed following Infliximab therapy.

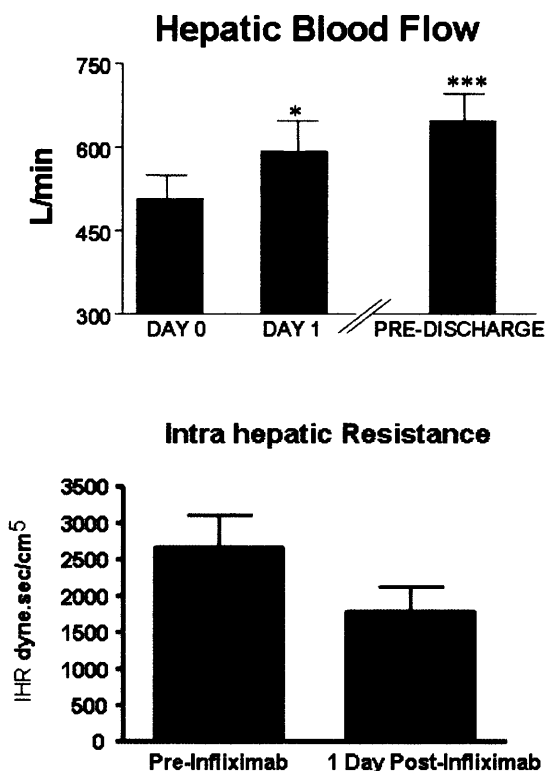


**Figure 6.2 (a)** There is a significant increase in mean arterial pressure (MAP) and **(b)** systemic vascular resistance following treatment with a single infusion of Infliximab (5mg/Kg). In the case of MAP, this improvement occurred within 24 hours and improved significantly further by the time of discharge



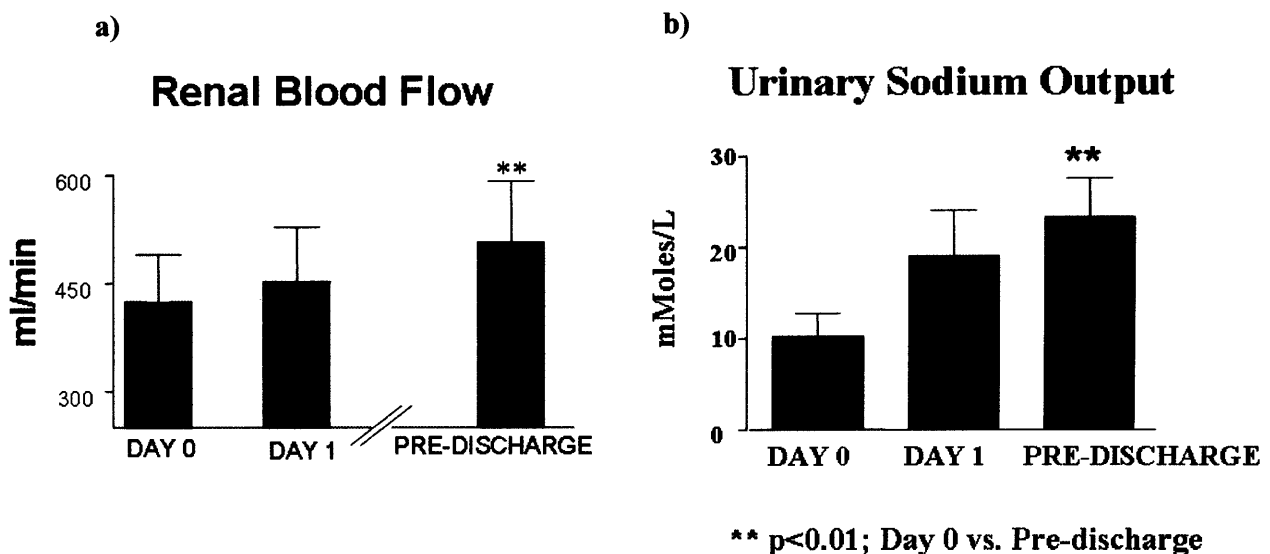
**Figure 6.2c** There is a general reduction in cardiac output (CO) following treatment of alcoholic hepatitis patients with Infliximab. However, it can be seen that in 2 patients (denoted by dotted lines) that the CO increased back to baseline values, despite manifesting a reduction in portal pressure

6.2.4.1.3 Hepatic Blood Flow: Although HBF was measured in all of the patients, data was analysed in the 6 patients who had a hepatic extraction of Indocyanine green (ICG) greater than 10% [mean % ICG extraction: baseline  $11.9 \pm 0.9\%$ ; Day 1  $12.5 \pm 0.7\%$ ]. A significant increase in HBF was observed in the 6 patients in whom it was measurable, from  $506.2 \pm 42.9$  ml/min at baseline to  $591.8 \pm 55.2$  ml/min on day 1, as shown Figure 6.3. This was associated with a decrease in intrahepatic resistance, evident 24 hours after a single infusion of Infliximab.



**Figure 6.3** There is a marked increase in hepatic blood flow within 24 hours following a single infusion of Infliximab, and this increased still further by the time of discharge from hospital. These changes occurred in the presence of lowered portal pressure following Infliximab therapy. The effects were coupled with a lowering of intrahepatic resistance after 1 day as shown in the lower panel of the figure.

6.2.4.1.4 Renal Blood Flow and Renal Function: Following treatment with Infliximab, no patient was found to develop renal impairment. There was in fact, a significant increase in RBF from the baseline value of  $424.3 \pm 65.1$  ml/min to  $506.3 \pm 85.7$  ml/min at the time of discharge ( $p < 0.01$ ). Figure 6.4a. This increase in RBF was mirrored by an increase in urinary sodium excretion from  $10.3 \pm 2.5$  mmol/L on day 0 to  $19 \pm 5.1$  mmol/L on day 1, as shown Figure 6.4b. This improvement in sodium excretion was sustained at the time of discharge ( $23.3 \pm 4.3$  mmol/L and was statistically significant ( $p < 0.01$ ). By contrast, although the urine output increased from  $895.8 (\pm 140)$  ml pre treatment to  $1122 \pm 109$  ml at the time of discharge, this failed to reach statistical significance ( $p = 0.21$ ).



**Figure 6.4 (a)** Intravenous Infliximab administration (5mg/Kg) caused a significant increase in renal blood flow prior to discharge (mean of 19 days); **(b)** change in renal blood flow was associated with an increase in urinary sodium excretion, which also reached statistical significance prior to discharge.

### **6.2.5 Discussion**

This study is the first to measure changes in portal and systemic haemodynamic in alcoholic hepatitis patients following treatment with Infliximab, and demonstrates an early, significant reduction in HVPG (mean of 38%;  $p < 0.001$ ), which was sustained over several weeks and was coupled with a significant increase in hepatic blood flow. This observation further highlights the critical role played by inflammation driven through  $TNF\alpha$  in the development of portal hypertension in AH, and the possible effects this may have on intrahepatic resistance. In addition, the results show a significant improvement in systemic haemodynamic; as evidenced by a reduction in cardiac output with a concomitant increase in systemic vascular resistance and MAP, which may help to explain the increase in renal blood flow and sodium excretion.

These results confirm those of a previous observation, (105) that treatment of AH patients with Infliximab is well-tolerated with 9 of the patients studied surviving, and demonstrating significant improvements in their prothrombin time and serum bilirubin levels, and consequently a decreased discriminant function ( $p < 0.01$ ). Improvement in CRP and white cell count 24 hours after Infliximab therapy was associated with a reduction in SIRS components, and circulating IL-6 and IL-8, highlighting an important role for  $TNF\alpha$  in sustaining the inflammatory response in these patients. This is further supported by data in our earlier collaborative study showing a decrease in intrahepatic IL-8 mRNA synthesis 28 days following Infliximab administration to AH patients. (105) The lack of significant reduction in  $TNF\alpha$  levels in the patients in whom it was measured following Infliximab administration, has been previously noted (105, 136) and may reflect the relatively small numbers of patients included in this study and/or the complex interaction between  $TNF\alpha$  and its bound and free receptors. Soluble TNF receptors have been

shown to be up-regulated in severe AH (108) whilst TNF levels in patients with AH are widely variable in the literature. They range from values as low as 5pg/ml to as high as 400 pg/ml (13, 94, 110, 111), suggesting that other factors such as concomitant infection, degree of endotoxaemia, genetic factors and soluble receptor expression may be important in determining the measured levels of TNF.

The significant reduction in HVPG following treatment with Infliximab found in the present study is likely to be due to a combination of an alteration in both the “forward” and “backward” components of portal hypertension. The changes in forward component, which regulates splanchnic inflow largely through a reduction in cardiac output and increase in systemic vascular resistance, have been documented in studies in animal models. (57, 66, 137) However, as shown in Figure 6.2c, in this study there was an increase in cardiac output in 2 patients despite a sustained reduction in their HVPG. This would appear to suggest that in addition to regulating splanchnic inflow, there is an equally important role for TNF $\alpha$  in the modulation of factors that regulate intrahepatic resistance that is, the “backward component” of portal hypertension.

This notion is further supported by the observation of a reduction in HVPG despite an increase in hepatic blood flow. Additionally, no significant change in HVPG was found in studies in portal hypertensive patients with cirrhosis demonstrating a reduction in cardiac output and a significant increase in systemic vascular resistance and MAP after systemic inhibition of nitric oxide synthase with the inhibitor N<sub>G</sub>-monomethyl-L-arginine (L-NMMA).(138, 139) This would suggest that modulation of systemic regulators of the forward component alone may be insufficient to significantly reduce HVPG.

The intrahepatic resistance is thought to have a “fixed” component relating to fibrosis and a change in cellular architecture and size, and a “variable” component

that can be modified by many factors including the activity of hepatic stellate cells (HSC). Following liver injury, HSCs undergo trans-differentiation to a myofibroblastic phenotype, significantly impairing segmental blood flow, whilst also proliferating and increasing production of the extracellular matrix. TNF $\alpha$  has been shown to be instrumental in activation of rat hepatic stellate cells and also increases the synthesis of extracellular matrix proteins (45), albeit human data is limited. Moreover, spontaneous apoptosis in activated HSCs is significantly down regulated by TNF $\alpha$ , thus prolonging the survival of activated HSC in chronic liver disease and maintaining increased intrahepatic resistance. (140) It is possible that in alcoholic hepatitis, where there is an overproduction of TNF $\alpha$ , (141) a prolonged activation of HSC may give rise to significant elevation of HVPG through increased intrahepatic resistance. It is interesting, therefore, to propose that the mechanism of reduction in intrahepatic resistance after administration of anti-TNF may result either from apoptosis of HSCs, their deactivation or from remodelling of extracellular matrix. However, there is no evidence to suggest massive HSC apoptosis can occur within 24 hours of Infliximab administration to account for the observed reduction in HVPG, or data to show that HSC activation can be reversed. Similarly there is no human data to support rapid remodelling of extracellular matrix, albeit this has been described within 24 hours using cultured HSC. (140)

An alternative explanation for the decrease in intrahepatic resistance following Infliximab administration, stems from research demonstrating decreased intrahepatic endothelial nitric oxide synthase (eNOS) activity in cirrhosis. (43, 53) Although the mechanisms for the local intrahepatic deficiency of NO and the differential tissue regulation of NO production remain ill-defined, there is evidence from cardiovascular research studies suggesting that methylarginine derivatives are important in regulating NOS activity following endothelial injury. (142, 143) Of

interest, asymmetrical dimethylarginine (an endogenous endothelial nitric oxide synthase inhibitor) is in turn regulated by dimethylarginine dimethylaminohydrolase, an enzyme whose activity can be modified by TNF $\alpha$ . (119) One may thus speculate that administration of Infliximab may reduce the intrahepatic concentration of eNOS inhibitors, resulting in a compartmentalised increase in intrahepatic NO. The potential role of ADMA is explored later in this thesis.

Another important finding in this study was the early improvement in hepatic blood flow and renal blood flow following Infliximab treatment. These findings coupled with the fact that there was no significant difference in central venous pressure or free hepatic venous pressure before and after treatment, discounts a reduction in central volume as a possible explanation for the observed reduction in HVPG. Furthermore, most treatments aimed at lowering portal pressure and splanchnic vasodilatation cause deleterious effects on renal perfusion, largely through a reduction in MAP. The significant acute and sustained increase in MAP reported in this study is likely to have played an important role in the increase in renal blood flow and sodium excretion observed. This improvement in renal blood flow may explain why none of the patients studied developed hepatorenal syndrome despite their severe presentations. A similar observation was also reported by Akriviadis et al in their placebo controlled trial of AH patients treated with Pentoxifylline, an inhibitor of TNF $\alpha$  synthesis.(144) This study found a significant reduction in hepatorenal syndrome related deaths (12% versus 43%) in the treatment arm compared with placebo, reflecting the importance of maintaining renal perfusion. A further possible explanation for the improvement in renal perfusion and function that was observed is that the reduction in HVPG may result in a favourable change in the “hepatorenal axis”, (145) independent of the improvement in MAP. This assertion is supported by the data from this study showing an improvement in natriuresis and

urine output, in addition to a significant increase in renal blood flow, concurrent with improvement in liver function and systemic haemodynamics.

Following the observations made in this study and its subsequent publication, there was a randomized controlled trial of Infliximab given 3 times at a dose of 10mg/Kg with concomitant corticosteroids versus corticosteroids alone, published by Naveau et al (106). In this French study, there were significantly increased deaths related to infection and a demonstration of reduced neutrophil responses in the combined treatment group, which led to a moratorium on the use of Infliximab applied to alcoholic hepatitis. However, there were many controversial elements to the protocol used in the Naveau study as noted by the use of multiple high dose Infliximab infusions in addition to high dose prednisolone, which place it at variance to the published data from other small studies. (146) A potential criticism of the study data presented here is that it did not have a control arm to compare with the effects of Infliximab. However, by way of justification, the inherent design of this study was to allow one to establish whether TNF $\alpha$  had a role in the development portal hypertension, and it did not aim to assess Infliximab treatment efficacy. Clearly, it would be difficult to justify intensive repeat haemodynamic assessments and a transjugular liver biopsy in patients not receiving any active therapy and thereby, there could be no 'placebo'. It is also important to note that the significant reduction in HVPG and MAP occurred within 24 hours of the administration of Infliximab, and was sustained thereafter until discharge. This would suggest that it would be highly unlikely for the improvements in HVPG and systemic haemodynamics to have occurred as part of the natural course of AH. Indeed, early work by Maddrey et al (89) reported HVPG data in AH patients treated with corticosteroids or placebo, and showed no significant difference in HVPG between the treatment groups over the 28-to-32-day study period. Moreover, the observed



improvement in haemodynamics coincided with the significant reduction in inflammatory indices 24 hours after Infliximab therapy, supporting a hypothesis that reducing a TNF $\alpha$  driven inflammatory cytokine cascade, restores the imbalance in vascular mediators that promotes a hyperdynamic state and perpetuates further elevation in portal pressure.

### ***6.3 Treatment with Albumin dialysis using The Molecular Adsorbents Recirculating System (MARS)***

#### ***6.3.1 Introduction***

AH often precipitates decompensation in patients with alcoholic cirrhosis and this may lead to associated organ failure. It has also been suggested that histological necrosis and an acute inflammatory infiltrate contributes to the worsening of portal hypertension in these patients (132). Our previous study on the effects of anti-TNF therapy and those of others(147) suggest that the inflammatory mediators of AH, especially pro-inflammatory cytokines such as TNF $\alpha$ , are especially important, and modulation of these factors may result in an amelioration of portal pressure. This implies that portal hypertension in the setting of acute AH may possibly be more labile and amenable to therapeutic interventions compared to that in alcoholic cirrhosis without evidence of hepatic inflammation. Moreover, an increased risk of variceal bleeding due to AH would have potentially more serious consequences with respect to outcome due to the associated decompensation. Albumin dialysis using the Molecular Adsorbents Recirculating System (MARS; Teraklin AG, Rostock, Germany) has been used mainly in the treatment of liver failure (148), and our group had previously shown its potential benefit in patients with severe acute AH. (149) Interestingly, a preliminary report has described an acute reduction of portal pressure

in four patients with acute-on-chronic liver failure (ACLF) after the first session of MARS, which increased before the second session but again underwent reduction, albeit less steep, after the second session (150). A noteworthy point is the fact that all four patients had evidence of hepatic inflammation (AH-3, non-alcoholic steatohepatitis-1). The aim of this study, was thus to evaluate the acute effects of albumin dialysis, using MARS, on portal haemodynamics in patients with severe acute AH. Patients treated with haemofiltration alone were investigated using the same protocol to establish whether changes observed were specific to albumin dialysis or just an effect of extracorporeal therapy.

### **6.3.2 Methods**

#### *6.3.2.1 Patient selection*

*Inclusion criteria:* (i) Patients were included with severe AH, which was defined by a history of alcohol abuse, clinical and laboratory stigmata of acute AH and supported by histological evidence. (ii) Acute-on-Chronic Liver Failure (ACLF), defined as acute deterioration in liver function over 2-4 weeks due to a defined precipitant leading to severe, progressive clinical deterioration despite supportive care (over 72 hours) with (a) increasing jaundice (bilirubin > 85  $\mu\text{mol/L}$ ), and (b) either hyperbilirubinaemia > 300  $\mu\text{M}$  and/or encephalopathy ( $\geq$  grade 2) (151) and/or renal failure. (iii) Clinically significant portal hypertension: (HVPG > 12 mmHg).

*Exclusion criteria:* Patients were excluded if they were <18 or >75 years old, adequate consent could not be obtained, were already enrolled in another study protocol, had uncontrolled variceal bleeding or uncontrolled infection over the past 48 hours, had known hepatic/extrahepatic malignancy, were pregnant, had co-existing HIV infection or severe cardio-respiratory disease. Concomitant or previous corticosteroid therapy was *not* an exclusion criterion.

### 6.3.2.2 *Study design*

Eleven patients were finally included and received a 6-hour session of extracorporeal therapy. Eight patients received MARS treatment in conjunction with haemofiltration. Three others did not consent to MARS but agreed to haemodynamic assessment and haemofiltration, and received a 6-hour session of veno-venous haemofiltration alone. HVPG changes at 6 and 24 hours were the primary endpoints.

**6.3.2.3 *Monitoring:*** The patients were evaluated clinically and biochemically and The Child Pugh score (87) and discriminant function (89) were calculated. The severity of encephalopathy was assessed using the West Haven criteria (151). Mean arterial pressure, electrocardiogram, heart rate and temperature, were monitored continuously during treatment. The patients were actively warmed to a core temperature of 37°C if temperature dropped during treatment. Intravascular volume was maintained using crystalloids, colloids or red cells as appropriate to maintain central venous pressure between 8 and 10cm H<sub>2</sub>O. Blood glucose was maintained between 5-7mmol/L with infusion of 50% dextrose.

**6.3.2.4 *Extracorporeal therapy:*** The MARS system consists of a blood circuit, an albumin circuit, and a renal circuit, in our studies using continuous veno-venous haemofiltration (Hospal BSM 22c, Hospal, Lyon, France). Blood is dialysed across an albumin-impermeable high-flux dialysis membrane (MARS Flux; Teraklin AG). The albumin circuit contains 600 ml of 20% human albumin, which passes through the dialysate compartment of the blood dialyser. It subsequently undergoes haemofiltration and passage through activated charcoal and anion exchange resin columns to remove acquired toxins. Heparin was used as required (if patient's INR <1.4) to prevent clotting in the extracorporeal circuit. The blood and albumin circuit

were run at 150 ml/min, with 1 L/hour fluid exchange, maintaining equal fluid balance. Each MARS session was continued for 6-8 hours duration.

6.3.2.5 Haemofiltration: Continuous veno-venous haemofiltration (Hospal BSM 22c) was performed with similar rates of blood flow (150 ml/min) and fluid exchange (1 L/hour) as that used for MARS.

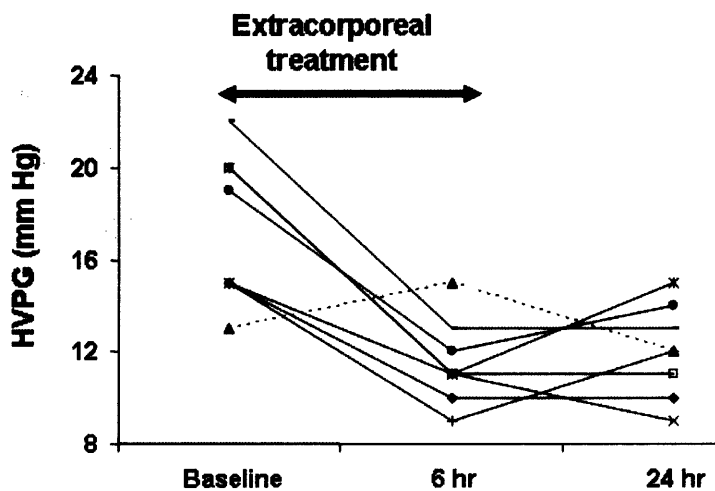
6.3.2.6 Haemodynamic measurements: Haemodynamic studies were performed at the time of trans-jugular liver biopsy prior to the extracorporeal session, and this followed an overnight fast and a 1-hour period during which the patient had been resting supine. The methodology applied for measurement of HVPG and systemic haemodynamics was as described previously (chapter 2, section 2.1.2.1-2.1.2.3). Patients were sedated for the procedure using midazolam (median dose of 3 [range 2-5] mg; Phoenix Pharma Ltd., Gloucester, UK).

### **6.3.3 Results**

Eleven patients with severe alcoholic hepatitis (median DF >68) were studied who had been assigned to treatment with MARS (n=8) or standard medical therapy (n=3), which included haemofiltration, as part of a larger trial on assessment of the efficacy of MARS [Teraklin AG, Rostock, Germany]. Patients in the 2 groups were comparable with respect to liver disease severity (discriminant function and MELD score) but patients in the treatment arm had a significant reduction in bilirubin within 24 hours ( $p<0.001$ ) and a trend towards a lower serum creatinine. Of interest, there were no significant differences in CRP, white blood cell count or liver function in between the groups after MARS treatment. None of the patients received any immuno-modulatory treatments as all had contra-indications (sepsis, recent GI bleed and/or renal failure).

6.3.3.1 *Hepatic Haemodynamics*: All included patients had a median HVPG of 16 (13–22) mmHg. Following MARS therapy, HVPG reduced significantly ( $p< 0.01$ )

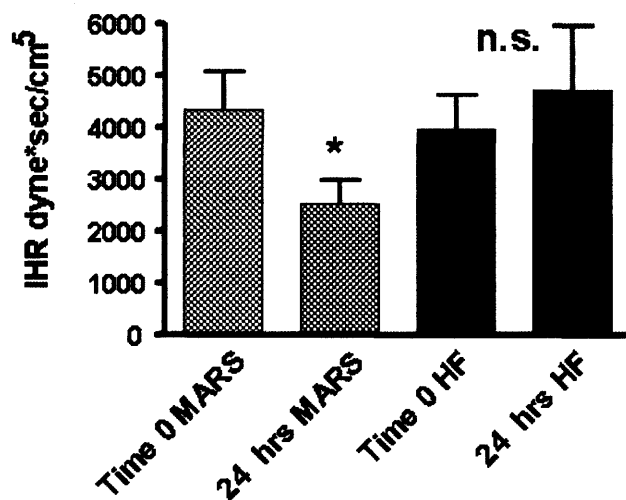
after 6 hours (the end of a treatment session), and remained at this level by 24 hours. The reduction in HVPG was greater than 20% reduction from baseline levels in 7/8 patients receiving MARS, both at 6 and 24 hours, as shown in Figure 6.5.



**Figure 6.5.** Hepatic venous pressure gradients (HVPG) before and after a 6-hour session of extracorporeal treatment with MARS. All but one MARS-treated patient showed a significant (>20%) reduction of HVPG (both at 6 and 24 hours).

No significant change in HVPG was observed in the patients receiving haemofiltration and supportive management alone. Six of the patients reached a target level of <12 mmHg at 6 hours. In two patients with repeated measurements during the session, a reduction in HVPG was apparent within the first 2 hours of commencement of treatment.

Patients treated with the extracorporeal MARS system had an overall increase in hepatic blood flow (as compared to no significant change in the patients treated with haemofiltration). This translated into a significant decrease in intra-hepatic resistance ( $p < 0.05$ ) in patients treated with MARS, as compared to a small increase in patients treated with haemofiltration alone, as seen in Figure 6.6.



\*  $p < 0.05$ ; n.s. - not statistically significant

**Figure 6.6.** MARS therapy induces a significant decrease ( $p < 0.05$ ) in intrahepatic resistance (IHR) within 24 hours compared to patients on haemofiltration alone, in whom there is no significant change from baseline.

#### 6.3.3.2 Systemic haemodynamics

MAP increased over the treatment period of 6 hours and this was sustained at 24 h ( $p < 0.05$ ), as shown in Table 6.2. Cardiac output showed a similar rapid sustained improvement. ( $p < 0.05$ ) The improvement in cardiac output, correlated well with a reduction of HVPG following MARS treatment ( $r = 0.7$ ,  $p < 0.05$ ). None of these haemodynamic parameters improved with haemofiltration and supportive therapy over the 24-hour study period.

**Table 6.2:** Changes in haemodynamics 6 hours and post 24 hours following intervention with MARS.

	MARS group (n=8)			Haemofiltration group (n=3)	
	Baseline	6 hours	24 hours	Baseline	24 hours
HVPG (mm Hg)	17 (13-22)	11 (9-15)**	12 (9-15)***	16 (15-17)	14 (14-19)
MAP (mm Hg)	72 (45-79)	80 (58-99)	76 (64-93)*	71 (65-91)	67 (65-78)
CO (l/min) <sup>†</sup>	8.4 (6.9-17.0)	7.8 (6.1-9.4)*	6.8 (5.5-8.9)**	10.3 (9.3-11.3)	9.5 (7.8-11.1)

<sup>†</sup> n=4 in MARS group

P-value vs. baseline: \* p<0.05; \*\* p<0.01; \*\*\* p<0.001

#### 6.3.4. Discussion

The results of this small study demonstrate an acute reduction in portal pressure with albumin dialysis using MARS which was sustained for up to 18 hours after stopping the treatment. Although the exact mechanism of this portal hypotensive effect of MARS remains unclear, the reduction in portal pressure is, at least in part, the result of effect on the forward component of portal hypertension (i.e. cardiac output), with a further contribution through lowering of intrahepatic resistance. The clinical importance of this observation stems from the fact that portal pressure reduction occurred to an extent which is deemed to reduce the risk of variceal bleeding/rebleeding, rapidly within a few hours of institution of MARS. Portal pressures greater than 12mmHg underlie the development of varices and a reduction to values  $\leq 12$ mmHg (152) or by  $>20\%$  of the baseline (29) are known to prevent rebleeding (153) In this study, within 6 hours HVPG fell by  $\geq 20\%$  in 7/8 patients, and reached 12 mm Hg in 6/8. It is very unusual for most conventional portal pressure reducing strategies to achieve such a rapid response. This reduction was sustained for at least 18 hours after stopping treatment.

Although it was demonstrated that there was a reduction in cardiac output, this was not simply due to a reduction of intravascular volume as a simple consequence of extracorporeal therapy. This is borne out by the facts that (i) central venous pressure was maintained between 8-10 cm H<sub>2</sub>O, under the study protocol, (ii) negative fluid balance was avoided, maintaining 1 litre/hr equal exchange in both the MARS and haemofiltration groups, and (iii) no significant change of haematocrit, serum albumin, creatinine or electrolytes occurred in either group following extracorporeal therapy. (iv) Finally, and most importantly, the volume of the extravascular circuit was very similar in both groups, and both systems (MARS and haemofiltration) were run at the same pump speeds with similar fluid exchange rates, in spite of which systemic and portal haemodynamics improved with MARS but not with haemofiltration.

Given the severity of presentation in these patients, as had been noted in our previous studies, the hepatic extraction of ICG in several patients was less than 10% and there-by unsatisfactory for calculation of hepatic blood flow. Notwithstanding this, hepatic blood flow data was available in 6 patients and a corresponding calculation of intrahepatic resistance (IHR), did show a significant reduction after MARS treatment, which was not evident in patients on haemofiltration alone. This would support a role for modulation of a vascular mediator such as NO in part explaining the observed reduction in HVP, through its effects on intra-hepatic resistance..

The lack of response seen with haemofiltration suggests that the portal pressure changes were mediated by the possible removal/alteration of an albumin-bound substance. The exact underlying pathophysiological mechanism may relate to removal of inflammatory mediators, alteration of NO metabolism, reduction of oxidative stress or modulation of the renin-angiotensin-aldosterone axis. Previous



studies from our group suggest that while alteration of NO metabolism or reduction of oxidative stress are a possibility, systemic inflammatory markers (leukocytosis, CRP) remain unchanged following MARS dialysis.(154)This might support a notion for the removal of a putative factor from the peripheral blood that mediates the hepatic haemodynamic changes observed in this study. Of interest, a recent study published on the effects of 2 different extracorporeal systems including MARS, has also demonstrated an improvement in systemic haemodynamic parameters, and altered levels of metabolites of nitric oxide, in addition to favourable changes in rennin activity and sympathetic drive. (155)

## ***Chapter 7***

***Is there a link between hepatic inflammation and  
circulatory dysfunction through altered regulation of  
nitric oxide synthase?***

## **7.1 Introduction:**

Taking the preceding data together, further experiments were conducted in order to try to elucidate whether the significant modulator of hepatic vascular tone controlled by inflammatory signals was nitric oxide, and how this may be altered by inflammation on the background of pre-existing liver disease.

### ***7.1.1 Nitric oxide generation during hepatic inflammation***

NO is believed to play an important role in both the normal regulation of hepatic microvascular resistance via its relaxing influence on the perisinusoidal stellate cells (156), and also in the context of endothelial dysfunction in experimental cirrhosis. (42-44) Increasing evidence points to a decrease in intrahepatic NO generation in the context of cirrhosis (43, 53) Reduced hepatic eNOS activity, with a resultant decrease in NO release, is likely to result in loss of the counteracting influence to potent vasoconstrictors such as endothelin-1 and angiotensin II, (41, 156) and thereby bring about an increase in the sinusoidal resistance. This is highlighted by studies demonstrating a paradoxical vasoconstrictor response to acetylcholine as a loss of endothelium-dependent vasodilatation through nitric oxide. (42)

An increase in splanchnic/systemic endotoxin load, which in liver disease is believed to parallel the degree of hepatic decompensation, (129) has been suggested to be the trigger for activating NOS activity with subsequent excess NO production (32) However, others have suggested that in states of increased endotoxaemia, endothelial nitric oxide synthase (eNOS) generated by regional vascular endothelial cells may be down-regulated. (157). This apparent paradox may relate to the effects of post-translational modification of NOS or through the actions of endogenous regulators, as the hepatic mRNA and protein expression of this enzyme have been found to be similar in control and cirrhotic animal models. (44, 158) However, general data

from human studies is limited and the role for inflammation in promoting these changes to eNOS functional activity, perhaps driven through increased levels of endotoxin, is poorly characterized.

Given the findings presented in this thesis on the vascular derangements in alcoholic hepatitis to this point, and the clear reversible nature of the intra-hepatic vascular defect with modulation of inflammation, the link between hepatic inflammation and circulatory dysfunction through altered regulation of nitric oxide synthase was investigated.

## **7.2 Studies in patients with alcoholic cirrhosis to assess NO generation, NOS protein and mRNA expression**

### ***7.2.1 Patient selection***

Plasma samples were drawn from 24 patients presenting with decompensated alcoholic cirrhosis, (increasing ascites, pedal oedema or progressive jaundice), with clinical and radiological evidence of cirrhosis, and a current history of alcohol abuse. Patients were excluded if they were <18 or >75 years and had evidence of: additional/another aetiology of liver disease; severe cardiac dysfunction or renal failure (creatinine >150µmol/L); hepatic/extra-hepatic malignancy; hepatic encephalopathy ≥Grade 2 (precluding informed consent), and if there was microbiological evidence of infection after 72 hours of broad-spectrum antimicrobials. Patients were studied at transjugular liver biopsy (days 1-3 of admission), performed to assess severity of liver dysfunction in those with ascites or/and coagulopathy. Classification to AH superimposed on cirrhosis (AH+C+) was made on subsequent histological examination using the criteria described in section 3.1.1.1 of chapter 3.

Blood samples were drawn from a peripheral artery and from the hepatic vein into pre-cooled tubes and the plasma obtained by centrifugation stored at  $-80^{\circ}\text{C}$ , until subsequent analysis for nitrite and nitrate. Liver tissue obtained with appropriate approved ethical consent at the time of transjugular biopsy, was divided such that some of the sample was fixed in formalin for routine histological assessment, and the remainder snap frozen until subsequent assessment for tissue NOS activity. For comparison to 'normal' tissue, clear hepatic resection margin tissue from 3 patients with colorectal hepatic metastases and no evidence of liver disease were used.

### **7.2.2 Results:**

The patients studied form a subset of those described in greater detail in Chapter 3 section 3.1.2.1. Ten patients had histological evidence of alcoholic hepatitis superimposed on cirrhosis, whilst 14 had cirrhosis without evidence of inflammation.

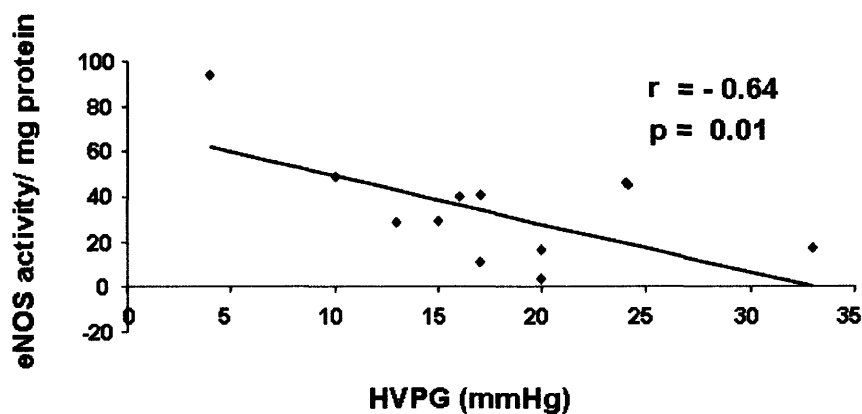
#### *7.2.2.1 Generation of nitrite from arterial and hepatic venous samples*

There were no statistically significant differences between measurements of nitrite in arterial samples compared to samples taken from the hepatic vein for either group of patients: Alcoholic hepatitis: artery ( $0.65 \pm 0.17 \mu\text{M}$ ) and hepatic vein ( $0.61 \pm 0.1 \mu\text{M}$ );  $p=0.8$  and alcoholic cirrhosis: artery ( $0.61 \pm 0.07 \mu\text{M}$ ) and hepatic vein ( $0.56 \pm 0.11 \mu\text{M}$ );  $p=0.7$ . In addition, there was no statistically significant difference between artery or hepatic venous nitrite values when comparing patients with and without additional inflammation (alcoholic hepatitis).

#### *7.2.2.2 Hepatic tissue NOS activity measures*

In contrast to measurements of nitrite, assessment of hepatic tissue NOS generated NO in a sub-group of patients using the method outlined in Chapter 2, section

2.4.3.3, demonstrated a significantly reduced total NOS activity in patients with inflammatory alcoholic hepatitis [n=6] compared to alcoholic cirrhosis patients [n=6] ( $38.5 \pm 12.6$  vs.  $91.4 \pm 31.8$  nmoles citrulline/h/mg liver tissue,  $p=0.04$ ). eNOS activity was calculated following  $\text{Ca}^{2+}$  chelation with EGTA (total NOS activity minus  $\text{Ca}^{2+}$ -independent activity), and also demonstrated significantly lower activity in AH patients ( $23 \pm 6.4$  vs.  $46 \pm 13$  nmoles citrulline/h/mg liver tissue,  $p=0.04$ ). The total and eNOS contribution to activity of alcoholic cirrhosis patients was slightly reduced compared to normal control tissues in which total NOS activity was  $133 \pm 53$  nmoles citrulline/h/mg liver tissue. Given the limited availability of normal tissue, any reliable statistical comparisons were constrained. The HVPG was shown to have a significant inverse association with hepatic tissue eNOS activity ( $R= - 0.64$ ;  $p=0.01$ ), (Figure 7.1) highlighting the importance of reduced NO generation to elevated portal pressure.

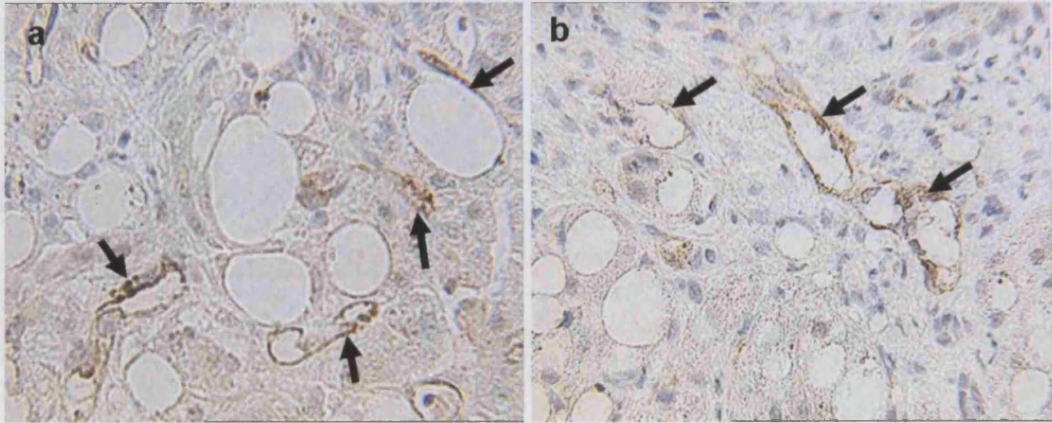


**Figure 7.1** This graph demonstrates the significant inverse correlation between hepatic venous pressure gradient and hepatic tissue eNOS activity in patients with alcoholic liver disease.

#### 7.2.2.3 Immunohistochemistry of NOS expression

Liver biopsy specimens assessed for tissue NOS activity were also studied for eNOS protein expression. Immunostaining of these tissue specimens with anti human eNOS demonstrated eNOS expression in both alcoholic hepatitis and alcoholic

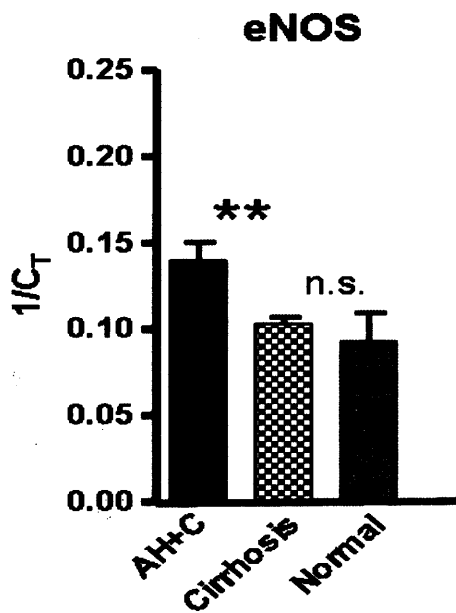
cirrhosis biopsies (Figure 7.2), albeit the method was semi-quantitative and thus, would not allow a clear difference to be defined between the 2 alcoholic liver disease groups



**Figure 7.2.** The figure above shows immunostaining of paraffin-embedded liver biopsy sections from an alcoholic hepatitis patient (a) and an alcoholic cirrhosis patient (b) using monoclonal anti-human eNOS (positive staining denoted by the position of the arrows).

#### 7.2.2.4 mRNA quantification of eNOS

Using quantitative RT-PCR, the transjugular liver tissue specimens from the decompensated alcoholic cirrhosis patients described above were evaluated for eNOS mRNA quantification. eNOS mRNA in the liver samples of inflammatory alcoholic hepatitis patients were significantly increased (as denoted by markedly lower cycle threshold [ $C_T$ ] values) compared to cirrhosis alone patients ( $7.2 \pm 0.4$  vs.  $9.8 \pm 0.4$ ,  $p=0.001$ ) and specimens from resection margins of 'normal' liver [ $n=3$ ] ( $11 \pm 1.8$ ,  $p=0.02$ ). No significant difference was found between eNOS mRNA expression levels in cirrhotic livers without inflammation and normal livers ( $p=0.25$ ), as shown in Figure 7.3, below.



**Figure 7.3** There is significantly higher eNOS mRNA expression in patients with cirrhosis with superadded inflammatory alcoholic hepatitis (AH+C) compared with alcoholic cirrhosis alone and samples of histologically normal livers. The values of expression of mRNA are denoted as relative expression 1/Cycle Threshold (1/C<sub>T</sub>) for normal liver, alcoholic cirrhosis and alcoholic cirrhosis with superimposed alcoholic hepatitis (AH+C). Thus, the greater the expression, the smaller the C<sub>T</sub> value

### 7.3 Studies in an acute liver failure animal model- Comparison of Sham and Galactosamine Liver tissue NOS activity

#### 7.3.1 Introduction

Galactosamine injection into rodent models is a well-established method of evoking acute hepatic necrosis, and this is believed to be the consequence of impaired RNA and protein synthesis. (159) Furthermore, if a further insult by injection of LPS is applied, there follows a marked inflammatory response as evidenced by very high serum aminotransferases and histological evidence of central necrosis. (160) It is also of interest that cultured hepatocytes from these animals show a marked decrease in generation of nitric oxide. (161)

Having observed a marked decrease in eNOS activity in patients with alcoholic liver disease, we wished to confirm these findings in a model with a known hepatic inflammatory insult, which is independent of the effects of alcohol, and to which a



defined further challenge with LPS could discern the effects of superadded inflammation.

### **7.3.2 Method**

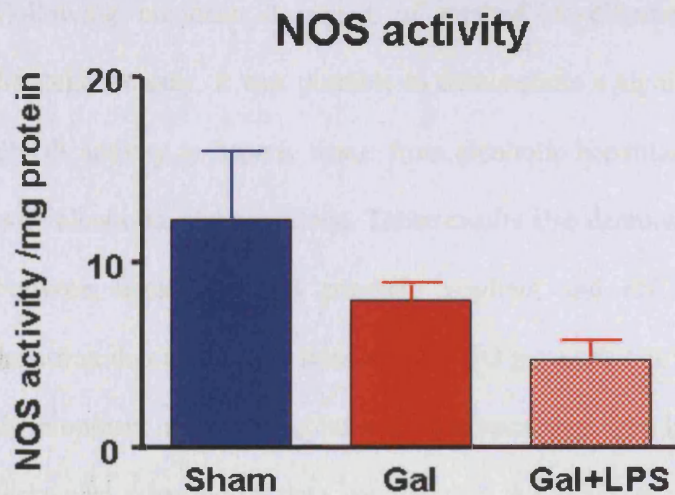
Rats were treated with galactosamine injection, with or without subsequent injection with LPS, as described in Chapter 2, section 2.3.2.

Hepatic tissue nitric oxide synthase generated nitric oxide was assessed using the methodology applied to human liver tissue homogenates as described in section 2.4.3.3 of Chapter 2.

### **7.3.3 Results**

Liver specimens acquired from 5 rats treated with galactosamine alone, were compared with 5 specimens from rats that had received galactosamine followed by LPS after 24 hours. Five sham rats were used as a normal control for inflammatory activity.

The galactosamine treated animals had a reduced total NOS activity compared to sham rats ( $7.9 \pm 0.9$  vs.  $12.3 \pm 2.7$  activity/ mg protein,  $p < 0.05$ ). Furthermore, adding further liver injury by the administration of LPS had the effect of an increased incidence of early deterioration (within 3 hours) in the animal's condition, warranting termination of the experiment. Under these conditions, there was an even greater reduction in NOS activity: ( $4.7 \pm 1$  vs.  $12.3 \pm 2.7$  activity/ mg protein;  $p < 0.01$ ). Figure 7.4.



**Figure 7.4.** Hepatic tissue NOS activity in sham, galactosamine treated rats and rats given LPS in addition to galactosamine. The addition of LPS to galactosamine treated animals resulted in a greater than 50% reduction in NOS activity compared to sham treated animals.

#### **7.3.4 Discussion**

These results do not show significant differences between measurement of nitric oxide metabolites in patients with alcoholic hepatitis and those with cirrhosis alone, nor when comparing between regional samples (artery compared to hepatic vein). It is important to note that measurement of plasma nitrate/nitrite in hepatic venous samples does not simply reflect the local hepatic generation of NO metabolites but also any spill-over from the splanchnic territory, through a contribution from portal venous blood. In addition, these assays are considerably dependent on local conditions including state of hypoxia, underlying nitrogen balance (nutritional status) and renal function, which can affect their sensitivities. As such, it was clear that a more 'reliable' NOS activity assay was needed which would reflect hepatic tissue NO generation.

Following on from a period of method development, and using a modified Strategene assay, it was possible to demonstrate a significant reduction in total and eNOS activity in hepatic tissue from alcoholic hepatitis patients compared to those with alcoholic cirrhosis alone. These results also demonstrated an inverse correlation between hepatic venous pressure gradient and eNOS activity, supporting the assertion that a deficient intra-hepatic NO generation is likely to be important in the development of increasing intra-hepatic resistance. The human NOS activity findings were also mirrored by data generated in the galactosamine rodent model, showing that the liver insult alone decreased NOS activity compared to sham, and that this was further exaggerated by the addition of LPS. However, this further deleterious reduction in hepatic eNOS activity in the context of hepatic inflammation may appear secondary to changes impacting directly on enzyme activity, or as result of the effect of inflammation acting on eNOS transcription or protein translation.

The above data shows that eNOS mRNA is increased in alcoholic hepatitis patients, whilst protein expression assessed semi-quantitatively through immunohistochemistry shows no difference in protein expression compared with cirrhosis alone. These findings have also been substantiated by studies in rodent models of cirrhotic bile duct ligation showing increased mRNA and preserved protein expression in the diseased state. (158) This implies that the reduced eNOS activity noted in patients as well as in the animal model was likely to be due to an effect directly on enzyme function, raising the possibility of action of inhibitors or dysfunctional molecular architecture influencing eNOS activity.

## ***Chapter 8***

***Is there evidence for altered levels of asymmetric dimethylarginine in liver failure and is this related to the severity of inflammation?***

## **8.1 Introduction:**

The previous demonstration of a reduction in eNOS activity in patients with alcoholic hepatitis in spite of an increased expression of eNOS mRNA and the apparent preservation of eNOS protein expression, raised the possibility of potential inhibitory regulators of NOS activity. From the cardiovascular literature, there has been increasing importance attributed to the role of asymmetric dimethylarginine (ADMA) as a regulator of vascular tone, and a potential risk factor for the development of hypertension, cardiovascular morbidity and poor outcome in patients with chronic renal disease. (118, 162-164) Although at the time of the study, there was no supportive ADMA-related physiology or pathophysiology liver literature available, some data has subsequently been published (165-168) The possibility that deterioration in vascular function in liver failure, whether this be acute or secondary to an acute decompensation of chronic liver disease, may be as a consequence of elevated levels of ADMA, is addressed in the following sections.

## **8.2 Study of patients with acute liver failure secondary to paracetamol overdose**

### ***8.2.1 Background***

In patients with acute liver failure (ALF) and fulfilling criteria for poor prognosis, rapid progression to multiorgan failure is common and without urgent liver transplantation, their mortality rates are nearly 90%. (169) The pathophysiological basis of the evolution of multiorgan failure in these patients is not clear. ADMA, an endogenous NO synthase (NOS) inhibitor, is synthesized by the action of protein-arginine-methyltransferases on arginine residues of nuclear proteins. ADMA is released following proteolysis, and most of this free circulating ADMA undergoes metabolism by dimethylarginine dimethylaminohydrolases (DDAH), an enzyme widely distributed in numerous tissues, including the liver. (170) Indeed, the liver

has been shown to be an important site of ADMA elimination through the action of DDAH, (167) and only a small amount of free ADMA is excreted in the urine. Recent data from patients with sepsis related multi-organ failure and concomitant hepatic dysfunction demonstrate high circulating levels of ADMA, which independently predict outcome. (171) Interventions such as tight glycaemic control associated with improved survival in sepsis related multi-organ failure have been linked with a reduction in circulating levels of ADMA. (172)

Levels of ADMA in patients with liver dysfunction vary widely, and the reasons for this are as yet, unclear. (166, 168) Studies from the cardiovascular literature suggest that inflammation in cell systems is associated with altered metabolism of ADMA through reduction in DDAH activity. (119) The impact of raised ADMA levels during inflammation may be further compounded by reduced availability of arginine if there is concomitant sepsis. (173)

There is an increasing body of literature supporting the view that the systemic inflammatory response, be it generation through hepatic inflammation or/and sepsis, is important in determining the outcome of ALF patients. (174-177) Given the inflammatory environment of ALF, a resultant high ADMA may contribute to vascular dysregulation and organ dysfunction. The aim of this study in patients with ALF was to test the hypothesis that a cytokine driven inflammatory response is important in modulating ADMA levels.

### **8.2.2 Methods**

Relatives of patients gave informed written assent in accordance with the Declaration of Helsinki of the World Medical Association, 1989, and the study approved by the local ethics committee.

*8.2.2.1 Patients:* Ten patients with severe ALF secondary to paracetamol overdose [age 31 (21-46); 7 female patients] presenting or transferred to Edinburgh Royal Infirmary, were included. All patients had equivalent severity of liver disease and renal dysfunction and fulfilled Kings College criteria for poor prognosis. Patients were managed using a standardised protocol of care and 8 of the 10 were treated for raised intracranial pressure. All patients underwent continuous veno-venous haemofiltration. Patients were enrolled into the study within the first 48 hours of admission to the intensive care unit with grade-IV HE, and all required mechanical ventilation. The patients were followed until liver transplantation or death.

*8.2.2.2 Sampling and Assays:* Blood was collected from an artery, and in 4 patients who had a transjugular liver biopsy, also from the hepatic vein. In patients undergoing liver transplantation, arterial blood was sampled immediately prior to and up to 4 hours post transplantation. In a single patient, arterial blood was sampled repeatedly during the different phases of the transplant operation. Sampling was performed in pre-cooled heparinized/ EDTA coated tubes (Becton Dickenson, Drogheda, Ireland). Routine haematology, biochemistry (including liver function tests) and C-reactive protein were measured in all patients. Additional plasma samples were separated by centrifugation and plasma stored at  $-80^{\circ}$  C for subsequent analysis of cytokines, chemokines and ADMA analysis. For comparative age matched [36 (31-48); 5 female] healthy control ADMA levels,

venous blood was drawn from an ante-cubital vein in 10 laboratory staff with no known liver disease or vascular pathology.

**8.2.2.3 Cytokines/chemokines:** TNF $\alpha$ , IL-6 and IL-1 $\beta$  were measured using standard commercial assay kits (BioSource, Nivelles, Belgium) as described in the methods in Chapter 2, section 2.4.1. The lower limit of detection was estimated to be 5pg/ml and the intra-assay coefficient of variation was 4.5-5.5 %.

**8.2.2.4 ADMA:** Plasma ADMA was measured using fragmentation specific stable isotope dilution electrospray mass spectrometry-mass spectrometry, as described in detail in Chapter 2, section 2.4.5.

### **8.2.3 Results**

The clinical characteristics and subsequent management in the 10 studied ALF patients are summarised in Table 8.1 below. All the patients fulfilled the Kings College Criteria for liver transplantation and required multiple organ support, including mechanical ventilation for severe encephalopathy and veno-venous haemofiltration for renal failure. Most patients required inotropic support and 8 of the patients required treatment with mannitol and fluid removal for the management of intracranial hypertension. All patients received broad spectrum antibiotics as prophylaxis, but despite this measure, culture positive infection was observed in 6 patients (*Staphylococcus aureus* in 4, *Klebsiella pneumonia* in 1 and *Candida albicans* septicaemia in 1). Two patients developed uncontrolled intracranial hypertension during follow up that remained unresponsive to medical therapy (Patients 9 and 10, depicted in Figure 8.4). Six patients underwent orthotopic liver transplantation (OLT) whilst the other 4 died (2 awaiting an organ for transplantation and 2 with contraindications for transplantation on psychosocial grounds and remained unlisted for OLT).



**Table 8.1 (Overleaf)**

Clinical characteristics and biochemical data for the 10 studied patients.

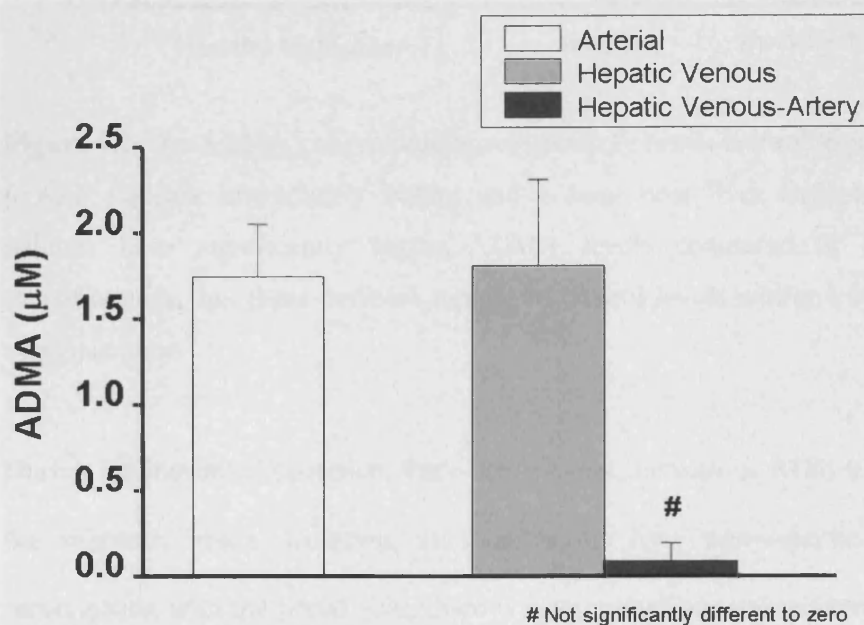
*Abbreviations:*

*POD-paracetamol overdose, \*after resuscitation, KCH-Kings College Hospital.*

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Age (years)	31	28	21	28	46	29	32	30	19	33
Sex	F	F	M	M	F	F	F	F	M	F
Aetiology of acute liver failure	POD	POD	POD	POD	POD	POD	POD	POD	POD	POD
Candidate for OLT	No	No	No	Yes	Yes	Yes	Yes	No	Yes	Yes
Other substances abuse	EtOH	EtOH	EtOH	No	No	No	No	No	No	No
Highest recorded prothrombin time (sec)	112	>100	>120	112	>100	>100	110	>100	>100	121
Alanine amino transferase (IU/L)	10290	8628	4584	7986	2461	10460	7640	8970	7561	8756
Bilirubin ( $\mu\text{mol/L}$ )	82	99	145	41	128	64	51	104	56	65
Time delay between poisoning and hospital admission	52 hr	38 hr	72 hr	not known	64 hr	58 hr	90 hr	72 hr	81hr	not known
Creatinine ( $\mu\text{mol/l}$ )	356	231	276	313	221	243	198	171	239	353
*Arterial hydrogen ion concentration (nmol/L)	46	89	46	41	54	56	78	56	59	61
SIRS score	2	2	1	2	3	3	3	4	4	4
CRP (ug/ml)	54	46	67	55	72	76	81	71	102	78
Mechanical Ventilation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Continuous venovenous haemofiltration	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Inotropic support	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes
Fulfil KCH criteria for OLT	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Liver Transplantation	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes

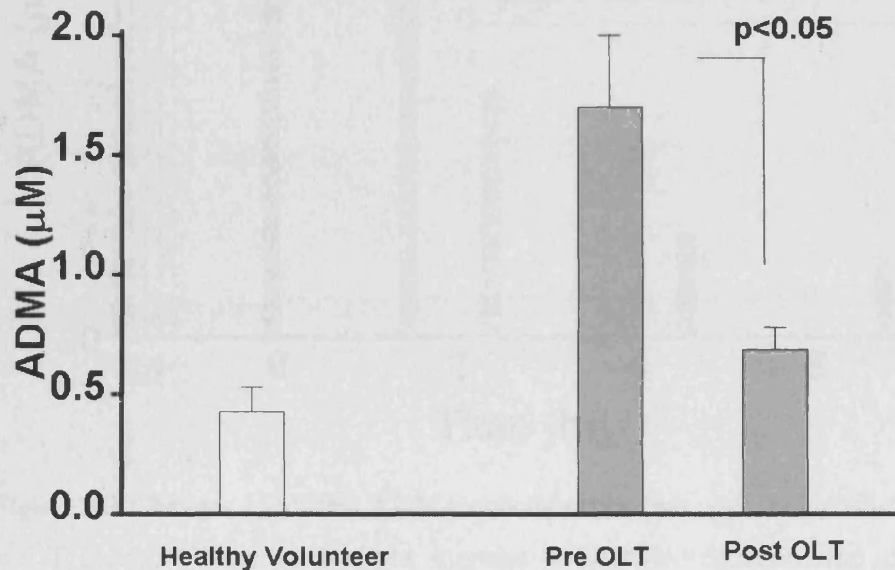
### 8.2.3.1 ADMA levels

ADMA levels on admission of the patients to intensive care were markedly elevated compared to levels in normal controls (patients: mean  $\pm$  SEM:  $1.75 \pm 0.3 \mu\text{mol/L}$ , controls:  $0.37 \pm 0.02 \mu\text{mol/L}$ ;  $p < 0.001$ ). There was no significant change in the levels of ADMA during the course of the illness (ADMA level:  $1.8 \pm 0.3 \mu\text{mol/L}$  at time of transplantation or death), compared with 1<sup>st</sup> measurement;  $p = 0.4$ . In the four patients that had hepatic vein catheterisation, no net-uptake of ADMA was detectable across the liver, as shown in Figure 8.1. However, as can be seen from the data in the figure, ADMA levels in both artery and hepatic vein from these 4 patients were significantly higher than the plasma ADMA value noted in age matched controls.



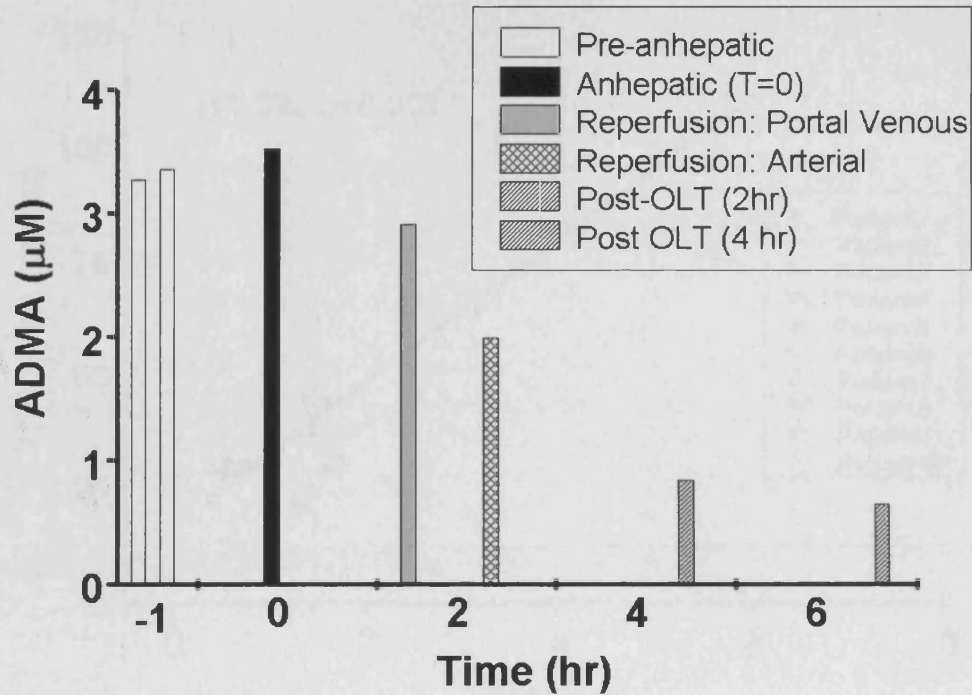
**Figure 8.1** The relative concentrations of ADMA are shown for the 4 patients who underwent hepatic venous catheterisation. It is evident that there was no net hepatic uptake of ADMA in these patients with ALF. ADMA levels in both artery and hepatic vein from patients were however, significantly higher than noted in plasma values from age matched controls [Control ADMA:  $0.37 \pm 0.02 \mu\text{mol/L}$ ].

Within 1 hour post liver transplantation, there was a significant reduction of ADMA levels to values which were only marginally higher than control levels (Figure 8.2).



**Figure 8.2** The ADMA concentrations are shown between control groups and in the 6 ALF patients immediately before and 1 hour post liver transplantation. The patients have significantly higher ADMA levels compared to controls pre-transplantation, but these decrease rapidly to control levels within 1 hour post liver transplantation.

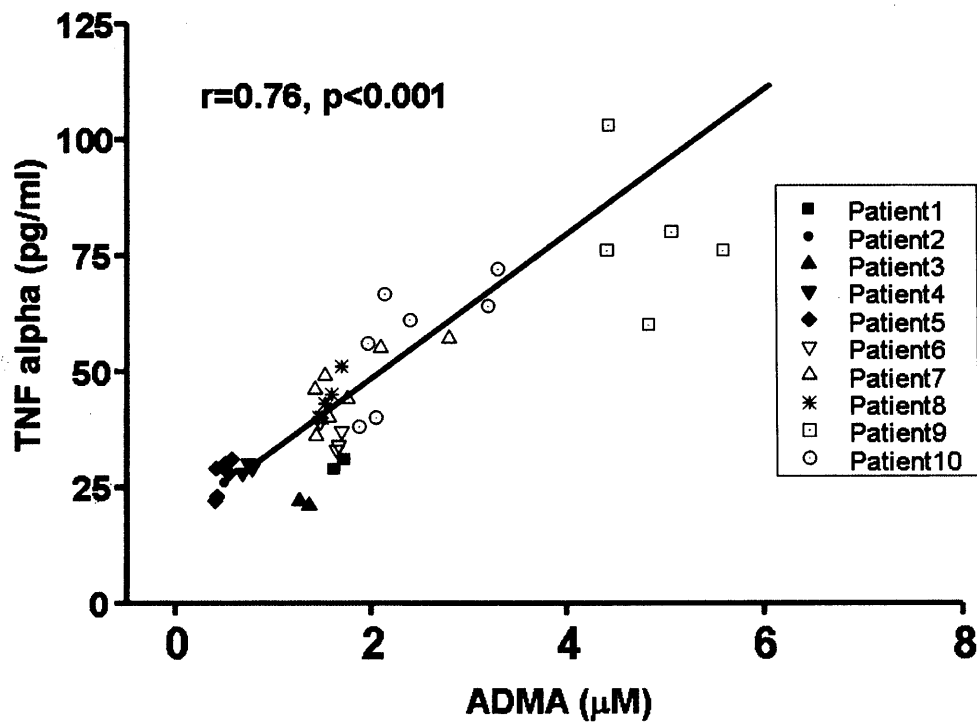
During the transplant operation, there was a slight increase in ADMA levels during the anhepatic phase. However, as soon as the liver was reperfused following reconnection with the portal vein, there was an immediate and marked reduction in ADMA concentration. This effect on reduction in ADMA level with reperfusion was further enhanced following return of hepatic artery perfusion. ADMA levels continued to progressively decrease until the time of final sampling, which was up to 4 hours following reperfusion of the transplanted liver, as shown below. (Figure 8.3)



**Figure 8.3** Changes in plasma ADMA concentration pre-, per- and post-operatively are illustrated. There is a slight increase in ADMA concentration during the anhepatic phase of the transplantation procedure, but following reperfusion with the portal vein and subsequently the hepatic artery, there is a significant and progressive reduction in ADMA, up to 4 hours post-reperfusion.

#### 8.2.3.2 Inflammatory markers

The pro-inflammatory cytokine concentrations in the patients were significantly higher than in controls, in whom levels were below detection threshold. Patient cytokine levels were as follows: TNF $\alpha$ :  $28.9 \pm 5$  pg/ml, IL-1 $\beta$ :  $32 \pm 6$  pg/ml and IL-6:  $93 \pm 8$  pg/ml. An important observation was that ADMA levels correlated strongly with the levels of the pro-inflammatory cytokines: TNF $\alpha$  (Figure 8.4:  $r=0.76$ ;  $p<0.001$ ); IL-1 $\beta$  ( $r=0.6$ ;  $p<0.01$ ) and IL-6 ( $r=0.56$ ;  $p<0.01$ ). Patients 9 and 10 depicted in the figure were noted to have the highest ADMA and TNF $\alpha$  concentrations, and these two patients developed uncontrollable intracranial hypertension resulting in death in these patients.



**Figure 8.4** There is a significant positive correlation between plasma ADMA levels and TNF $\alpha$  concentrations, with  $r=0.76$  and a P value of  $<0.001$ . Patients 9 and 10 are noted to have the highest ADMA and TNF $\alpha$  concentrations, and these two patients developed uncontrollable intracranial hypertension and died.

SIRS score (86) was variable between the patients, ranging from 2 to 4 and in general, those with higher values of SIRS had higher ADMA levels. CRP was elevated in all patients but there was no direct correlation between CRP values and ADMA levels or SIRS score.

### **8.3 Does inflammation superimposed on a model of acute liver injury further impact on ADMA? -Study of a rodent model of liver failure**

#### ***8.3.1 Background***

The aim of this study was to investigate the effects of liver injury on the levels of ADMA using a rat model of acute toxic injury by intraperitoneal galactosamine injection, before and 3 hours after LPS injection. Having shown that inflammation in the context of liver injury in humans is associated with elevated ADMA, this study would also help determine whether inflammation exerts additional effects on levels of dimethylarginines in a model with prior acute liver injury.

#### ***8.3.2 Methods***

The methods for this experimental galactosamine model are described in section 2.3.2 of Chapter 2. Plasma aminotransferases in the animals were measured using standard kits on a Cobas Integra analyser (Roche Diagnostics Ltd, west Sussex, UK). ADMA measurements were performed using the method described in Chapter 2, section 2.4.5.

#### ***8.3.3 Results***

##### ***8.3.3.1 Biochemistry and inflammatory indices***

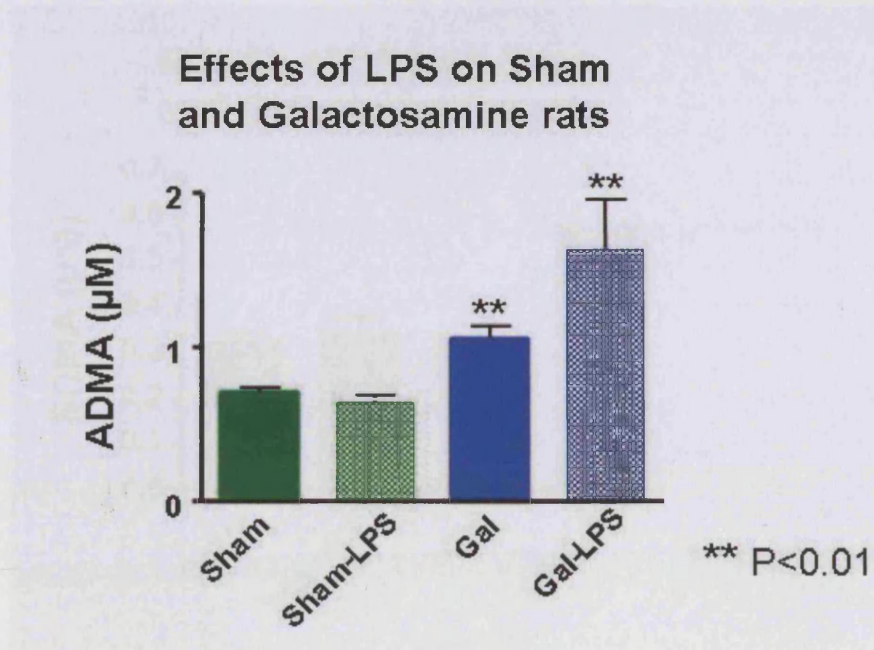
Galactosamine animals [n=5] had markedly elevated plasma aminotransferases compared with sham animals: [n=5] (ALT:  $1707 \pm 644$  vs.  $58 \pm 11$  IU/ml;  $p < 0.05$ ), which were further exaggerated by injection of LPS. There were no significant differences between sham and galactosamine treated animals with and without LPS, in regard to plasma bilirubin. However, the galactosamine animals that were also administered LPS did have significantly higher plasma creatinine levels compared to

sham animals ( $44 \pm 6$  vs.  $25 \pm 1.6$   $\mu\text{mol/L}$ ;  $p < 0.05$ ) or sham animals that were also administered LPS ( $26 \pm 1.1$   $\mu\text{mol/L}$ ). Previous studies in the literature had confirmed the severity of inflammatory insult, largely driven through a  $\text{TNF}\alpha$  mediated pathway, in these animals, whilst also showing elevated levels of IL-6 and interferon  $\gamma$ , (178, 179) and thus, these cytokines were not assayed in these animals.

#### *8.3.3.2 Plasma ADMA*

ADMA levels were found to be significantly higher in galactosamine injected animals compared to sham ( $1.1 \pm 0.08$  vs.  $0.7 \pm 0.03$   $\mu\text{mol/L}$ ;  $p < 0.01$ , Figure 8.5). Furthermore, ADMA levels were found to be even greater after administration of LPS to galactosamine pre-treated animals with mean ADMA levels increasing to  $1.6 \pm 0.3$   $\mu\text{mol/L}$ , albeit that there was no statistical difference ( $p = 0.14$ ) compared to values amongst the galactosamine alone animals. The likely explanation for lack of statistical differences between the galactosamine groups was the considerable variation of levels within this small group of animals. Of interest, the levels of ADMA in sham rats subsequently administered LPS, was no different from that measured in sham animals ( $0.65 \pm 0.05$  vs.  $0.7 \pm 0.03$   $\mu\text{mol/L}$ ,  $p = 0.4$ ).

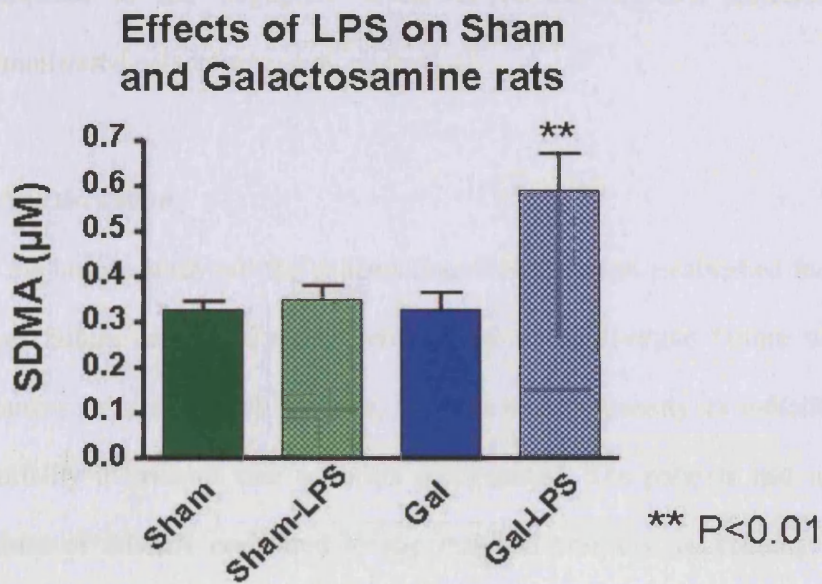




**Figure 8.5** Galactosamine treated rats show markedly elevated plasma ADMA concentrations, which was further enhanced by LPS injection

#### 8.3.3.3 Plasma SDMA

Given the high creatinine levels in the galactosamine + LPS administered animals and evidence in the literature for high symmetric dimethylarginine (SDMA) values in renal dysfunction, the levels of this dimethylarginine homologue were also measured. SDMA levels were considerably elevated in the galactosamine animals also administered LPS compared to sham animals ( $0.6 \pm 0.08$  vs.  $0.33 \pm 0.02$   $\mu\text{mol/L}$ ;  $p < 0.01$ ). There were no differences in SDMA levels between sham and galactosamine alone animals ( $p = 1$ ) and sham animals administered LPS ( $p = 0.8$ ), as seen in Figure 8.6



**Figure 8.6** Galactosamine treated rats show markedly elevated plasma SDMA concentrations only after administration of LPS 21h after galactosamine injection, which appears to mirror the increase in plasma creatinine observed in these animals. There were no significant differences in SDMA levels between galactosamine alone and sham and sham-LPS treated animals

#### 8.3.3.4. Plasma arginine levels

Arginine levels were also measured for each of the animal groups, as arginine is the main substrate for NOS for which the dimethylarginines act as competitive inhibitors. Plasma arginine was significantly reduced in galactosamine administered animals compared with sham ( $4 \pm 1.2$  vs.  $175 \pm 19$   $\mu\text{mol/L}$ ;  $p < 0.01$ ). The addition of LPS to sham animals did result in a decrease in arginine levels to  $98 \pm 20$ ,  $p < 0.05$  compared with sham without LPS. However, though the addition of LPS to galactosamine animals, as might have been anticipated, did cause a significant reduction in plasma arginine compared to sham, ( $16 \pm 11$  vs.  $175 \pm 19$   $\mu\text{mol/L}$ ;  $p < 0.01$ ), this did not significantly alter plasma arginine concentrations further when

compared to the negligible levels of plasma arginine detected in animals administered galactosamine alone ( $p=0.4$ ).

#### **8.3.4 Discussion**

In the human study, all the patients described manifest established features of acute liver failure namely, a rapid development of multi-organ failure with associated changes in intra-cranial pressure, an increased propensity to infection and a high mortality in patients that were not transplanted. The patients had higher baseline values of ADMA compared to age matched controls, in keeping with previous observations in acute and chronic liver failure patients, (165, 168) and these levels reduced significantly in those patients that were transplanted. However, the most important finding of this study was a good correlation between ADMA levels and pro-inflammatory cytokines, lending support to the hypothesis that inflammation may be important in regulating ADMA metabolism.

It has been suggested that the liver is a major site for ADMA metabolism through the action of the enzyme DDAH. (167) However, DDAH activity has been shown to be modulated by the effects of oxidative stress and inflammation in model systems. (119, 180) Indeed under experimental conditions of cytokine driven iNOS up regulation, there is a relative inactivation of DDAH through S-nitrosylation of a cysteine residue (Cys 249), leading to accumulation of ADMA with consequent inhibition of NOS. (181) In patients following liver transplantation, increases in ADMA have been noted prior to the onset of acute rejection, a process that manifests both biochemically and histologically as expressing increased indices of inflammation. (168) Other data supporting an association between inflammation and ADMA have been described in chronic kidney disease patients, in whom renal transplantation is associated with a significant improvement in brachial artery flow

mediated dilatation concomitant with a decrease in CRP and ADMA; the kidney being also a significant site of DDAH activity. (182)

The data described in this human study also shows that the failing liver loses its ability to extract ADMA in contrast to normal livers, where significant hepatic dimethylarginine elimination has been demonstrated. (183) It has been hypothesized that the “local” accumulation of ADMA may contribute to the development of multi-organ failure, through its inhibitory action on NOS in regional circulatory beds. (184) This is a realistic possibility to explain the observation of low systemic vascular resistance and relative mean arterial pressure, despite high plasma ADMA levels, as has been described in decompensated cirrhosis. (165, 166) It is of interest that improvement in organ function following transplantation for ALF is associated with a rapid decline in ADMA levels, which this study shows to occur within the first hour after transplantation. This may suggest that the new graft is more capable of efficiently clearing ADMA since DDAH activity is likely to be preserved. Additionally, the decrease in ADMA may also result from a reduction in the inflammatory cytokine burden post transplantation, since removal of the necrotic failing liver has been shown to be associated with a sharp and sustained reduction in inflammatory cytokines. (185) These results also suggest that hepatic function and perfusion contribute to ADMA regulation as evidenced by an initial small increase in ADMA during the anhepatic phase of the transplant operation, consistent with complete absence of hepatic DDAH activity. Following restoration of portal and hepatic arterial flow with new graft placement, there was a significant reduction in ADMA levels, in an environment of reduced inflammatory drive.

The study in rats administered galactosamine to induce acute liver injury, and an additional group in which a further inflammatory insult was super-imposed in the form of LPS injection, allowed one to address whether inflammatory injury in

addition to that imposed by the primary insult (galactosamine) further elevates levels of dimethylarginines. The results of this study did suggest an additional effect on elevation of plasma ADMA by administration of LPS to the galactosamine injured model. However, when LPS was administered to sham animals, there was no significant effect on ADMA levels which may suggest that a 'priming' effect of the initial liver injury may be required to then manifest the effects of inflammation on the metabolism or/and synthesis of ADMA (Chapter 9). Furthermore, it has been suggested that there is a difference in the handling of dimethylarginines between humans and rodents. (186) In a study by Nijveldt and co-workers, injection of LPS into normal animals was also found not to precipitate a rise in plasma ADMA. (187) The explanation put forward by the authors was an increased uptake of the dimethylarginines through the  $\gamma^+$  cationic co-transporter system, induced by LPS.

Another interesting observation following the measurement of plasma arginine levels in this animal model study, was that administration of LPS to sham animals resulted in significantly reduced levels of plasma arginine. This reduction in plasma arginine was even more significant after administration of galactosamine suggesting that any NOS dependent NO generation might be further compromised by reduced availability of the substrate for this enzyme, after the liver insult. This coupled with the observed further elevation in ADMA (the competitive inhibitor for arginine) in galactosamine rats and galactosamine animals treated with LPS, would be likely to exert even greater deleterious effects on vascular modulation in systems that were dependent on NO generation to maintain vascular tone. (188, 189) It is also important to note that local generation of NO serves numerous functions in addition to maintaining vascular tone, including as an anti-oxidant, (190) a modulator of leukocyte adhesion (191) and an inhibitor of platelet aggregation. (192) Thus, high levels of ADMA may compound inflammatory injury in vascular beds during

progressive liver failure, through lack of NO availability and control of interaction with leukocytes and platelets.

Another important finding from this study was evidence for an association between inflammation and the development of organ failure, manifest as elevated creatinine in animals with galactosamine + LPS compared to galactosamine alone. These animals were also shown to have high SDMA levels. Association between SDMA and markers of renal function including creatinine have recently been identified, with SDMA proving to be a reliable marker of renal function. (193) Thus this study might suggest that SDMA, despite having no significant hepatic metabolism through DDAH (unlike the requirement by ADMA), may also be affected by superadded inflammatory injury in liver failure. The data from this study leads directly to the question of whether elevated dimethylarginines could have a significant biological effect in the progression of liver failure to multi-organ injury, and whether they could act as markers to assess disease progression, or/and in monitoring the effects of treatment responses.

## ***Chapter 9***

***Does elevated ADMA in liver disease relate to increased portal pressure and is it of biological importance?***

## 9.1 Background

Patients presenting with severe AH on the background of cirrhosis manifesting as an acute hepatic decompensation, can be incorporated within the recently described entity known as acute-on-chronic liver failure (ACLF). (194) In ACLF, the acute decompensation in liver function occurs secondary to precipitating events such as sepsis, upper gastrointestinal bleeding, ischemia or an additional superimposed liver injury such as AH. ACLF is characterised by multi-organ failure and a high risk of mortality, albeit with the potential for reversibility if the patient's condition can be supported adequately. The severity of portal hypertension is likely to be an important determinant of mortality in these patients through association with variceal bleeding, progressive renal failure and hepatic encephalopathy.

A potential link between changes in portal blood flow and poor outcome in severe AH has been suggested. (131) Furthermore, elevated portal pressures in patients with decompensated cirrhosis independently predict survival, (195) although the pathophysiological mechanisms behind these haemodynamic disturbances are incompletely defined. As previously discussed, factors modulating intrahepatic resistance, an important contributor to the severity of portal hypertension, include liver ultra structural changes secondary to cirrhosis in addition to variable components such as myo-fibroblastic stellate cell activity and vaso-active mediators such as NO. (196) Data from animal studies strongly support the notion that in portal hypertension, there is a deficiency of intrahepatic NO resulting from a reduction in the activity of eNOS. (42, 44) Hepatic NO delivery in these animal models reduces the severity of portal hypertension through a reduction in intrahepatic resistance. (197)



ADMA, an endogenous NOS inhibitor, is thought to be an important contributor to a detrimental reduction in NOS activity in disease, including effects on vascular tone. (142) The liver is an important site of ADMA metabolism, both in its production due to the high rate of protein turnover and more importantly, its elimination through the action of DDAH (167). Recent data suggests that patients with decompensated cirrhosis have higher ADMA levels compared to compensated disease, and that these levels increase further with evolving liver failure (166, 168). Inflammation may also increase ADMA levels and exacerbate vascular dysfunction by reducing NOS activity, as evidenced by studies in cultured endothelial cells exposed to TNF $\alpha$  or oxidised LDL, showing a reduction in DDAH activity and an increase in ADMA. (119)

To explore the potential biological role of the dimethylarginines further during the evolution from liver failure to multi-organ failure, a study was conducted to assess patients with chronic alcoholic liver disease in whom there was a progression to ACLF following a precipitating event, namely AH. The earlier data presented in this thesis had already suggested that these patients have significant systemic and regional haemodynamic disturbances associated with evidence of NO dysfunction, and a high incidence of progression to organ dysfunction. This study assessed whether: (i) ADMA and SDMA were increased in AH patients compared to patients with alcoholic cirrhosis alone; (ii) there was a relationship between hepatic ADMA concentrations and severity of portal hypertension and (iii) dimethylarginines could be useful as potential markers of clinical outcome in ACLF.

## **9.2 Methods**

Included consenting patients had evidence of acute decompensation of alcoholic liver disease (increasing ascites, pedal oedema or progressive jaundice), clinical and radiological evidence of cirrhosis, and a current history of alcohol abuse. Patients

were excluded if they were <18 or >75 years and had evidence of: additional/another aetiology of liver disease; severe cardiac dysfunction or renal failure (creatinine >150µmol/L); hepatic/extra-hepatic malignancy; hepatic encephalopathy ≥Grade 2 (precluding informed consent), and if there was microbiological evidence of infection after 72 hours of broad-spectrum antimicrobials.

Patients were studied at transjugular liver biopsy (days 1-3 of admission), performed to assess severity of liver dysfunction in those with ascites or/and coagulopathy. Classification to patients with decompensated cirrhosis with superadded AH at risk of ACLF (AH+C+) was made on subsequent histological examination using previously defined criteria,(198) in addition to a compatible alcohol history and clinical signs of decompensated cirrhosis. Patients received supportive therapy including nutrition and vitamin supplementation according to local guidelines. Local treatment protocols were initiated for development of complications including, sepsis (antimicrobials after culture); hepatorenal syndrome (terlipressin 0.5-2mg intravenously, up to six times daily with 60g salt-poor albumin); organ failure (full intensive care support including haemofiltration or/and ventilation, as indicated). No patient received immuno-modulatory treatment during the study.

**9.2.1 Measurements:** Haemodynamic studies were performed after overnight fasting and a one hour supine resting period, in sedated patients. HVPG was measured as described previously in section. 2.1.2.1 of Chapter 2.

**9.2.2 Sampling and Assays:** Blood was collected from an artery and hepatic vein in pre-cooled heparinized/ EDTA coated tubes. Routine haematology, liver biochemistry and CRP were measured. Plasma creatinine was assessed by mass spectrometry. (85) Additional plasma was separated by centrifugation and stored at –80° C for subsequent analysis of cytokines and ADMA. Hepatic tissue was either

formalin-fixed or snap-frozen in liquid nitrogen and stored at -80°C for subsequent assessment of ADMA concentration and PRMT 1 and DDAH II protein estimation. Blood samples drawn from a “control” group of 12 healthy volunteers (9 male), with no history of alcohol excess nor any past medical history, were used to evaluate “normal” ranges for ADMA and SDMA.

*9.2.2.1 Cytokines/chemokines:* TNF $\alpha$ , IL-6, IL-8 and soluble TNF receptors I and II (TNF-R1, TNF-R2) were measured using standard commercial assays (BioSource, Nivelles, Belgium) as described in Chapter 2, section 2.4.1. The lower limit of detection was 5pg/ml and the intra-assay coefficient of variation ranged from 4.5 % to 5.5%.

*9.2.2.2 ADMA levels:* Plasma ADMA was measured using fragmentation specific stable isotope dilution electrospray tandem mass spectrometry, as described in section 2.4.5 of the methods chapter. Within assay imprecision for ADMA was 2.1% at a concentration of 0.37 $\mu$ mol/l. Inter assay imprecision for ADMA was 7.4%, 5.8%, and 5.1% at concentrations of 0.39, 1.15, and 3.96  $\mu$ mol/l, respectively.

*9.2.2.3 DDAH and PRMT Western blot analysis:* Detailed methodology for Western blot assessment of DDAH II and PRMT 1 protein expression is provided in the Chapter 2, section 2.4.6.

## 9.3 Results

### 9.3.1 Patient clinical chemistry and inflammatory cytokine profile:

In this study, 52 patients were included with a mean Pugh score (87) for the group of  $10 \pm 0.3$ , and an elevated mean HVPG of  $19 \pm 1$  mmHg. The patients were the same cohort previously studied in detail in relation to characterisation of their inflammatory status, as described in Chapter 3. Twelve patients were excluded due to: viral hepatitis (7), severe renal dysfunction (3), lack of histological evidence of cirrhosis (1), and overwhelming sepsis in 1 patient. Using the histological criteria for defining alcoholic hepatitis listed in the methods above, the 52 included patients were then divided into those with alcoholic hepatitis and cirrhosis [AH+C+] (n=27), and those with alcoholic cirrhosis alone [AH-C+] (n=25). Table 9.1. Severity of necro-inflammation in AH+C+ biopsies was graded by applying a scoring system similar to that suggested for non-alcoholic steatohepatitis, (107) with all patients having moderate-severe grading agreed upon by 2 independent histopathologists. In addition to histological criteria, all AH+C+ patients had acute severe alcoholic hepatitis as evidenced by a mean discriminant function score of  $46 \pm 6$ , and reflected in the significantly elevated plasma bilirubin levels and prothrombin times compared to AH-C+ patients, Table 9.1. Mean serum creatinine was within normal reference ranges for both groups, albeit mean values were higher in AH+C+ patients,  $p=0.01$ . Twenty AH+C+ patients had moderate/severe ascites compared with ten AH-C+ patients;  $p=0.008$ . Twelve AH+C+ patients had grade 2 or more hepatic encephalopathy compared to four AH-C+ patients;  $p=0.02$ .

As has been outlined above in Chapter 3 and can be seen from Table 9.1 from the baseline characteristics, in this cohort of patients the inflammatory indices were significantly elevated in AH+C+ patients and they had significantly higher portal

pressures. Data for plasma IL-8 and IL-10 are not described despite being measured owing to marked variability in measurable levels which were largely below the assay detection limit in this cohort of patients.

**9. 3.2 Patient Outcome:** There were thirteen in-patient deaths in the fifty-two studied patients, twelve with AH+C+ and one with only cirrhosis. Of the AH+C+ deaths, eight developed renal failure, four of whom also had concomitant sepsis. Two patients died from uncontrolled GI bleeding, whilst two other patients failed to recover from spontaneous bacterial peritonitis with gram negative organisms, with development of multi-organ failure. (Table 9.2). Amongst the fifteen AH+C+ survivors successfully discharged from hospital within 28 days, six had evidence of infection during their admission, one with renal failure responded to supportive therapy and two had episodes of controlled variceal bleeding. AH+C+ patients who died had no significant difference in discriminant function ( $p=0.3$ ), Pugh ( $p=0.2$ ) or MELD ( $p=0.6$ ) scores compared with surviving AH+C+ patients. In the cirrhotic only (AH-C+) group, one in-patient died from renal failure despite supportive therapy. Of the remaining 24 AH-C+ patients successfully discharged from hospital, five had an episode of infection during their admission, and there was 1 controlled variceal bleed. AH-C+ patients had significantly lower Pugh ( $p<0.001$ ) and MELD ( $p<0.001$ ) scores than AH+C+ survivors ( $n=15$ ).

**Table 9.1:** Baseline clinical chemistry and inflammatory characteristics for all studied patients and their sub-classification into patients with cirrhosis and alcoholic hepatitis (AH+C+) and cirrhosis with no inflammation (AH-C+)

	<b>All Patients (n= 52)</b>	<b>Alcoholic Hepatitis (AH+C+, n=27)</b>		<b>Cirrhosis Only (AH- C+, n=25)</b>
<b>Age (years)</b>	50 [32-69]	47 [32-69]	ns	51 [38-67]
<b>Bilirubin (µmol/L)</b> [3-17]	169 ± 26	249 ± 39	***	56 ± 13
<b>Prothrombin (secs)</b> [10-12]	15.6 ± 0.7	18 ± 1	**	13.4 ± 0.6
<b>ALT (U/L)</b> [8-50]	55 ± 9	60 ± 15	ns	47 ± 9
<b>Creatinine (µmol/L)</b> [66-112]	76.5 ± 9.7	92.5 ± 11	*	59.8 ± 5
<b>Pugh Score</b>	10 ± 0.3	11.5 ± 0.3	***	8.4 ± 0.4
<b>MELD Score</b>	7.4 ± 1.7	15 ± 2.3	***	0.26 ± 1.4
<b>HVPG (mmHg)</b> [<5]	19 ± 1	22 ± 1.9	**	15.5 ± 1.6
<b>CRP (mg/L)</b> [0-5]	33.5 ± 6.5	50 ± 7	***	17 ± 3
<b>WBC (x10<sup>9</sup>/L)</b> [3-10]	10.2 ± 1.4	13.7 ± 1.5	***	6.4 ± 0.5
<b>SIRS</b> [0]	0.9 ± 0.2	1.6 ± 0.4	***	0.3 ± 0.1
<b>TNFα (pg/ml)</b> [0-5]	21 ± 11	46 ± 20**		4 ± 0.5
<b>TNFR1 (ng/ml)</b> [0-1.6]	726 ± 76	885 ± 93	**	455 ± 74
<b>TNFR2 (ng/ml)</b> [0-3.5]	1237 ± 141	1798 ± 128	***	759 ± 136
<b>IL-6 (pg/ml)</b> [0-5]	29.5 ± 9.6	55.7 ± 19	*	9.3 ± 2

\* p<0.01;\*\* p<0.001;\*\*\* p<0.0001;ns- no significant difference  
[Normal values]: Reference ranges for normal values in our laboratory

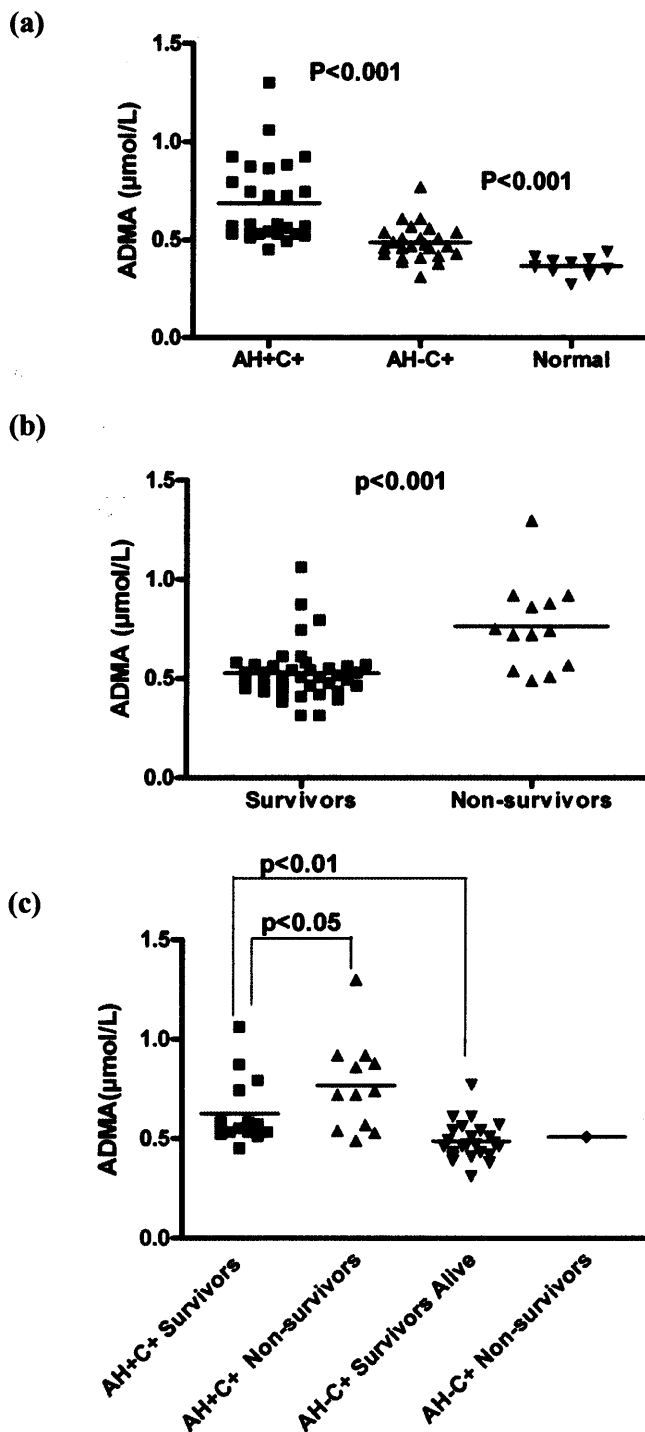
SIRS= Systemic Inflammatory Response Scores (temperature >38°C; a heart rate > 90 beats/minute; tachypnoea >20 breaths/minute; white blood cell count of >12 x 10<sup>9</sup>/L or < 4 x 10<sup>9</sup>/L or the presence of more than 10% immature neutrophils)  
(86)HVPG= Hepatic Venous Pressure Gradient

**Table 9.2** Outcome characteristics amongst studied patients

	<b>Alcoholic hepatitis Deaths (n=12)</b>	<b>Alcoholic hepatitis Discharged (n=15)</b>	<b>Cirrhosis Only Discharged (n=24)</b>
<b>Renal Failure</b>	8	1	0
<b>Gastrointestinal Bleed</b>	2	2	1
<b>Infection</b>	6 (2 with SBP)	6	5
<b>Pugh score</b>	11.9 ± 0.5	11.1 ± 0.4	8.4 ± 0.4 ***
<b>MELD</b>	16.2 ± 3.9	14.2 ± 3.1	0.7 ± 1.7 ***
<b>Discriminant Function</b>	53 ± 13	41 ± 6	-

**\*\*\* p<0.001 :Alcoholic cirrhosis compared with Alcoholic hepatitis patients discharged from hospital**

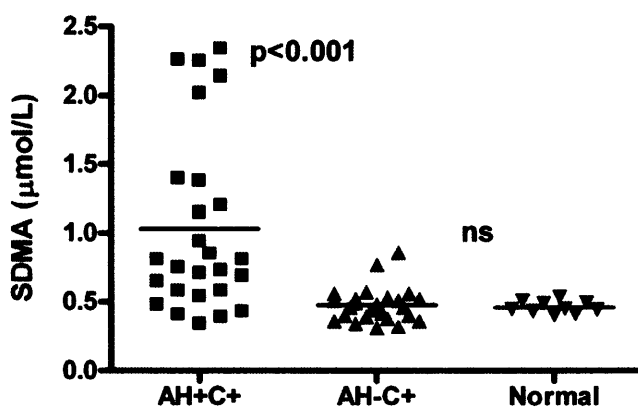
**9 3.3 ADMA:** Mean plasma ADMA levels were higher in the 52 patients compared to 12 healthy controls [age: 38(31-55); 9 male]:  $0.59 \pm 0.03$  vs.  $0.37 \pm 0.01 \mu\text{mol/L}$ ;  $p < 0.001$ . Furthermore, AH+C+ patients had significantly higher ADMA values compared to the AH-C+ group:  $0.69 \pm 0.04$  vs.  $0.49 \pm 0.02 \mu\text{mol/L}$ ;  $p < 0.001$ . Figure 9.1a. Hepatic tissue ADMA levels were also considerably higher in AH+C+ patients compared to AH-C+ ( $97 \pm 23$  vs.  $27 \pm 13 \mu\text{mol/mg protein}$ ,  $P < 0.05$ ), and correlated with portal pressure,  $r = 0.61$ ,  $p < 0.05$ . Plasma ADMA levels were significantly greater in patients that died during their admission [ $n=13$ ] compared with survivors [ $n=39$ ] ( $0.77 \pm 0.08$  vs.  $0.53 \pm 0.02 \mu\text{mol/L}$ ;  $p < 0.001$ ); Figure 9.1b. Further sub-analysis revealed significantly greater ADMA levels between discharged AH+C+ patients and non-inflamed AH-C+ patients ( $0.62 \pm 0.04$  vs.  $0.49 \pm 0.02 \mu\text{mol/L}$ ,  $p < 0.01$ ). Furthermore, ADMA levels were higher in AH+C+ patients that died compared to AH+C+ survivors,  $P < 0.05$ . Figure 9.1c.



**Figure 9.1** panel (a) shows that patients with cirrhosis and superadded alcoholic hepatitis (AH+C+) had significantly higher ADMA levels ( $p < 0.001$ ) compared to patients with cirrhosis alone (AH- C+). All cirrhotic patients had significantly higher ADMA levels compared with normal volunteers ( $p < 0.001$ ). (b) In-patient survivors with decompensated cirrhosis [n=39] had significantly lower ADMA levels than those that died [n=13];  $p < 0.001$ . (c) Surviving alcoholic hepatitis patients (AH+C+) had greater ADMA levels compared with the cirrhosis alone (AH- C+) survivors [n=24];  $p < 0.01$ . ADMA levels were significantly higher in AH+C+ patients that died [n=12] compared with AH+C+ survivors;  $p < 0.05$ )



**9.3.4 SDMA:** Mean plasma SDMA levels for the 52 patients although higher, were not significantly greater than in controls:  $0.77 \pm 0.08$  vs.  $0.46 \pm 0.01 \mu\text{mol/L}$ ,  $p=0.08$ . However, levels of SDMA were significantly higher in AH+C+ patients compared to AH-C+ ( $1.03 \pm 0.1$  vs.  $0.48 \pm 0.03 \mu\text{mol/L}$ ;  $p<0.001$ ). SDMA levels amongst AH-C+ patients were similar to healthy volunteers ( $p=0.67$ ) [Figure 9.2a]. Levels of SDMA were significantly higher in patients that died compared with all survivors, ( $1.6 \pm 0.35$  vs.  $0.6 \pm 0.05 \mu\text{mol/L}$ ;  $p<0.001$ ) [Figure 9.2b]. Further sub-analysis of AH+C+ patients demonstrated a significantly greater SDMA in patients that died compared with AH+C+ survivors, in whom the mean SDMA was  $0.8 \pm 0.1 \mu\text{mol/L}$ ;  $p<0.05$  [Figure 9.2c]. It is noteworthy that there was also a significant difference in SDMA between AH+C+ survivors and non-inflamed AH-C+ survivors in whom the mean SDMA level was  $0.48 \pm 0.03$ ;  $p<0.01$ .



**Figure 9.2a** Patients with alcoholic hepatitis (AH+C+) had significantly higher SDMA levels ( $p<0.001$ ) compared to cirrhosis alone (AH- C+) patients. There were no differences in SDMA levels between AH- C+ patients and healthy volunteers.

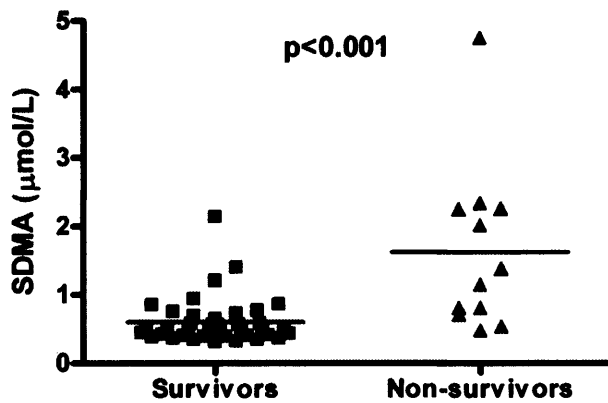


Figure 9.2b demonstrates that in-patient survivors with decompensated cirrhosis [n=39] had significantly lower SDMA levels than those that died [n=13];  $p < 0.001$

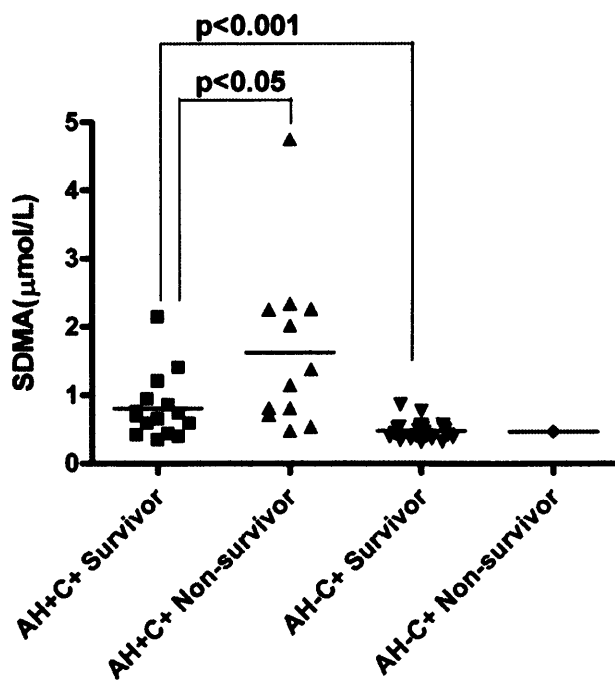


Figure 9.2c demonstrates the greater plasma SDMA levels in alcoholic hepatitis (AH+C+) patients that died [n=12] compared to those that survived [n=15];  $p < 0.01$ . AH+C+ survivors also had higher SDMA values compared with AH-C+ (non-inflammatory cirrhotic) survivors (n=24);  $p < 0.01$ .

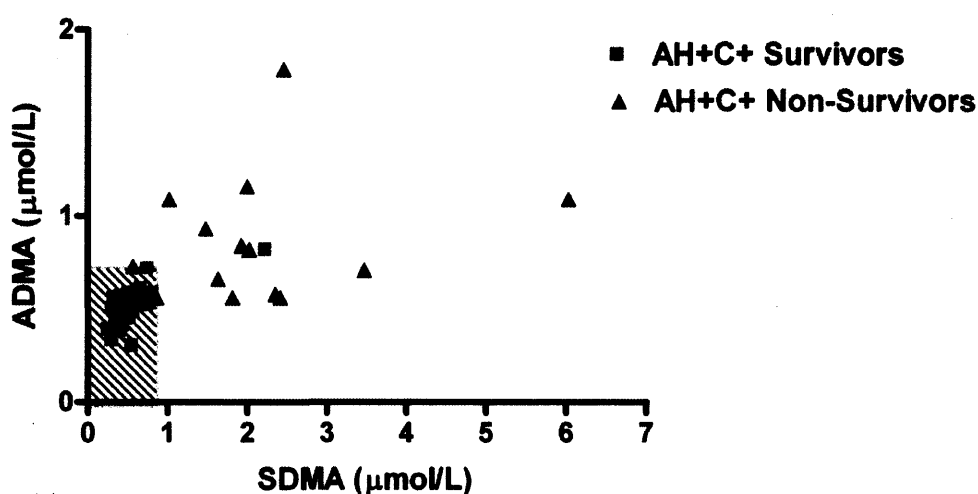
**9.3.5 Predictors of Survival:** Baseline measurements (day 0-3) of ADMA, SDMA, Pugh Score, calculated MELD and DF, were used to compare the predictive utility of these measures in determining in-patient survival, by area under the curve (AUC) for receiver operator curves (ROC). The AUC for each predictive measure for all patients, and a separate analysis for AH+C+ patients, is shown in the Table 9.3 below.

**Table 9.3** Comparison of prognostic scores with dimethylarginine levels to predict outcome in all studied alcoholic cirrhosis patients and in a subgroup with alcoholic hepatitis using receiver operator curves

Scoring system/ Dimethylarginine level	All Patients		Alcoholic Hepatitis (AH+C+)	
	Cut-off value (%Sensitivity; Specificity )	AU ROC	Cut-off value (%Sensitivity; Specificity)	AU ROC
<b>Pugh</b>	10.5 (63; 75)	0.81 ± 0.07	11.5 (60; 64)	0.65 ± 0.11
<b>DF</b>	36 (76; 73)	0.78 ± 0.08	39 (53; 70)	0.55 ± 0.13
<b>MELD</b>	10.3 (74; 73)	0.76 ± 0.08	18.9 (60; 40)	0.53 ± 0.12
<b>ADMA</b>	0.57 (74; 77)	0.83 ± 0.06	0.65 (73; 75)	0.74 ± 0.09
<b>SDMA</b>	0.7 (77; 85)	0.87 ± 0.06	0.89 (73; 67)	0.82 ± 0.08
<b>DAS</b>	1.23 (77; 92)	0.9 ± 0.05	1.52 (73; 83)	0.85 ± 0.07

This table clearly shows that when comparing sensitivities and specificities for each scoring system and their related AUROC, ADMA, SDMA and their summated score- DAS (see below) perform as well if not better than other currently applied scores in AH (MELD,DF, and Pugh).

Also of note, when ADMA was compared with SDMA in AH+C+ patients as shown in Figure 9.3, at the cut-off value for SDMA of 0.9 µmol/L derived from its ROC, all deaths predicted by using an ADMA cut-off 0.65 µmol/L were not accounted for (denoted by the shaded area), suggesting a potential benefit of utilizing the discriminatory benefit of both these dimethylarginines.

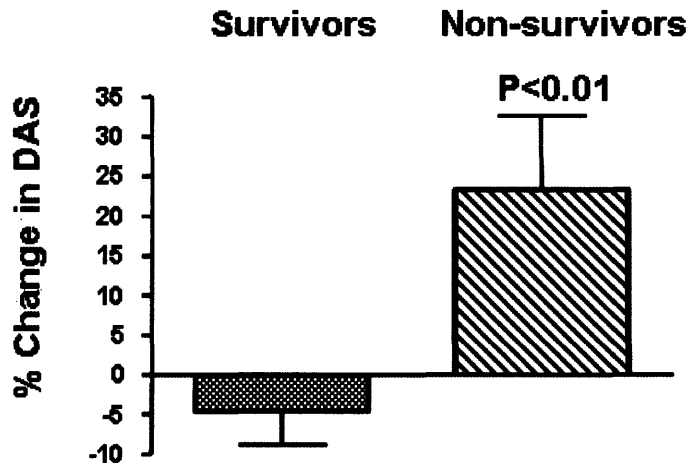


**Figure 9.3** A plot of ADMA vs. SDMA in alcoholic hepatitis (AH+C+) patients demonstrates that at the cut-off value for SDMA of 0.9  $\mu\text{mol/L}$  derived from receiver operator curves, a number of deaths as predicted by ADMA using a cut-off 0.65  $\mu\text{mol/L}$ , are not accounted for as denoted by the shaded area.

A simple mathematical summation of ADMA and SDMA was devised to obtain a combined 'dimethylarginine score'-DAS [DAS=ADMA+SDMA] in order to see if this would improve the predictive utility of an individual dimethylarginine level in determining survival. The calculated DAS was significantly higher in AH+C+ patients compared with AH-C+ ( $1.9 \pm 0.2$  vs.  $0.96 \pm 0.04$ ;  $p < 0.001$ ) Furthermore, the DAS value was significantly higher in non-surviving AH+C+ patients compared to survivors,  $2.5 \pm 0.38$  vs.  $1.4 \pm 0.16$ ;  $p < 0.01$ . It was interesting to note that DAS improved the specificity, whilst maintaining a high sensitivity of predicting outcome in all patients, and appeared at least as good in determining outcome as Pugh, DF or MELD scores, Table 9.3.

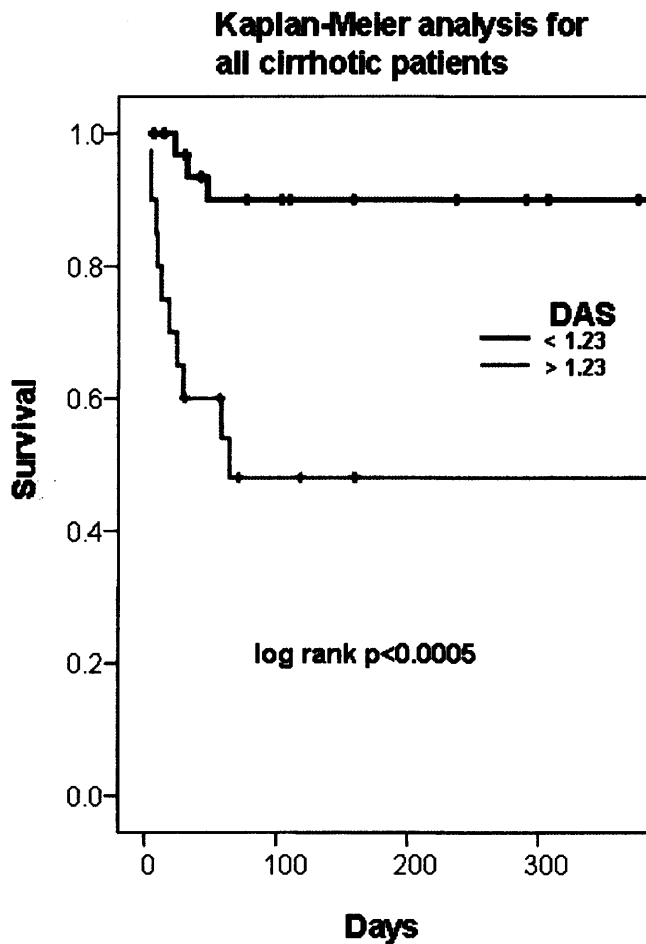
Repeat dimethylarginine measurements were made from blood taken during admission in the 12 AH+C+ patients that died compared to values from 18 surviving patients, including 6 AH+C+ patients. In patients that died, there was a significant

percentage increase in DAS ( $p < 0.01$ ), whilst a small decrease in DAS was noted in patients who survived and went on to be discharged from hospital, Figure 9.4.



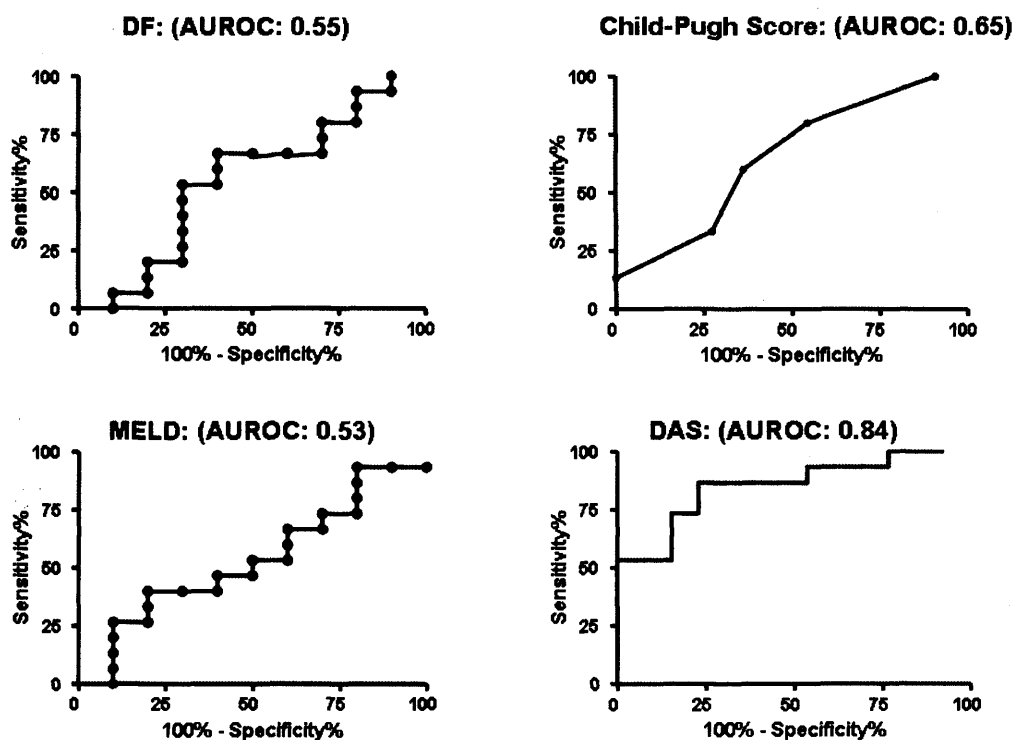
**Figure 9.4** The percentage increase in DAS [ADMA+SDMA] value in non-surviving alcoholic hepatitis patients [n=12] compared to values from 18 surviving patients, including 6 alcoholic hepatitis patients, in whom there was a net decrease in DAS value ( $p < 0.01$ ).

A Kaplan-Meier plot for all studied patients (Figure 9.5) demonstrates a significantly decreased in-hospital survival in patients with an admission DAS value  $> 1.23$ ; log rank of  $p < 0.0005$ . Thus patients with a higher DAS score have a significantly increased risk of in-hospital mortality.



**Figure 9.5.** A Kaplan-Meier survival analysis comparing in-hospital mortality in all studied alcoholic cirrhotic patients, based on admission DAS value < 1.23 or > 1.23 ( $p < 0.0005$ ). A higher DAS score significantly increases the risk of in-hospital mortality.

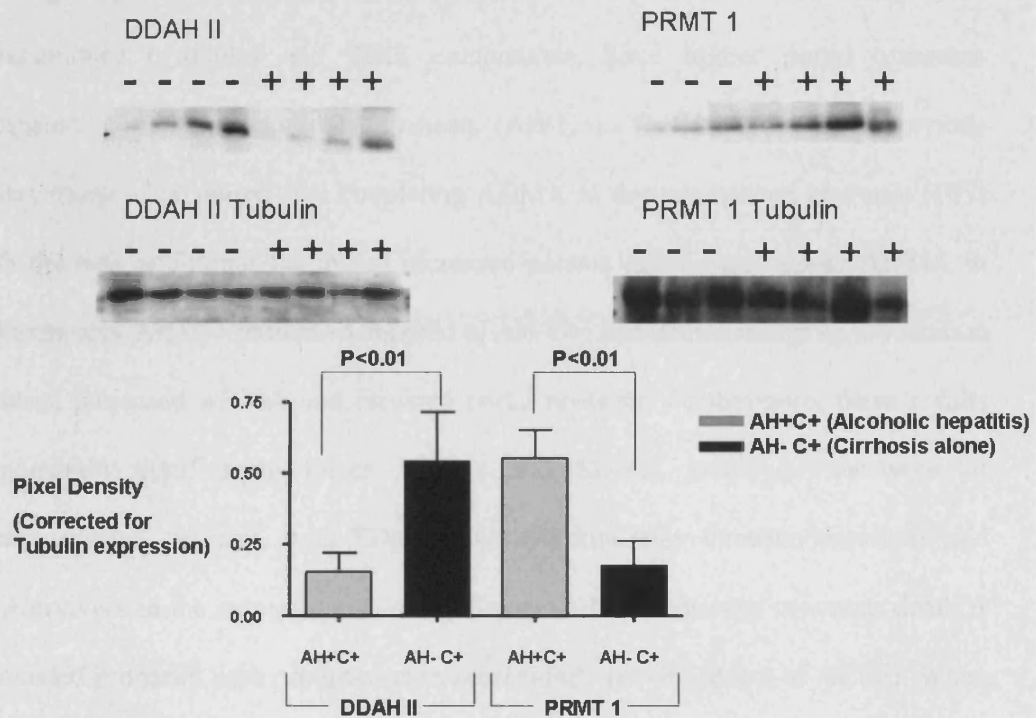
Furthermore, a DAS cut-off of 1.52 appeared to be a more useful predictor than all other scoring systems when evaluating survival in AH+C+ patients, as shown in Figure 9.6. From closer inspection of the area under the curve of the ROC curves for DF, Child-Pugh, MELD and DAS, shown below, it is evident that in the subgroup of AH+ C+ patients, the DAS value provides the most reliable predictor of mortality (AUROC = 0.84), using a DAS cut-off of 1.52. This provides a sensitivity of 73% and a specificity of 83%.



**Figure 9.6.** The Receiver Operator Curves (ROC) curves for discriminant function (DF), Child-Pugh, MELD and Dimethylarginine Score (DAS) are shown with the area under the curve (AUROC) listed. It is evident from these figures that in this group of alcoholic hepatitis patients, the DAS value provides the most reliable predictor of mortality (AUROC =0.84), using a DAS cut-off of 1.52; with a sensitivity of 73% and a specificity of 83%

**9.3.6 DDAH II and PRMT-1 expression:** Representative Western blots for dimethylarginine-dimethylaminohydrolase-II (DDAH-II) and protein-arginine-methyltransferase-1 (PRMT-1) expression in the studied AH+C+ and AH-C+ patients, together with their corresponding  $\alpha$ -Tubulin protein blots, to control for gel loading are shown in Figure 9.7. A quantification of DDAH II and PRMT-1 densities on these Western blots of all assessed patient biopsies is represented by the column bars having been corrected for  $\alpha$ -Tubulin expression. PRMT-1 data for 7 patients is shown due to limited homogenate extracted from 1 AH-C+ patient that was insufficient to allow for adequate gel loading. Hepatic DDAH II expression was

significantly lower ( $p < 0.01$ ) in the 4 AH+C+ patients in whom it was measured, as compared with 4 AH-C+ patients. In contrast, expression of PRMT-1 was significantly increased in AH+C+ patient livers' ( $p < 0.01$ ).



**Figure 9.7** Quantification of DDAH II and PRMT-1 densities on Western blots of all assessed patient biopsies is represented in the column bars for alcoholic hepatitis (AH+C+) and cirrhosis alone (AH-C+) patients, having been corrected for  $\alpha$ -Tubulin expression. AH+C+ patients have significantly lower DDAH II expression ( $p < 0.01$ ) and significantly higher PRMT-1 ( $p < 0.01$ ) expression



#### **9.4 Discussion**

This study confirms that patients with alcoholic cirrhosis with superimposed histologically proven inflammatory alcoholic hepatitis (AH+C+) and high pro-inflammatory cytokines and SIRS components, have higher portal pressures compared to patients with only cirrhosis (AH-C+). This study extends previous observations of increased free circulating ADMA in decompensated cirrhosis (165) with the new additional finding of increased plasma and hepatic tissue ADMA in inflammatory AH+C+ patients compared to AH-C+, and demonstrates an association between increased ADMA and elevated portal pressure. Furthermore, these results demonstrate significantly lower ADMA and SDMA levels in survivors of decompensated cirrhosis, with SDMA also discriminating between survivors and non-survivors in the sub-group of inflammatory AH+C+ patients, in whom death is associated primarily with progression to renal failure (development of ACLF). When current validated scoring systems (Child-Pugh, DF and MELD) used to determine in-patient mortality in alcoholic hepatitis or following intervention (terlipressin and albumin for deteriorating renal function or haemofiltration) were applied to the described cohort of patients, their predictive utility was limited. It is possible that the limited numbers of patients studied may have reduced the predictive utility of these scores compared with the published literature. However, it is important to note that the MELD score is dependant on measured creatinine and INR both of which will vary between laboratories and are subject to significant error in this group of patients. This is reflected by the variability in the cut-off values for MELD described in the literature, for predicting outcome in AH. (199-201). Similarly, DF has a limited range of predictive use and it is likely that the clinical variables associated with Pugh score in our patients, who were studied by the same observers were more

internally consistent. Notwithstanding this, circulating levels of ADMA, SDMA or their sum DAS, demonstrated a greater utility in predicting those patients that were at risk of dying.

There is general agreement in the literature that increased intrahepatic resistance in cirrhosis is contributed to by reduced local NO availability, secondary to decreased hepatic eNOS activity. (42, 44) ADMA is a competitive, endogenous inhibitor of eNOS produced during proteolysis, (142) and it has been suggested that the liver is an important site of ADMA metabolism. (167) Elevated ADMA has been shown to be associated with endothelial dysfunction in numerous conditions (202), and its increased urinary excretion described in patients with chronic active hepatitis. (165) Moreover, ADMA has been found to be elevated in end-stage cirrhotics compared to healthy controls and in liver failure patients prior to orthotopic liver transplantation, the levels falling significantly, post transplantation (168) These studies imply that with progression of liver disease and portal hypertension ADMA rises, and following liver transplantation, there is a rapid decline suggesting a correction of the derangement in ADMA metabolism associated with reduced inflammatory indices. (185) This study demonstrates significantly increased plasma and hepatic tissue levels of ADMA in AH+C+ patients compared to AH-C+ patients. The effects of inflammation appear to be above and beyond those expected for decompensated alcoholic cirrhosis alone (166). These data would support the hypothesis that following an increase in hepatic inflammatory mediators, there is an alteration in hepatic ADMA metabolism or/and generation.

ADMA homeostasis is maintained by its metabolism through the enzyme DDAH, which is particularly abundant in the liver and kidney. (203) DDAH activity has been shown to be reduced by inflammatory stimuli such as TNF $\alpha$ .(119) It follows

that when levels of TNF $\alpha$  are markedly increased, as seen in AH+C+ patients, one might expect higher liver ADMA levels through impaired metabolism, and this in turn may decrease local hepatic NOS activity. This assertion is supported in part by the observation of increased hepatic tissue ADMA coupled with decreased liver DDAH II protein expression (the most abundant DDAH subtype found in the liver) in AH+C+ patients in this study, in whom HVPG was significantly increased.

An alternative explanation for the findings in this study of increased ADMA in AH+C+ patients might be through increased ADMA production. ADMA is generated by the action of PRMTs, a family of enzymes that methylate the side-chain nitrogen of arginine within proteins(204) with the subsequent catabolic release of the resulting methylated arginine residues. Several types of PRMT have been described with Type I being responsible for the generation of N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) and ADMA, and Type II, L-NMMA and SDMA. Upon proteolysis, which is increased in hyper metabolic states such as cirrhosis and inflammation, (205) significant amounts of ADMA are generated (206). The rate of ADMA and SDMA generation is thus likely to be dependent on the presence and activity of PRMTs and the rate of protein breakdown. In keeping with this, this study demonstrates an increase in expression of PRMT-1 in AH+C+ patients in whom there is increased inflammation, compared to AH- C+ patients. This finding is further supported by data from studies on human endothelial cells exposed to oxidised LDL-cholesterol, in which up-regulated ADMA release is associated with increased PRMT expression (118).

Although this study was neither powered nor specifically designed to look at the role of the dimethylarginines in determining survival, one of the most important findings to emerge from the study was the association of elevated ADMA and SDMA levels

with in-hospital mortality for the whole group of alcoholic cirrhotic patients (25%) and specifically, in AH+C+ patients, in whom the mortality was 44%, in keeping with previous published series. (207) Current outcome predictive scores in alcoholic hepatitis focus on biochemical (bilirubin, prothrombin time and creatinine) and clinical variables (degree of ascites and grade of encephalopathy) but do not specifically factor in the changes in pro-inflammatory state or haemodynamic disturbances associated with this condition. (89, 200)

This study has identified the potential use of dimethylarginine levels as measurable biological markers (with minimal inter-assay variability), that may predict early in-patient mortality with a better sensitivity (73%) and specificity (83%) than currently used prognostic scores. The data presented here also suggests a further temporal increase in dimethylarginine levels in those with poor outcome but firm conclusions on the discriminatory value of repeated measurements are limited by the small number of patients that have been studied. It is possible that ADMA levels partly reflect the inflammatory state in AH in which vascular dysfunction occurs, including within the renal circulatory bed, heralding the onset of organ failure, and thus progression to ACLF. Increasing ADMA has been associated with a decreased effective renal plasma flow and increased renovascular resistance, (208) which would lead to increased retention of SDMA. Indeed, SDMA has recently been shown to be elevated in hepatorenal syndrome patients compared to patients with cirrhosis without renal failure. (209) A combined dimethylarginine (DAS) score thus factors in both inflammation and incipient renal dysfunction (the main cause of mortality in the AH+C+ patient cohort described in this study) and might explain the better predictive utility of the DAS score identified in this study.

## ***Chapter 10***

### ***Other regulators that may account for a decrease in hepatic eNOS activity***

## 10.1 Introduction

There is an increasing body of literature in cirrhotic animals suggesting a reduction in the activity of hepatic endothelial nitric oxide synthase (eNOS), (42, 44) and this is despite apparently normal hepatic eNOS protein expression.(43) The resultant reduction in hepatic nitric oxide (NO) generation contributes to increased intrahepatic resistance and elevated portal pressure. Indeed, a reduction in intrahepatic resistance, and thereby severity of portal hypertension, through the delivery of NO to the liver in model systems, either pharmacologically, (59) or through gene transfer techniques,(57, 58) highlights the importance of hepatic NO generation in the pathophysiology of portal hypertension.

Studies in relation to decreased hepatic eNOS activity have, to date, focussed on inhibitors of eNOS activity such as caveolin-1(43, 210) and ADMA (162), or on processes affecting the post-translational modification of eNOS. (58) Recently, a more complex subcellular regulation of NO generation has been described through interaction of eNOS with specific proteins involved with trafficking within the cell. One such protein, eNOS traffic-inducing protein (NOSTRIN), was first identified by Prof Muller-Esterl's lab in a yeast two-hybrid approach. (56) Subsequent work by this group has demonstrated an interaction and co-localisation between eNOS and NOSTRIN in cultured vascular endothelial cells and eNOS-expressing cell lines, and that NOSTRIN over-expression results in a redistribution of eNOS into intracellular vesicle-like structures whilst significantly inhibiting NO release. (56) Similarly, other eNOS interacting proteins such as NOSIP may be instrumental in displacing eNOS from plasma membrane caveolae with a consequent inhibition of NO generation. (55)

The principal aim of this study was to determine whether the greater severity of portal hypertension previously described in this thesis in patients with inflammatory alcoholic hepatitis compared to patients with cirrhosis alone, was associated with differences in gene expression for the NOS-regulatory proteins caveolin-1, NOSIP, and NOSTRIN.

## **10.2 Methods**

**10.2.1 Patients:** Patients were included in this study if they had a clearly documented history of alcohol consumption of greater than >80 g of alcohol/day for men and >60 g/day for women up until the time of admission. Admission to hospital was precipitated by decompensation, defined by the development of progressive jaundice, derangement of coagulation and liver function tests, or/and the development or worsening of ascites. All eligible patients underwent transjugular liver biopsy and portal pressure assessment as clinically indicated, and a sample of liver biopsy tissue was snap frozen in liquid nitrogen for further evaluation, in accordance with given informed consent and approval by the local ethics committee. Patients were excluded if they were <18 or >75 years and had any of the following: renal dysfunction (plasma creatinine >130  $\mu\text{mol/L}$ ), severe cardiovascular or cerebrovascular disease, active or latent infection, hepatic or extra-hepatic malignancy and treatment with vasoactive agents or corticosteroid therapy in the prior month.

Liver tissue was assessed by an independent histopathologist for evidence of alcoholic cirrhosis, and further criteria applied (balloon degeneration of hepatocytes; Mallory bodies; neutrophilic infiltrates and apoptotic acidophilic bodies) to define a sub-group of patients with alcoholic hepatitis. (198) Liver tissue was also obtained

from 6 additional patients undergoing hepatic resection for metastatic disease of a colonic primary lesion, who had no significant history of alcohol intake, and no evidence of liver disease or derangement of liver function tests. Histological inclusion criteria of the 6 selected specimens included a clear resection margin with no evidence of cellular atypia and no evidence of cirrhosis.

**10.2.2 Hepatic portal venous pressure measurements:** The methods applied for assessment of portal pressure are detailed in section 2.1.2.1 of Chapter 2.

**10.2.3 mRNA and protein expression assays:** The methodology for Quantitative real-time PCR and Immunohistochemistry and Western blotting are detailed in the Methods in Chapter 2, sections: 2.4.7.1-3.

**10.2.4 Cell culture and transfection:** Human hepatocellular carcinoma (Hep3B) cells were cultured in DMEM/10% FCS with or without 1  $\mu$ M retinoic acid for up to 6 days. Chinese hamster ovary (CHO) cells stably expressing eNOS (CHO-eNOS) were cultured in DMEM/10% FCS containing 200nM methotrexate. Transient transfections with pcDNA-3.1B-NOSTRIN $\alpha$  and pcDNA-3.1B-NOSTRIN $\beta$  were performed with Nanofectin (PAA, Coelbe, Germany) according to the manufacturer's instructions. Cells on coverslips were fixed in methanol 20 h post-transfection, treated with monoclonal anti-NOSTRIN, and analysed by confocal laser-scanning microscopy.

**10.2.5 Co-immunoprecipitation:** Human liver was frozen in liquid nitrogen, ground and lysed in OG buffer (50 mM Tris HCl, pH 7.4; 2 mM EDTA; 1 mM EGTA; 50 mM NaF; 150 mM NaCl; 1 mM Na<sub>3</sub>VO<sub>4</sub>; 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>; 1 mM DTT; 60 mM N-octylglycoside; protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)). Co-immunoprecipitation was performed using polyclonal rabbit anti-NOSTRIN (AS532). Western blots of co-immunoprecipitates and lysates were



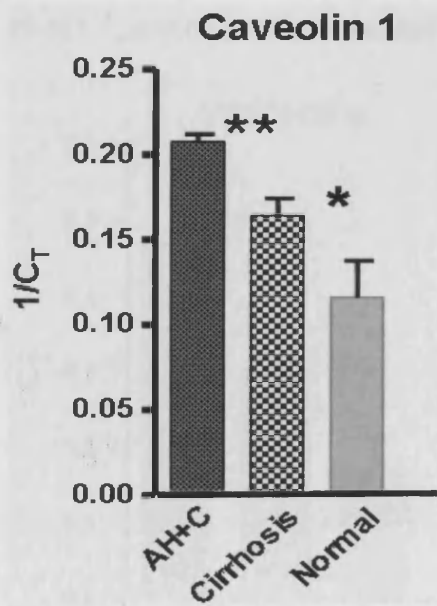
probed with mouse anti-eNOS, anti-caveolin-1 (Transduction Laboratories, Lexington, USA) and anti-NOSTRIN (see above).

### **10.3 Results**

#### ***10.3.1 Characterisation of Caveolin-1 and NOSIP:***

Biopsy specimens from 20 decompensated alcoholic cirrhotic patients were included, with 10 of these fulfilling histological and clinical criteria for alcoholic hepatitis superimposed on cirrhosis. All the alcoholic hepatitis patients had a moderate to severe grading of necro-inflammation histologically and as was previously observed, these patients had significantly higher inflammatory indices compared to patients with alcoholic cirrhosis alone. By quantitative RT-PCR, caveolin-1 mRNA expression levels were significantly higher in alcoholic hepatitis patients' livers compared to cirrhotic livers without inflammation ( $4.6 \pm 0.3$  vs.  $6.3 \pm 0.4$ ,  $p=0.007$ ), and when compared to normal liver ( $9.7 \pm 2.1$ ,  $p=0.02$ ). The expression of mRNA is denoted as relative expression  $1/\text{Cycle Threshold}$  ( $1/C_T$ ) in Figure 10.1 below. Given limitations in available material from a transjugular liver biopsy, it was difficult to obtain quantitative protein characterisation using techniques such as Western blotting for Caveolin-1 expression in the biopsy tissue. However, there is historical and accepted evidence in the literature for increased Caveolin-1 expression in human cirrhosis (211) and in animal models of alcoholic (212) and bile duct ligated cirrhosis (43)

Of note, there were no significant differences observed in NOSIP mRNA expression levels between the alcoholic patient groups (with and without additional inflammatory alcoholic hepatitis) and resection specimens with histologically normal liver tissue ( $p=0.3$  and  $0.5$ , respectively).

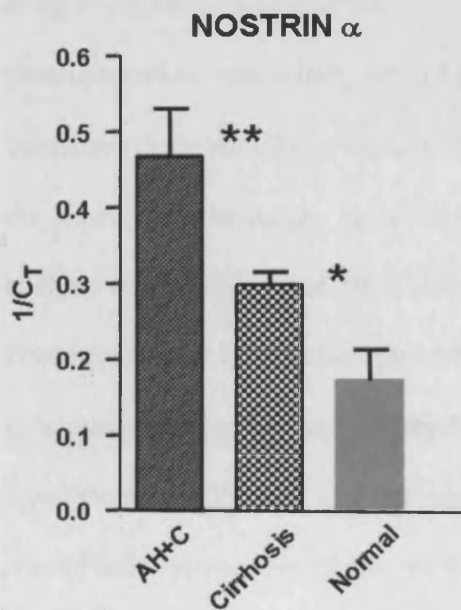


**Figure 10.1** The mRNA expression (represented as  $1/C_T$ ) for Caveolin-1 was significantly higher in alcoholic hepatitis (AH+C) patients compared to patients with alcoholic cirrhosis alone ( $p < 0.01$ ). No significant difference was noted in Caveolin-1 mRNA between cirrhosis and histologically normal biopsies.

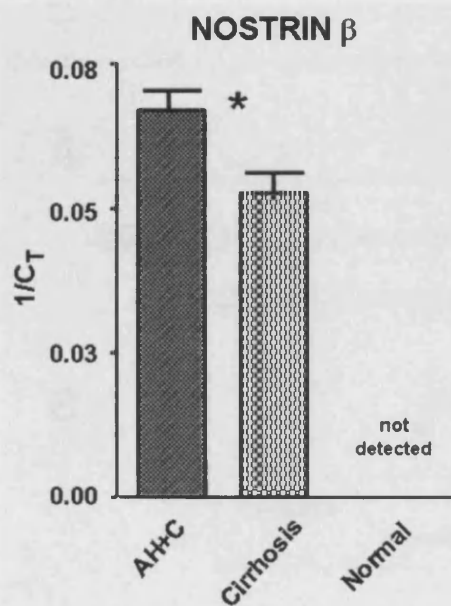
### 10.3.2 Characterisation of NOSTRIN

Quantification of mRNA for NOSTRIN expression revealed significantly higher levels in liver samples of alcoholic hepatitis patients compared to those with only alcoholic cirrhosis ( $2.3 \pm 0.3$  vs.  $3.4 \pm 0.2$ ,  $p = 0.009$ ; Figure 10.2A). Liver samples of alcoholic cirrhosis patients also had significantly higher NOSTRIN mRNA expression levels compared to normal tissue, ( $6.8 \pm 2$ ,  $p = 0.04$ ). Furthermore, a second mRNA species hybridising with NOSTRIN-specific probes was discovered, representing a novel isoform of NOSTRIN that lacks exon-2. The shortened isoform was designated NOSTRIN $\beta$  whereas the parent full-length NOSTRIN was referred to as NOSTRIN $\alpha$ . Importantly, NOSTRIN $\beta$  mRNA was significantly elevated in alcoholic hepatitis patients as compared to those without inflammation ( $15.1 \pm 0.7$  vs.

19.6±1.7, p=0.04), while it was not detected at all in normal tissue (Figure 10.2.B).



**Figure 10.2A:** NOSTRIN $\alpha$  mRNA levels were significantly higher in alcoholic hepatitis (AH+C) patients compared with alcoholic cirrhosis alone (p<0.01); cirrhotic specimens had higher NOSTRIN $\alpha$  mRNA levels compared to normal liver specimens (p<0.05).

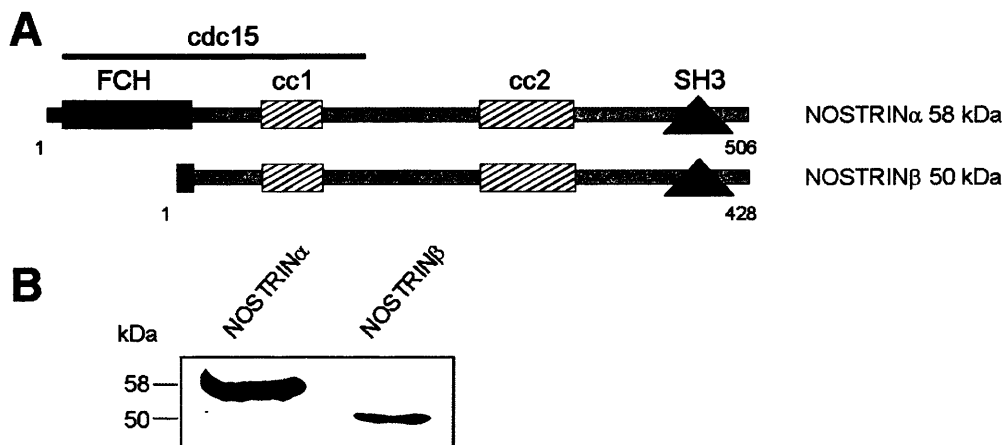


**Figure 10.2B:** NOSTRIN $\beta$  mRNA levels were significantly higher in alcoholic hepatitis (AH+C) patients compared to those with cirrhosis alone (p<0.01); NOSTRIN $\beta$  mRNA was undetectable in normal liver tissue.

Sequence analysis of the NOSTRIN $\beta$  cDNA revealed that skipping of exon-2 generates a premature stop codon in exon-3 such that an internal methionine codon present in exon-4 is used for alternative transcription initiation at the NOSTRIN $\beta$

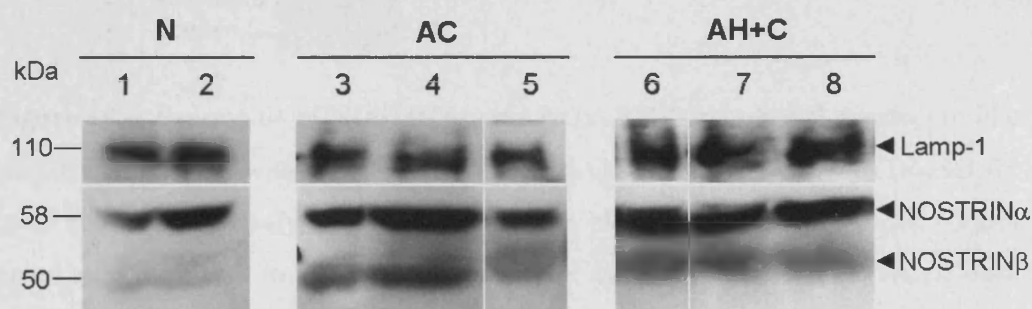
mRNA. This results in a shortened isoform lacking the first 78 amino acid residues at the N-terminus of NOSTRIN.

In collaboration with colleagues in Frankfurt, experiments were devised in order to determine the potential relevance of NOSTRIN $\alpha$  and NOSTRIN $\beta$ 's association with the vascular endothelium in relation to physiology/patho-physiology. Firstly, to analyse for expression of NOSTRIN protein, Western blots were performed on lysates of human liver tissue specimens with a monoclonal antibody binding to the C-terminal portion that is shared by NOSTRIN $\alpha$  and  $\beta$  (Figure 10.3A). On Western blots, the two NOSTRIN isoforms clearly differed by their size, as demonstrated by recombinant expression of the proteins in Chinese Hamster Ovary (CHO) cells. NOSTRIN $\alpha$  was detected at 58 kDa, and NOSTRIN $\beta$  at 50 kDa (Figure 10.3B), and thus they could be easily distinguished from each other.



**Figure 10.3** (A) NOSTRIN $\alpha$  displays a characteristic domain structure comprising an N-terminal Fes/CIP homology (FCH) domain, two coiled-coil domains (cc1 and cc2), and a C-terminal Src homology (SH3) domain. NOSTRIN $\beta$  lacks the N-terminal 78 amino acid residues and thus most of the FCH domain present in NOSTRIN $\alpha$ , (B) In Western blotting, recombinant expression of NOSTRIN $\alpha$  and NOSTRIN $\beta$  in Chinese Hamster Ovary (CHO) cells show molecular masses of 58 and 50 kDa, respectively.

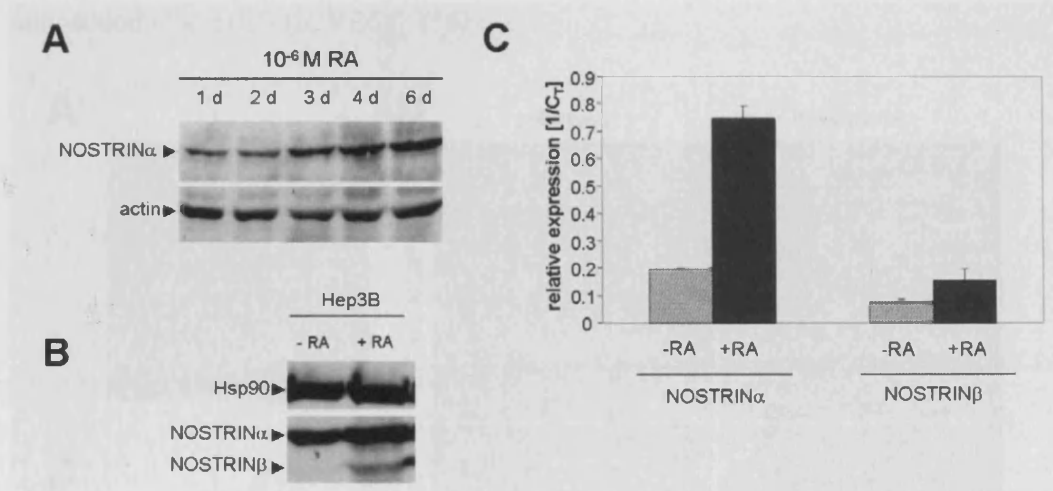
In the liver samples, NOSTRIN $\alpha$  protein expression was readily detectable at the expected size in all patient samples analysed (Figure. 10.3C). NOSTRIN $\beta$  was present in all alcoholic liver disease patient biopsies but was not found to be expressed in normal hepatic tissue. Due to the strict size limitation of the available trans-jugular biopsy specimens, it was not possible to perform quantitative protein analyses. On the Western blots, the protein levels of Lamp-1 (lysosome associated membrane protein-1) were used to ensure that roughly equal amounts of total protein were analysed (Figure 10.3C).



**Figure 10.3C** Western blotting analysis of human liver samples for expression of NOSTRIN $\alpha$  and NOSTRIN $\beta$ . To estimate total protein loading, Lamp-1 is shown as a marker. NOSTRIN $\alpha$  was detected in all liver samples analysed, whereas expression of NOSTRIN $\beta$  was restricted to patients with AC (alcoholic cirrhosis alone and no inflammation) or AH+C (alcoholic hepatitis), and was not seen in normal liver tissue (N).

Study of hepatic (Hep3B) cell lines demonstrated endogenous NOSTRIN $\alpha$  but not NOSTRIN $\beta$  was present. (Figure 10.4A). Moreover, NOSTRIN expression was shown to increase in Hep3B cells with the addition of retinoic acid (RA) in a time-dependent manner (Figure 10.4A). After six days of RA treatment, low levels of a 50 kDa band were detected by the monoclonal NOSTRIN antibody, fitting the molecular weight of NOSTRIN $\beta$  (Figure 10.4B). In line with this finding, elevated

mRNA levels were found for both NOSTRIN isoforms in RA-stimulated vs. non-stimulated Hep3B cells using qRT-PCR (Figure 10.4C).

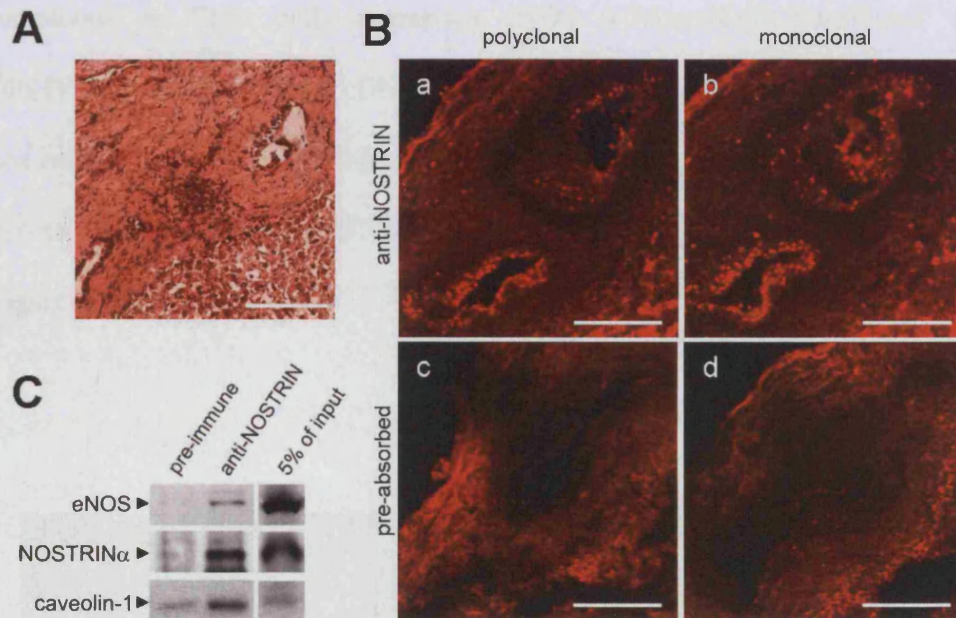


**Figure 10.4.** Expression of NOSTRIN $\alpha$  and NOSTRIN $\beta$  in cultured hepatocyte-like Hep3B cells treated with 1  $\mu$ M retinoic acid (RA) or mock-treated with DMSO for up to six days, and analysed by (A, B) Western blotting and (C) qRT-PCR. Values given are normalized to ubiquitin C and shown as relative expression (1/C<sub>T</sub>). Both experimental methods show a clear up-regulation of NOSTRIN $\alpha$  and NOSTRIN $\beta$  expression after RA treatment. Note that (B) shows a blot after prolonged exposure.

### 10.3.3 Interaction partners of NOSTRIN

Immunohistochemistry performed on human liver tissue sections demonstrated a close association between NOSTRIN and hepatic vascular endothelium, with a cuff of positive staining around vascular structures (Figure 10.5A and B). This staining pattern was lost following pre-absorption of the NOSTRIN antibody with GST-NOSTRIN. Importantly, identical observations were made with both monoclonal (Figure 10.5B, a+c) and polyclonal anti-NOSTRIN (Figure 10.5B, b+d). This finding suggested that NOSTRIN and eNOS may indeed interact in liver endothelium. To confirm this observation, a co-immunoprecipitation was performed on human liver lysates and probed with mouse anti-eNOS, anti-NOSTRIN and anti-caveolin-1.

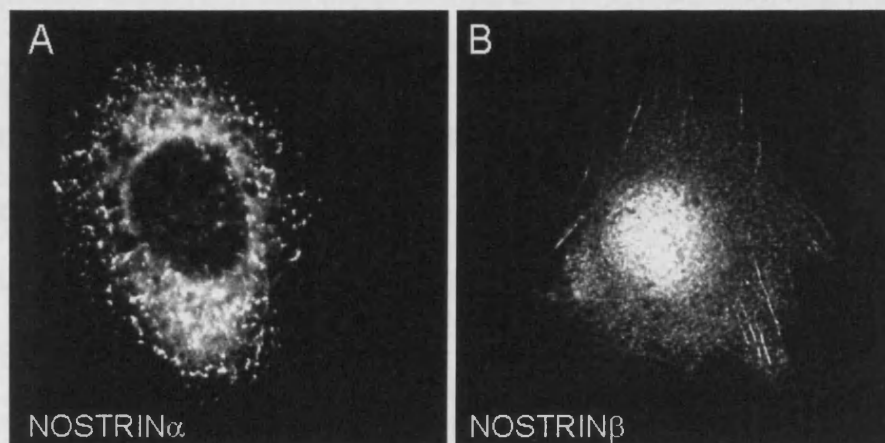
eNOS and caveolin-1 were shown to co-precipitate with NOSTRIN but not with pre-immune serum (Figure 10.5C), as has previously been observed in human umbilical vein endothelial cells (HUVECs). (56)



**Figure 10.5** NOSTRIN localisation and its interaction partners in liver tissue (**A**, **B**) These panels show liver tissue with H+E-staining (**A**) or immunohistochemistry (**B**); Polyclonal (**a** + **c**) and monoclonal (**b** + **d**) anti-NOSTRIN was used for staining. The figure demonstrates a close association between NOSTRIN and hepatic vascular endothelium, with a cuff of positive staining (monoclonal and polyclonal) around vascular structures. This staining pattern is lost after pre-absorption of the NOSTRIN antibody with GST-NOSTRIN, used as a control. (Bars: 50  $\mu$ M), (**C**) Co-immunoprecipitation was performed using rabbit anti-NOSTRIN (AS532). Western blots of co-immunoprecipitates and human liver lysates were probed with mouse anti-eNOS, anti-NOSTRIN and anti-caveolin-1. eNOS and caveolin-1 co-immunoprecipitate with NOSTRIN from total liver lysates, but not with pre-immune serum.

#### 10.3.4 Comparison of NOSTRIN $\alpha$ and $\beta$ isoforms

To analyse NOSTRIN $\beta$  more closely, the localisation of the recombinantly expressed protein was determined using immuno-fluorescent labelling and confocal microscopy in CHO cells expressing eNOS (CHO-eNOS) transfected with NOSTRIN  $\alpha$  or NOSTRIN  $\beta$  cDNA, by our collaborators in Frankfurt. NOSTRIN $\alpha$  localised to vesicular and tubular membrane structures throughout the cytoplasm (Figure 10.6A), whereas NOSTRIN $\beta$  displayed a pre-dominantly nuclear staining (Figure 10.6B).



**Figure 10.6** Subcellular localisation of NOSTRIN $\alpha$  and NOSTRIN $\beta$ : Chinese Hamster Ovary (CHO)-eNOS cells were transfected with NOSTRIN $\alpha$ - (A) or NOSTRIN $\beta$ -cDNA- (B), fixed with methanol and analysed by immunofluorescence. The two isoforms clearly differ in their subcellular localisation; NOSTRIN $\alpha$  primarily cytoplasmic and NOSTRIN $\beta$  nuclear.



#### **10.4 Discussion:**

The results from these elements of study show that expression of several eNOS regulatory proteins become altered in cirrhosis. Initially, NOSIP, an intracellular regulator of eNOS, was tested to evaluate any alterations in its expression pattern but no significant differences in mRNA expression levels were found between the cirrhotic and non-cirrhotic patient groups. In contrast, it could be shown that NOSTRIN $\alpha$  (the full-length parent protein) was expressed in normal liver and in a hepatocyte cell line (Hep 3B), and through immunohistochemistry on patient liver biopsies, found to be associated with vascular structures suggesting interactions with vascular endothelium. It was also possible to demonstrate that NOSTRIN $\alpha$  expression was significantly higher in cirrhotic liver compared with the normal liver indicating that NOSTRIN $\alpha$  expression was likely to be subject to regulation under pathological conditions. Furthermore, alcoholic hepatitis patients demonstrated even higher levels of NOSTRIN $\alpha$  mRNA compared to patients with cirrhosis alone, whilst also having the lowest eNOS activity and higher portal pressures, as determined by our earlier studies. At present the mechanism by which NOSTRIN $\alpha$  is up-regulated in cirrhosis and further increased during inflammation remains unclear. However, the association between inflammation and increased expression of NOSTRIN $\alpha$  may suggest a potential role for NOSTRIN $\alpha$  in regulating eNOS activity during hepatic inflammation, impacting on the severity of portal hypertension. It was also possible to confirm previous observations of a potential role for caveolin-1 in regulating the activity of hepatic eNOS, (43) by demonstrating increased caveolin-1 expression in patients with cirrhosis.

Full-length NOSTRIN has been demonstrated to physically interact with eNOS via its C-terminal SH3 domain. (56) Over-expression of NOSTRIN $\alpha$  in CHO-eNOS

cells results in a significant decrease in eNOS activity, in conjunction with a redistribution of eNOS from the plasma membrane to intracellular sites. (56) Further studies have revealed that during internalisation of eNOS, NOSTRIN $\alpha$  serves as a homo-trimeric platform to recruit proteins like dynamin and Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) which play a crucial role in endocytic processes. (213) Moreover, NOSTRIN $\alpha$  can directly bind to caveolin-1 independent of its interaction with eNOS, and the interaction between NOSTRIN $\alpha$  and eNOS is significantly increased in the presence of the caveolin-1 scaffolding domain. (214) Thus, enhanced interaction between NOSTRIN $\alpha$ , eNOS and caveolin-1 may silence eNOS activity during subcellular transport. (214) In this study, an interaction between eNOS, NOSTRIN and caveolin-1 was confirmed in a co-immunoprecipitation assay from lysed human liver specimens. Furthermore, immunohistochemistry clearly demonstrated association of NOSTRIN with the hepatic vascular endothelium.

The nature of a physiological stimulus regulating the interaction of NOSTRIN $\alpha$  with its binding partners is currently unknown. There are some indications, however, that NOSTRIN $\alpha$  may be regulated by changes at the transcriptional level. Treatment of murine F9 cells with retinoic acid induces expression of a murine NOSTRIN orthologue.(215) In the present study, it has been shown that exposure of Hep3B cells to retinoic acid increases the expression of NOSTRIN in a time -dependent manner. Thus, in an environment of hepatic stellate cell activation following pro-inflammatory stimuli, the subsequent release of lipid droplets containing retinoic acid (216, 217) may contribute to the increased expression of NOSTRIN. This requires further testing in an appropriate model system. Moreover, a recent report shows that increased expression of NOSTRIN $\alpha$  correlates with reduced eNOS

activity in placental tissue of women suffering from pre-eclampsia (218), supporting the concept that up-regulated NOSTRIN expression in an environment of increased oxidative stress and unbalanced cytokine milieu (219) translates into attenuated NO production.

This study also describes a novel NOSTRIN variant, NOSTRIN $\beta$ , lacking the first 78 residues at the N-terminus of parent NOSTRIN, and demonstrates that this is appreciably expressed in the liver of AH+C patients at both gene and protein level. NOSTRIN $\beta$  is also expressed in low amounts in cirrhotic livers but not detectable in non-cirrhotic livers. In regard to understanding the functional impact of the “loss” of the N-terminal portion containing almost the entire FCH region of NOSTRIN $\alpha$ , the FCH regions of NOSTRIN-related proteins and of NOSTRIN itself are known to be involved in their binding to membranes (220, 221). Consistent with this finding, we show here that the subcellular distribution patterns of NOSTRIN $\alpha$  and NOSTRIN $\beta$  differ considerably; in over-expression systems such as in Chinese Hamster Ovary cells, NOSTRIN $\alpha$  is distributed over the cytosol and associated with subcellular membranes, whilst NOSTRIN $\beta$  is localised predominantly to the nucleus. This difference in localization may well be explained by the absence of a significant portion of the membrane-associating FCH region. (221) Consequently, the molecular target(s) and possibly also the function of NOSTRIN $\beta$  is likely to be different from that of NOSTRIN $\alpha$ . Of interest, a study by McNaughton et al, (84) demonstrated a translocation of eNOS to hepatocyte nuclei in end-stage cirrhotic patients compared to normal controls. This observation may be the result of an increased expression of mainly nuclear NOSTRIN $\beta$  but this assertion requires further investigation.

## ***Chapter 11***

### ***Summary***

## **11. Summary**

Patients with alcoholic hepatitis continue to have a high mortality with currently available supportive management, often related to complications associated with severe portal hypertension. Current knowledge on the factors that aggravate portal hypertension in these patients is poorly understood. Moreover, our current pharmacological armamentarium to treat severe portal hypertension in these patients is limited to agents that attempt to decrease portal inflow by producing splanchnic vasoconstriction, but such agents are poorly tolerated and may further compromise liver perfusion. There is, therefore, a significant clinical need to understand the pathophysiological elements that contribute to elevated portal pressure in alcoholic hepatitis and relate this to the known inflammatory mechanisms that have been evaluated largely in animal models. An improved understanding of these processes would help in the design of more targeted therapies.

The studies described in this thesis were designed to systematically investigate the differences in inflammatory status and vascular dysfunction between patients with underlying alcoholic cirrhosis and those who develop in addition, superadded inflammatory alcoholic hepatitis. Subsequent studies in patients and experimental animal models explored the effects of inflammation on nitric oxide generation and its regulators, and their potential biological significance to observations of elevated portal pressure and outcome in patients.

Initial studies confirmed that inflammation, when superimposed on chronic liver injury, manifests as a more advanced histological stage of disease commensurate with more severe liver biochemical dysfunction and high markers of a systemic inflammatory response. These features occur on a background of increased oxidative

stress and are associated with more advanced features of liver decompensation when the patient presents. Poor outcomes were observed both in humans and in a bile-duct ligated rodent model of chronic liver disease. Given the high incidence of sepsis in alcoholic cirrhosis and the existing literature suggesting neutrophil dysfunction in alcoholic hepatitis, the contribution of neutrophil dysfunction to the effects of inflammation and its outcome in alcoholic cirrhosis were also investigated. These studies suggested that in cirrhosis there was an increase in unstimulated neutrophil oxidative burst and that this was further exaggerated in the context of inflammation, suggesting a prior 'priming' of neutrophil responses by the presence of inflammation. However, further bacterial challenge resulted in blunted oxidative burst responses associated with decreased phagocytic capacity and an increased incidence of infection, organ failure and mortality in alcoholic hepatitis patients. Thus neutrophil dysfunction in alcoholic hepatitis may contribute to the inflammatory environment through increased priming, but paradoxically, there is inadequate neutrophil phagocytic function in these patients to offset an increased bacterial load, resulting in a higher incidence of sepsis and poor outcome.

The next phase of work addressed circulatory differences in alcoholic cirrhosis patients in the context of inflammation. It confirmed a more severe hyperdynamic circulation in alcoholic hepatitis, whilst also demonstrating a markedly higher portal pressure in these patients which correlated with the level of systemic inflammatory response. Moreover, hepatic blood flow was shown to be further compromised in alcoholic hepatitis and the calculated intrahepatic resistance markedly elevated. This suggested that in the context of increasing inflammation in cirrhosis, there was an important element contributed by increasing resistance within the liver, and this was likely to be a variable component modulated by vasoactive mediators as have been

previously identified. (38) Subsequent studies applying a single infusion of an anti-TNF $\alpha$  monoclonal antibody (Infliximab) or albumin dialysis using the MARS system, demonstrated marked reductions in portal pressure within 24 hours of treatment. This was associated with an improvement in hepatic blood flow in the absence of changes in central venous filling, suggesting an action on intra-hepatic resistance. The study with Infliximab also demonstrated an improvement in renal blood flow and urinary sodium excretion post therapy, which may imply that the mechanisms affecting regional vascular tone, and thereby function, were unlikely to be limited just to the liver.

Following these observations and data in the literature highlighting the importance of nitric oxide in the maintenance of hepatic vascular tone, studies were conducted to define the expression and function of eNOS in patients. These studies involved patients with and without inflammation in alcoholic cirrhosis, in addition to comparisons with an animal model of acute liver injury to which a further inflammatory insult (LPS) was added. These studies showed that there was an enhanced expression of eNOS mRNA, but no significant difference in protein expression with additional inflammation. In contrast, hepatic tissue eNOS activity was markedly reduced in alcoholic hepatitis and correlated inversely with portal pressure. This effect of inflammation on eNOS activity was confirmed in the animal model, and raised the possibility of action by inhibitors of eNOS activity, resulting in an intrahepatic 'endotheliopathy'.

Given the weight of evidence for ADMA as a potential cause of endothelial dysfunction, studies were conducted to determine the levels of ADMA in liver disease and to ascertain if they were modified by the presence of superadded inflammation. In patients with paracetamol induced acute liver failure, ADMA levels

were significantly elevated compared with controls, and correlated with the plasma levels of pro-inflammatory cytokines. Furthermore, at the time of liver transplantation, restoration of hepatic perfusion via anastomosis of the portal and hepatic veins was accompanied by a marked decrease in ADMA, suggesting a restoration of hepatic clearance of ADMA and specifically, DDAH activity which is required for ADMA metabolism. A further study in a galactosamine rat model with an additional endotoxin insult confirmed the effects of superadded inflammation on the dysfunctional liver in promoting increased ADMA levels, when compared to galactosamine alone or sham animals treated with LPS.

The biological relevance of elevated ADMA in liver disease was established from a study in patients with decompensated alcoholic cirrhosis, showing higher ADMA levels in patients with alcoholic hepatitis, and in those patients who subsequently died from organ failure. SDMA was also significantly increased in patients with organ failure and subsequently a dimethylarginine score ('DAS' =ADMA +SDMA) was defined, which provided a significantly improved predictive utility for mortality compared with scores used in current clinical practice such as MELD and Child-Pugh. Patients with alcoholic hepatitis also had reduced expression of DDAH II and increased PRMT-1 expression, suggesting decreased metabolism and increased synthesis of ADMA, respectively. Hepatic tissue ADMA also correlated with the severity of portal pressure gradient, supporting a notion that elevated ADMA in the context of inflammation in liver disease decreases eNOS activity resulting in increased intrahepatic resistance.

A final phase of work addressed other novel regulators of eNOS activity and established increased expression of caveolin-1 and NOSTRIN in patients with alcoholic hepatitis, compared to patients with alcoholic cirrhosis alone. Further



characterization of NOSTRIN noted the presence of a shortened variant with a mainly nuclear localization, which was not found in normal liver specimens. NOSTRIN was shown to co-precipitate with eNOS and caveolin-1 suggesting a complex relationship of protein interactions which determine eNOS function, which may be modified by the presence of inflammation.

In conclusion, the studies described in this thesis confirm a clear association between inflammation in alcoholic liver disease and vascular dysfunction, and importantly increased intrahepatic resistance. Furthermore, it was established that inflammation promotes a significant decrease in eNOS activity and it is suggested that this might be the result of the effects of inflammation on specific NOS regulators, especially ADMA and NOSTRIN. Whether these findings can be extrapolated to all types of inflammatory liver injury, and whether the putative regulators of NOS activity are causal in the vascular dysfunction will be important areas for future study, the findings of which may help to devise more targeted therapy for portal hypertension.

## ***Chapter 12***

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*Appendix A*  
*Future Directions*

## **Future Directions:**

Amongst the most important findings in the context of the studies presented in this thesis have been the observations of significantly elevated ADMA in patients with a more severe expression of disease (alcoholic hepatitis and acute liver failure) and in whom portal pressure is considerably raised, and also the identification of increased expression of NOSTRIN and its short-variant. These observations and their potential impact on the pathophysiology of liver disease are worthy of further study:

Firstly, it is important to address whether the observed elevation of ADMA is causally related to the progression of injury and/or the development of increased intrahepatic resistance, as this could not be confirmed by the studies performed so far. In the next phase of study, it would be interesting to address the question of a causal relationship by application to a rodent model of liver injury, such as galactosamine. The experimental questions to be considered should include:

1. Does an infusion of ADMA into a galactosamine model evoke deterioration in liver biochemistry, expression of disease/short term survival and measured vascular parameters? How do these changes compare with the effects of inflammation (injection of LPS) into a galactosamine model?
2. Can the effects of an ADMA infusion be reversed by administration of intravenous arginine, i.e. support for the concept that the ADMA:arginine ratio is important? Use of stable isotopes to label arginine and ADMA in conjunction with selective liver vessel cannulation would help in better defining the contribution of arginine in this setting.



3. What is the level of hepatic injury and vascular dysfunction in a heterozygous DDAH *knock-out* animal, treated with galactosamine injection? If the injury is sub-lethal, can the condition be improved by administration of arginine?
4. What are the potential mechanisms by which high ADMA results in poor outcome?- Is there evidence for impaired liver regeneration in the context of reduced NO signalling and pro-inflammatory drive? This could also be tested in model systems such as partial hepatectomy, and by experimentation via incubation with hepatocyte cell lines.
5. Considering the translation to man- Does an infusion of arginine lower portal pressure in patients with alcoholic liver disease through effects on intrahepatic resistance? –A pilot study would be set up with efficacy and dose ranging studies.
6. Are ADMA and SDMA reliable markers of outcome in patients with decompensated cirrhosis, who are at risk of progressing to multi-organ injury?- A large well powered prospective study with repeated measurements over the follow-up period, and continuous data collection would need to be set-up to evaluate these markers, with appropriate statistical analysis to confirm the utility of a DAS score.

Secondly, with regards to the observation of increased NOSTRIN  $\alpha$  and  $\beta$  in alcoholic liver disease, it is clearly going to be important to substantiate whether these findings can be extrapolated to other forms of liver injury through studies in different animal models and to ask the fundamental questions:

1. Which cell types express NOSTRIN and is it possible to over-express both variants in these relevant cell types?- Assess expression within endothelial cells, parenchymal cells and hepatic stellate cells.
2. What factors govern NOSTRIN up-regulation?
3. What are the functions of NOSTRIN  $\alpha$  and  $\beta$ ? – Studies to be conducted using over-expression systems or by modification of the NOSTRIN gene through application of a newly developed *knock out*
4. Is NOSTRIN important for the regulation of NOS activity in the endothelial cell? What is the mechanism of translocation of eNOS to the nucleus and is this of biological importance?

These questions are to be pursued systematically as part of on-going studies to develop this area of research further.

***Appendix B***

***Publications arising from the work in this thesis***

**Mookerjee RP, Sen S, Davies NA, Hodges SJ, Williams R, Jalan R**  
**TNF  $\alpha$  is an Important Mediator of Portal and Systemic Haemodynamic**  
**Derangements in Alcoholic Hepatitis *Gut* 2003, 52(8):1182-1187**

Sen S, Mookerjee RP, Cheshire LM, Davies NA, Williams R, Jalan R. Albumin dialysis reduces portal pressure acutely in patients with severe alcoholic hepatitis *J Hepatol.* 2005 Jul;43(1):142-8.

**Mookerjee RP, Malaki M, Davies NA, Hodges SJ, Dalton RN, Turner C, Sen S, Williams, R, Leiper J, Vallance P, Williams R, Jalan R.** Increasing dimethylarginine levels are associated with adverse clinical outcome in severe alcoholic hepatitis. *Hepatology* 2007; 45 (1) 62-71

**Mookerjee RP**, Dalton RN, Davies NA, Hodges SJ, Turner C, Williams R, Jalan R. Inflammation is an important determinant of levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) in acute liver failure. *Liver Transpl* 2007; 13 (3) 400-405

**Mookerjee RP**, Wiesenthal A, Icking A, Hodges SJ, Davies NA, Schilling K, Sen S, Williams R, Novelli M, Muller-Esterl W, Jalan R. Increased gene and protein expression of the novel eNOS regulatory protein NOSTRIN and a variant in alcoholic hepatitis. *Gastroenterology* 2007; 132: 2533-41



**Mookerjee RP, Stadlbauer V, Lidder S, Wright GAK, Hodges SJ, Davies NA, Jalan R.** Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome. *Hepatology* 2007; 46: 831-40

Davies NA, Hodges SJ, Pitsillides A, Mookerjee RP, Jalan R, Mehdizadeh S.  
Hepatic guanylate cyclase activity is decreased in a model of cirrhosis: a quantitative  
cytochemistry study. *FEBS Lett* 2006;580:2123-2128

*This article resulted from a phase of developmental work, in an attempt to establish a working assay to measure tissue nitric oxide generation. Although the technique proved too time consuming and complex for measurement in human samples from transjugular biopsies, the results on defective guanylate cyclase activity in a cirrhotic model provides evidence for an alternative target for future therapy beyond the derangements in nitric oxide synthase activity.*

***Appendix C***

***List of figures and tables***

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