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Albumin dialysis in liver failure

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ROLE OF VARIOUS CONTRIBUTORS

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(Sambit Sen)

ABSTRACT

Albumin-bound toxins accumulating due to hepatic dysfunction are believed to lead to multi-organ dysfunction in liver failure, contributing to a poor prognosis. Thus, liver support devices utilising albumin dialysis have been developed, and the Molecular Adsorbents Recirculating System (MARS) is the one being investigated most thoroughly. This series of studies was designed to systematically investigate its role in liver disease.

The initial studies evaluated its clinical impact in patients with severe alcoholic hepatitis. MARS therapy was safe and feasible. Improvement of hyperbilirubinaemia and hepatic encephalopathy were the most consistent findings. Effects on systemic haemodynamics or renal function were insignificant. A rapid and sustained portal hypotensive effect was observed, suggesting that albumin dialysis may be a useful adjunctive therapy for variceal bleeding in patients with liver failure. An apparent reduction in mortality was observed, though the studies were not powered to evaluate survival.

The next study investigated the pathophysiological basis of these changes in acute-on-chronic liver failure. Albumin dialysis improved encephalopathy, accompanied by reduced oxidative stress, without significant changes in arterial ammonia or cytokines. The improvement in encephalopathy independent of ammonia highlights the importance of other mediators such as oxidative stress in its pathogenesis.

A porcine model of acute liver failure (ALF) was used to study the effect of MARS on the cerebral changes. Attenuation of intracranial hypertension was observed, as was a reduction of cerebral oedema (in white matter), without alterations of arterial ammonia, cerebral blood flow, cytokines or oxidative stress. Thus, while hyperammonaemia is probably essential for the initial development of cerebral oedema and intracranial hypertension in ALF, cerebral hyperaemia and inflammation are not. Regional differences exist for brain oedema, perhaps with therapeutic imlications. Factors in addition to hyperammonaemia are also important.

Finally, the effect of albumin dialysis on protein-bound drugs was studied. MARS efficiently removed both albumin-bound (phenytoin, midazolam) and non-albumin-bound (fentanyl) substances. The mechanism is probably by uptake of free drug, with constant re-equilibration of free and bound components. Albumin dialysis may be important in treating non-dialysable protein-bound substance intoxications.

Therefore, albumin dialysis does bring about measurable biochemical and pathophysiological changes, translating to clinical improvements. Ongoing randomized controlled trials will provide a definitive answer regarding impact on mortality.

1. BACKGROUND

1.1 The Pathophysiologic Basis of Acute-on-Chronic Liver Failure

1.1.1 Introduction

Acute-on-chronic liver failure (ACLF) remains an extremely poorly defined entity, largely because of the considerable heterogeneity in the mode of presentation. It is meant to encompass those patients with previously well-compensated chronic liver disease in whom an acute decompensation of liver function occurs due to the effects of a precipitating event such as complications of sepsis, upper gastrointestinal (UGI) bleeding, ischaemia or additional superimposed liver injury due to alcohol, hepatotoxic drugs or hepatitis virus infection. This should be contrasted with the chronic hepatic decompensation that may occur in patients with end-stage cirrhosis as a result of progression of underlying disease. While both entities can lead to various clinical manifestations, most notably, renal dysfunction and hepatic encephalopathy (HE), the underlying mechanisms of decompensation are probably quite different. Progression of the primary liver disease is responsible for chronic decompensation, and is irreversible in most cases. On the other hand, the precipitant altering the delicate balance in a well-compensated chronic liver disease patient is more important in ACLF. Hence, probably the most important difference between the two entities is the potentially reversible nature of ACLF if precipitants can be controlled. At present, attention is focused at supportive therapy aimed at the failing organs in the hope that the liver would recover if the patient can be supported through this acute deterioration. With the concept that acute deterioration in liver function is important in producing the manifestations of liver failure, strategies to enhance recovery of liver function should become the central theme rather than solely focusing upon improving the function of the failing end-organs.

The literature is difficult to interpret with regard to what features are important in defining the condition of ACLF. The parameter best represented in the prognostic models of chronic liver disease is the serum bilirubin level. The end-organs that are most frequently involved and of major importance in determining outcome are the kidneys and the brain. There will also be a variable component related to dysfunction of other organ systems. Therefore, a form of overall scoring such as the Acute Physiology and Chronic Health Evaluation (APACHE) II system or the Sequential Organ Failure Assessment (also known as the Sepsis-related Organ Failure Assessment) (SOFA), which are well established in intensive care medicine, may help better identify and grade the severity of illness of patients who may come within the category of ACLF[1,2].

The following working definition has been suggested, which comprises the essential features of the syndrome: 'Acute deterioration in liver function over a period of 2-4 weeks, usually associated with a precipitating event, leading to severe deterioration in clinical status,

with jaundice and HE and/ or hepatorenal syndrome (HRS), with a high SOFA/ APACHE II score'[3].

The numerous factors that may precipitate acute decompensation of chronic liver disease can be divided into two groups. First, the acute component of the liver injury may be due to the effects of a known hepatotoxic factor such as superimposed viral infection with a hepatotrophic virus, drug reaction, ingestion of a hepatotoxin or excessive alcohol consumption. Second, the liver injury may be the result of precipitating factors such as variceal bleeding or sepsis, which, though not necessarily acting as specific hepatotoxins, have important secondary end-organ-damaging effects, and also damage the liver.

1.1.2 Prognosis of ACLF

It is important to be able to determine the prognosis in ACLF, especially over the short term, with respect to the use of temporary liver support or even transplantation in these patients. This has, however, proved difficult. The Child- Pugh score has conventionally been used for this purpose[4]. However, this is probably not an ideal system in the context of these patients as it only looks at parameters associated with liver function and largely ignores parameters related to renal, respiratory and cardiovascular function (which are important in determining outcome, for example, with HRS or sepsis). It is also not particularly versatile at detecting changes on a day-to-day basis, which is important in ACLF. For example, a rise of serum bilirubin from 100 to 300 μmol/L would not make any difference in the score. For this reason, newer scoring systems have been developed, which seem to be more appropriate in this setting.

The Model for End-Stage Liver Disease (also known as the Mayo End-Stage Liver Disease) (MELD) score is one such, which is a continuous system with no ceiling or floor in the score and the coefficients in the scale are derived statistically so that appropriate weights are given to variables according to their relative importance. It also takes renal function into account. It has been found to be reliable for predicting outcome in patients with decompensated cirrhosis, with 3-month predicted mortality changing from 27% at a score of < 20 to 76% at a score ≥ 20[5]. From the 27th of February, 2002, it is being used by the United Network for Organ Sharing for determining priority for donor organs in patients awaiting transplant[6]. However, the number of patients defining the 'sick' end of the spectrum in the original study where the system was developed was very few (only 10 patients analysed with a score ≥ 30). This raises doubts as to its validity in ACLF patients. Another system which has been studied is the APACHE (II and III) system, which takes into account a host of parameters required for the monitoring of a patient in an Intensive Care setting [1]. However, this has not been validated for sequential monitoring of patients, which is a disadvantage among the critically ill. We believe that probably the most suitable system is the SOFA

scoring system. This takes into account parameters representing respiration, coagulation, cardiovascular and central nervous systems, and renal and liver functions (Table 1). This is versatile as well in that it is designed to look at day-to-day changes in an Intensive Care setting. One study has shown it to be more accurate in ACLF than either APACHE II or the Child- Pugh score [2]. A score of 8 has been suggested as a suitable cut-off value, with predicted hospital mortality below and above it being 4% and 88% respectively. Moreover, the mortality (both at hospital discharge and at 6 months) increases dramatically when number of organ failures (defined by ≥ 3 SOFA points for that organ system) increases from one to two.

Table 1: The SOFA scoring system (PaO2 - arterial oxygen tension, FIO2 - fractional inspired oxygen, MAP - mean arterial pressure)

SOFA score	**0**	**1**	**2**	**3**	**4**
Respiration PaO2/FIO2 (mmHg)	> 400	≤ 400	≤ 300	≤ 200 with respiratory support	≤ 100
Coagulation Platelets (x 10^3/mm^3)	> 150	≤ 150	≤ 100	≤ 50	≤ 20
Liver Bilirubin (mg/dl) (μmol/l)	< 1.2 (< 20)	1.2-1.9 (20-32)	2.0-5.9 (33-101)	6.0-11.9 (102-204)	> 12.0 (> 204)
Cardiovascular Hypotension	No hypotension	MAP < 70 mmHg	Dopamine ≤ 5 or dobutamine (any dose)(*)	Dopamine > 5 or epinephrine ≤ 0.1 or norepinephrine ≤0.1(*)	Dopamine > 15 or epinephrine > 0.1 or norepinephrine > 0.1(*)
Central nervous system Glasgow Coma Score	15	13-14	10-12	6-9	< 6
Renal Creatinine(mg/dl) (μmol/l) or urine output	< 1.2 (< 110)	1.2-1.9 (110-170)	2.0-3.4 (171-299)	3.5-4.9 (300-440) or < 500 ml/day	> 5.0 (> 440) or < 200 ml/day

(*) Adrenergic agents administered for at least 1 h (doses given are in μg/kg per min)
(***From:*** **Vincent JL, Moreno R, Takala J, Willatts S, De Mendonca A, Bruining H, Reinhart CK, Suter PM, Thijs LG.** The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. *Intensive Care Med* 1996; 22: 707-10.)

1.1.3 Manifestations of ACLF

ACLF manifests itself through altered function of all organ systems, but primarily the circulation, brain, kidneys and liver.

Liver: Hyperbilirubinemia, with clinical jaundice, is almost invariably present. Reduction of hepatic synthetic functions is important as well, with hypoalbuminemia resulting in oedema and increasing ascites. Decreased production of clotting factors, sometimes in association with thrombocytopenia (due to hypersplenism), may result in a haemorrhagic diathesis.

Circulatory changes: Circulatory changes hold the centre-stage in the development of ACLF, and also are part of the manifestation of the condition itself. It is likely that any alteration of hepatic perfusion, in a patient with a chronically diseased liver, may lead to acute decompensation. This may be as part of generalized haemodynamic changes (for example, gastrointestinal bleeding, sepsis, dehydration) or may be a local alteration (for example, portal vein thrombosis or after porta-caval shunting).

Characteristic changes occur in the circulation of cirrhotics (which become more exaggerated during ACLF), including increased cardiac output, a dilated and hyporesponsive peripheral circulation, increased portosystemic shunting and portal pressure, and a reduced renal blood flow [7, 8]. These phenomena are thought to be secondary to a reduction in vascular responsiveness and desensitisation to vasoconstrictors or to the effects of vasodilating factors or both. As they are reversible with liver transplantation, it seems likely that a common mechanism underlies their development, and that this is related to the severity of liver dysfunction.

The involvement of nitric oxide (NO), a profound vasodilator, has generated considerable interest as a potential mediator in the hyporesponsiveness observed in chronic liver disease [9]. Studies on rats subjected to endotoxin challenge suggest that NO formed from *L*-arginine by calcium-dependent constitutive NO synthase (NOS) is important in the maintenance of microvascular integrity in endotoxaemic states [10]. NO, by promoting vasodilatation, may maintain local organ blood flow and have a protective role [10]. Similarly, studies carried out on pre-ascitic cirrhotic patients using the forearm blood flow model, have shown reduced vasoconstrictor responses to sub-systemic infusions of angiotensin II [11]. These studies suggest that angiotensin II stimulates endothelial NOS (eNOS), mediating the attenuated vasoconstrictor response to exogenous angiotensin II in these patients.

In addition, widespread expression of a calcium-independent inducible NOS can be detected several hours after a challenge with endotoxins or cytokines [12, 13]. This is associated with microvascular damage in cardiac, pulmonary, renal and hepatic tissues and may reflect the initiating pathological events in the microvasculature, leading to multi-organ failure

which is so characteristically found in septic patients. Studies of delayed administration of L-N^G-monomethyl-arginine (*L*-NMMA) at a time of known induced NOS expression, following endotoxin challenge in rats, caused a dose-dependent reduction in vascular endothelial damage in the heart, lung and kidney. These effects were reversed by *L*-arginine administration [14, 15].

Cirrhotic patients have elevated renin and angiotensin II secretion rates, and increased sodium retention in response to the application of 20 mm Hg lower body negative pressure, when compared with controls [16]. These changes are to be expected in the context of splanchnic arterial vasodilatation, resulting in arterial underfilling of the renal vasculature, leading to avid renal sodium and water retention in spite of an expanded total plasma volume. The fact that induction of NOS is the likely explanation for the splanchnic arterial vasodilatation is supported by studies showing upregulated NOS in cirrhotic rats compared with portal vein-ligated animals [17]. Furthermore, when the hyperdynamic circulation is reversed by NO inhibition, there is an accompanying reversal of the elevated plasma renin and angiotensin levels, hyponatraemia and reduction in ascites in experimental cirrhosis [18]. This has been borne out in studies on cirrhotic patients as well, where inhibition of NOS corrected the altered systemic hemodynamics and improved renal function and sodium excretion [19] (Fig 1).

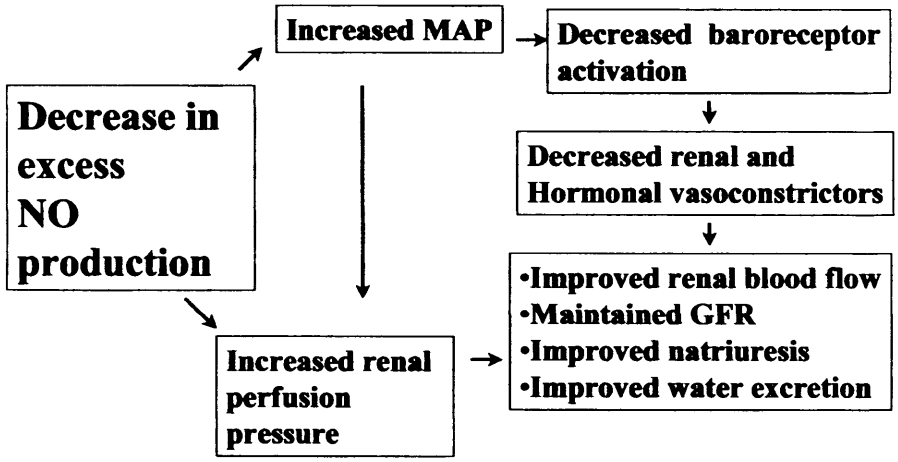


Fig 1: Mechanism of action of improved haemodynamics by inhibition of NOS [NO- nitric oxide, NOS- NO synthase, MAP- mean arterial pressure, GFR- glomerular filtration rate]

From studies measuring induced NOS (iNOS) activity in septic shock, it has been noted that NOS activity differs in various organs and is mainly dependant on the pro-inflammatory cytokines, tumour necrosis factor (TNF) α and interleukin-1β [20]. In vitro, cytokines induce expression of NOS activity in hepatocytes and vascular smooth muscle cells

[21], and it has been shown that NOS activity in vivo is compartmentalised to putrescent areas in septic shock patients, compared to normal controls [22]. The lack of significant expression of iNOS activity away from the nidus of infection might explain the deleterious effect of non-selective NOS inhibitors in septic shock. Furthermore, studies on mice after partial hepatectomy have shown that iNOS induction occurs preferentially in regenerating hepatocytes and protects them from cytokine-induced apoptosis and endotoxaemia. This hepatoprotective factor in regenerating liver is lost in iNOS-deficient mice, supporting the concept of compartmentalisation of NO-mediated effects. If this hypothesis of compartmentalised NO production is correct, it will explain why there is a preferential dilatation of the splanchnic circulation with relative vasoconstriction of the renal and cerebral circulation in patients with ACLF.

Evidence implicating the role of eNOS in the development of the hyperdynamic circulation of cirrhosis has been emerging. A recent study has shown that hepatic sinusoidal endothelial cells express eNOS in vitro and in vivo and produce NO under basal conditions. This production is greater in response to more flow. Moreover, blockage of NO production by *L*-NMMA resulted in a rise of portal pressure, indicating that eNOS modulates portal pressure [23]. Another study has shown the increased expression of eNOS in the aorta and arterial system of cirrhotic rats, suggesting that it may have a role in the development of systemic haemodynamic changes in cirrhosis [24].

Kidneys: Hepatorenal syndrome (HRS) is the development of renal failure in the setting of progressive deterioration of liver function with poorly controlled ascites, and marked alterations in splanchnic and systemic haemodynamics. In HRS, the histological appearance of the kidneys is normal and the kidneys often resume normal function following liver transplantation. HRS is characterised by severe renal hypoperfusion due to an increase in renal vascular resistance. It is one of the most dangerous complications of ACLF, from which the chances of recovery are extremely poor [25]. In some patients, renal dysfunction may be precipitated by gastrointestinal bleeding, sepsis, use of non-steroidal or nephrotoxic agents, hypovolaemia or excessive use of diuretics [26]. Such cases, though not HRS in the strict sense of the term [27], will form an important part of the syndrome of ACLF as defined previously.

Two different forms of HRS are described[27]. Although their pathophysiology is similar, their manifestations and outcomes are different. Type 1 HRS is an acute form of HRS in which renal failure occurs spontaneously in patients with severe liver disease and is rapidly progressive. The development of type 1 HRS has a poor prognosis with 80% mortality at two weeks. Type 2 HRS usually occurs in patients with diuretic resistant ascites. Renal failure has

a slow course, in which it may deteriorate over months. It is associated with a poor prognosis, although the survival time is longer than that of patients with type 1 HRS.

The pathogenesis of HRS is multi-factorial, although the circulatory dysfunction of ACLF is probably central to its development (Fig 2). The splanchnic vasodilation induces the highly activated sympathetic nervous system to produce renal vasoconstriction and reduction in renal plasma flow [28]. The renin-aldosterone-angiotensin axis is stimulated in cirrhosis and produces afferent arteriolar vasoconstriction [29]. Further renal vasoconstriction is produced by increased endothelin-1, expressed in the hepatic sinusoids, the concentration of which correlates with the severity of renal dysfunction. The compensatory mechanism, mediated by renal prostaglandins, fails to maintain renal perfusion in HRS [30]. HRS is usually associated with a decrease in the mean arterial pressure and this is a known risk factor for its development [25]. In normal circumstances, the renal blood flow is autoregulated but in patients with HRS who have an activated neurohumoural system the autoregulatory curve (renal blood flow/renal perfusion pressure) is shifted to the right [31]. This means that a small reduction in the arterial pressure produces a pronounced reduction in renal blood flow.

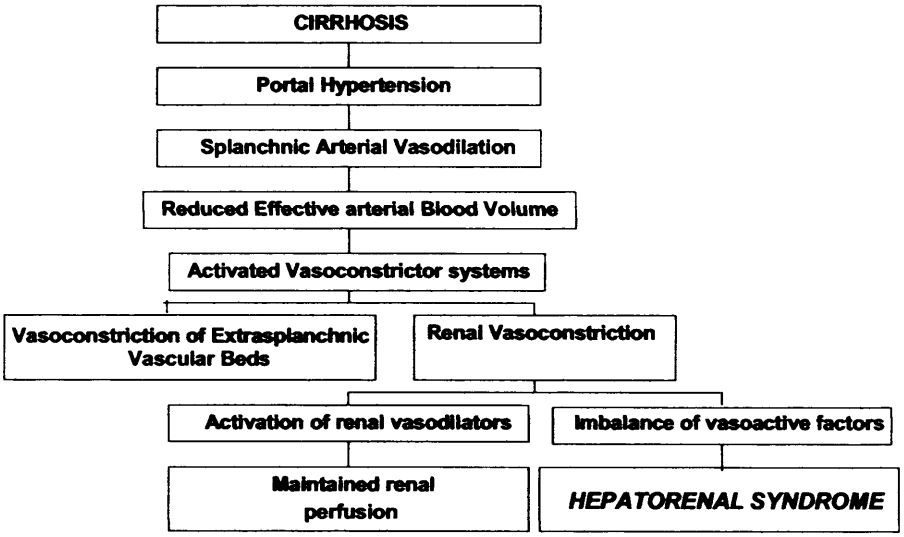


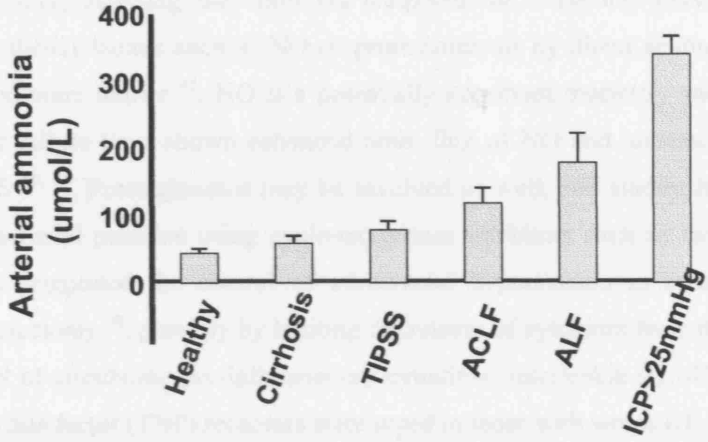
Fig 2: Peripheral vasodilation hypothesis of ascites and renal dysfunction

It is therefore possible to envisage that precipitants such as sepsis produce HRS by worsening the vasodilatation which results in further activation of the vasoconstrictor neurohormones. Excessive use of diuretics (through decreased renal perfusion) and non-steroidal anti-inflammatory drugs (through decreased production of prostaglandins) would also compromise renal homeostasis and lead to renal dysfunction.

Brain: HE is a potentially reversible neuropsychiatric syndrome that occurs in patients with significant liver dysfunction and is characterised by altered sleep-wake cycle,

varying degrees of confusion and disorientation, asterixis, hyper-reflexia, and slowing of the dominant rhythm on electroencephalography [32]. Apart from jaundice, this is probably the most apparent major manifestation in ACLF.

Although ammonia remains central in the pathogenesis of HE (Fig 3), a number of different mechanisms have been proposed and the present view is that these may interact with ammonia to influence the neurotransmitter pathways. Some studies have shown the arterial ammonia level to correlate directly with severity of liver disease [33] as well as with degree of HE [34]. Indeed, in the context of acute liver failure, the occurrence of cerebral herniation seems to correlate well with the arterial ammonia concentration [35].



(Olde Damink, Deutz, Dejong, Soeters, Jalan; 2001)

Fig 3: Ammonia is central in the pathogenesis of Hepatic encephalopathy: Arterial ammonia in healthy controls, patients with cirrhosis, with transjugular intrahepatic porto-systemic stent shunt (TIPSS) placement, with acute-on-chronic liver failure (ACLF), with acute liver failure (ALF) and with intracranial pressure (ICP) > 25 mm Hg

Of the other mechanisms which seem to be of importance, alterations in the blood brain barrier, resulting in altered amino acid transport [36], is one. Patients with HE also have reduced cerebral blood flow and glucose and oxygen consumption [37]. However, the latter is likely to be an epiphenomenon, secondary to the global depression in CNS function, rather than the cause of HE.

Data in patients with ACLF are scarce, but some recent clinical and pathophysiologic studies in patients suggest that cerebral oedema with the resulting rise in intracranial pressure can occur in chronic liver disease and presents as neurological deterioration [38]. It has also been found to develop following transjugular intrahepatic porto-systemic stent shunt placement in cirrhosis [39]. Cirrhotic patients may show development of minimal HE and low-grade cerebral edema, which appear to be the consequences of the metabolism of ammonia in

the brain [40]. The pathophysiologic basis of HE in ACLF may be similar to that in acute liver failure (Fig 4). In this connection, recent studies have focused attention towards the inflammatory basis of intracranial hypertension and HE in acute liver failure [41], which may also be relevant considering the frequency of infections in ACLF. Takada et al [42] showed in a pig model of acute liver failure that animals administered lipopolysaccharide (LPS) and amatoxin intraportally developed greater intracranial hypertension than animals given amatoxin alone, even though ammonia levels were similar in both groups. Patients with acute liver failure who had higher systemic inflammatory response syndrome (SIRS) scores or were overtly infected had markedly greater severity of encephalopathy and were also more likely to develop intracranial hypertension [43, 44]. One important mechanism may involve peripheral cytokines, affecting the brain via peripheral or autonomic nerve, through production of endothelial factors such as NO or prostanoids, or by direct action after crossing an altered blood-brain barrier [45]. NO is a potentially important mediator, and animal models of acute liver failure have shown enhanced brain flux of NO and increased expression of neuronal NOS [46, 47]. Prostaglandins may be involved as well, and studies have shown the control of intracranial pressure using cyclo-oxygenase inhibitors such as indomethacin [48, 49]. Reports have suggested the control of intracranial hypertension in acute liver failure following hepatectomy [50], possibly by limiting the release of cytokines from the necrotic liver. A higher level of circulating pro-inflammatory cytokines, interleukin (IL)-1b, IL-6 and soluble tumor necrosis factor (TNF) receptors were noted in those with worse HE [51].

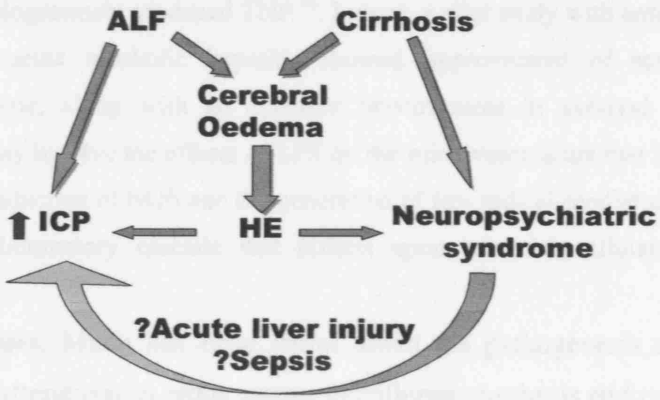


Fig 4: Hepatic Encephalopathy (HE): Common pathogenic mechanisms in acute liver failure (ALF) and Cirrhosis [ICP- intracranial pressure]

1.1.4 Role of a precipitant in producing acute decompensation

As yet there are no studies available which have tried to demonstrate a possible mechanism. The commonest precipitants are sepsis and UGI bleeding, in addition to ischaemia or superimposed liver injury due to alcohol, drug reactions or virus infection.

Sepsis: Sepsis is an important factor that can produce liver damage by LPS-induced hepatocyte apoptosis, as well as by ischaemic injury following the associated circulatory disturbances. Sepsis may occur either in the form of spontaneous bacterial peritonitis, septicaemia or with infections such as pneumonia or urinary tract infection. Several lines of investigations suggest that prophylaxis of patients at high risk of infection reduces mortality[52]. Presence of bacterial infection in bleeding cirrhotic patients has been independently associated with early mortality and failure to control bleeding[53]. Pathophysiogical data either in animal models or patients with cirrhosis with hepatic decompensation precipitated by infection are scarce. One study demonstrating the role of LPS has already been discussed earlier[42]. Another study has suggested that liver damage induced in D-galactosamine-sensitized mice by endotoxin was initiated by TNF-induced hepatocyte apoptosis[54]. Although severe sepsis can rarely produce a syndrome that resembles ALF[55], the current data suggests that LPS has a synergistic effect with other hepatotoxins in inducing hepatic injury.

LPS is a potent inducer of the release of TNF and its hepatocyte apoptotic effect is enhanced both in vitro and in vivo when sensitising concentrations of a hepatotoxin are present [56]. A neutralising antibody to LPS has been shown to reduce the extent of hepatocellular damage during administration of hepatotoxin in rats [57]. Experiments using anti-TNF antiserum, have suggested that the mechanism of the hepatotoxin-mediated injury may be modulated through an indirect mechanism involving sensitisation of the hepatocytes towards the endogenously produced TNF [58]. Indeed, a pilot study with anti-TNF antibodies in patients with acute alcoholic hepatitis showed improvement of serum bilirubin and prothrombin time, along with an apparent improvement in survival [59]. These indirect mechanisms may involve the effects of LPS on the microvasculature that is mediated through alterations in induction of NOS and the generation of free radical-mediated injury. These may set up an inflammatory cascade that inflicts upon essential cellular function such as respiration.

Metabolic factors: Much has been learnt about the pathogenesis of alcoholic liver disease, with alterations in redox status, glutathione synthesis and protein metabolism all being involved [60]. One can therefore postulate that metabolic changes would have a role to play in the further worsening seen in ACLF. UGI bleeding is one such scenario where this seems to be the case. Overall, about 30% of patients with cirrhosis die from the consequences of oesophageal variceal bleed despite adequate control of acute bleeding. In addition to the associated ischaemic injury to the liver [61], these deaths may occur due to the metabolic consequences of the bleed, which precipitate further hepatic decompensation and further life-threatening complications

such as spontaneous bacterial peritonitis, sepsis, renal failure and encephalopathy [37, 62-64].

In patients with cirrhosis, ingestion of erythrocytes produces a larger increase in ammonia than ingestion of whole blood or plasma [58]. In normal pigs an erythrocyte meal resulted in extremely low plasma isoleucine concentrations (25% of normal fasting levels) and an increase in almost all other amino acids compared with values obtained in animals receiving an iso-nitrogenous control meal [65]. Furthermore, after ingestion of erythrocytes, ammonia production by the gut and plasma urea concentrations were significantly higher than in the control animals [65]. These findings are explained by the unique amino acid composition of the haemoglobin molecule, which is totally devoid of the branched-chain amino acid (BCAA), isoleucine, and contains large amounts of the two other BCAAs, valine and leucine [66, 67]. Therefore, an UGI bleed presents the gut with protein of very low biologic value. The absorbed (blood) amino acids cannot be used for protein synthesis. Moreover, leucine activates further breakdown of BCAA [68]. Consequently, these amino acids are degraded in the gut wall producing ammonia or released into the portal vein, stimulating urea synthesis.

Isoleucine deficiency is likely to diminish protein synthesis, just as it impairs DNA synthesis, cell proliferation and cell function [69, 70]. This could lead to a net catabolic state, in which the patients break down proteins in order to provide the lacking amino acid isoleucine. This catabolism may further increase ammonia concentrations (induce encephalopathy), influence the function of rapidly dividing cells (e.g. immune cells) and short half-life proteins (e.g. clotting-factors). The deficient protein synthesis and increased catabolism resulting from gastrointestinal bleeding may increase the risk of further bleeding and infection. In the pig model, the catabolic cascade, e.g. ammonia- and ureagenesis, following a simulated bleed was significantly lower in the group that were given isoleucine intravenously compared to control animals [71].

1.1.5 Impact of a better understanding of underlying pathophysiology on approach to therapy

It is likely that ACLF develops as a result of a chain of events leading to hepatic injury through one of several diverse mechanisms. Sepsis, one of the more common precipitants, together with the associated hepatic dysfunction, produce a pro-inflammatory environment, which leads to the circulatory disturbances and finally to multi-organ failure in patients with ACLF. Gut/liver-derived pro-inflammatory cytokines, free radicals and NO are probably the key mediators. The ischaemic effects of UGI bleeding contribute as well. At present, our focus of therapy is directed at improving the function of the individual end-organs along with control of any precipitant such as control of bleeding with endoscopic therapy and sepsis with antibiotics, with limited options for the management of the failing

liver. However, ACLF is potentially reversible and with a better understanding of the responsible mechanisms, it should be possible to correct it.

Thus one can envisage the use of agents altering the pro-inflammatory cytokine milieu (for example, antibodies to TNF α) and reducing oxidative stress (a role for anti-oxidants). Alternatively, or in addition, one may attempt to remove the toxins (for example, ammonia, nitric oxide and endotoxins), which mediate the development of circulatory disturbances and multi-organ failure in ACLF. In effect, this should lead to a temporary improvement of the patient's condition, thereby buying time and allowing the liver to recover spontaneously. Here lies the role of 'cleansing devices' in liver failure, the liver assist devices. The Molecular Adsorbents Recirculating System (MARS) is one such device, which has been found to be of benefit in small studies in improving HRS [72], systemic haemodynamics [73], HE [74] and cerebral perfusion [75] in patients with ACLF. This indicates that MARS improves the toxin load, along with the severity of hyperbilirubinaemia, in this group of patients. However, only with further studies can a clearer picture emerge of the role that it may play in the treatment of ACLF in the future.

1.2 Albumin Dialysis in Liver Failure

1.2.1 Introduction

Liver failure, whether occurring *de novo* without pre-existing liver disease (acute liver failure, ALF) or as an acute episode of decompensation superimposed on a chronic liver disorder (acute-on-chronic liver failure, ACLF), carries a high mortality. The lack of the detoxification, metabolic, and regulatory functions of the liver leads to life threatening complications, including kidney failure, hepatic encephalopathy (HE), cerebral oedema, severe hypotension and susceptibility to infections culminating in multi-organ failure[76, 77]. The only established therapy for patients with ALF is liver transplantation (LTx), but currently one-third of these patients die whilst waiting for a transplant and the organ shortage is increasing[78]. Many patients with ACLF are not eligible for LTx, and current management focuses on treatment of the individual organ dysfunction. However, liver failure, whether of the acute or acute-on-chronic variety, is to some extent reversible. A supportive therapy which can tide over the acute period of crisis (and act as a bridge to liver transplantation in cases of ALF) can possibly be life-saving[76], and the search for an effective liver support system continues. Essentially, two types are under development: bio-artificial devices, using hepatocytes to perform the functions of the failing liver; and artificial devices, including the Molecular Adsorbents Recirculating System (MARS), utilizing the principles of albumin dialysis. Only the latter are being extensively studied at present.

1.2.2 Importance of albumin in liver diseases

The mechanisms underlying the development of the multi-organ dysfunction of liver failure are, as yet, poorly understood. The 'toxin hypothesis' implicates a variety of toxins which accumulate as a result of impaired hepatic metabolism/ detoxification. Ammonia, protein breakdown products (aromatic amino acids, tryptophan, indole, mercaptan, phenol) and endogenous benzodiazepines, among others, are implicated in the development of hepatic encephalopathy. Nitric oxide (NO) and prostanoids are believed to be important in the pathogenesis of circulatory and renal dysfunction. Pro-inflammatory cytokines probably have wide-ranging influences, and oxidative stress has effects ranging from increased capillary permeability to modulating cell death[76]. However the vast majority of these toxins (except possibly ammonia) are water-insoluble and albumin-bound (Table-2), and conventional renal replacement therapy cannot effectively remove them.

Intravenous albumin administration is of benefit in the treatment of patients with cirrhosis[79-81], and improves survival in those with spontaneous bacterial peritonitis[82] or with hepatorenal syndrome[83, 84], but the benefits exceed what could have been expected if it was acting simply as a volume expander. Indeed, it has been suggested that albumin is an important molecule involved in detoxification and binds various substances[85], and is perhaps more important in liver diseases than was previously thought[86]. This is the basis for the use of albumin as a binding and scavenging molecule in devices based on albumin dialysis, and therefore the trial of such devices in patients with liver failure.

Table 2: Some of the endogenous albumin-bound toxins, which accumulate in liver failure

Aromatic amino acids	Bile acids
Bilirubin	Copper (Wilson's disease)
Digoxin-like substances	Endogenous benzodiazepines
Indols	Mercaptans
Middle- and short-chain fatty acids	Nitric oxide
Phenols	Prostacyclins
Tryptophan	

1.2.3 The Molecular Adsorbents Recirculating System (MARS)

In albumin dialysis, blood is dialysed against an albumin-containing solution across a suitable membrane[87-89]. The albumin-bound toxins are potentially taken up by the binding sites of the dialysate albumin and thus removed from blood. However, the expense involved in using a volume of albumin comparable to the volume of dialysate used in conventional haemodialysis/ haemodiafiltration (i.e., as a single pass albumin dialysis system) would be prohibitive.

Stange and Mitzner from the University of Rostock, Germany, who designed the Molecular Adsorbents Recirculating System (MARS) (Teraklin AG, Rostock, Germany)[90, 91]

in 1993, circumvented this problem by converting the albumin circuit into a closed circuit and recirculating a fixed volume of dialysate (Fig 5). Briefly, the system consists of three compartments - a blood circuit, an albumin circuit and a renal circuit (haemofiltration/ haemodialysis). Blood flows through a hollow fibre dialysis module, where it is dialysed across an albumin-impregnated high-flux polysulfone dialysis membrane. 600ml of 20% human albumin in the albumin circuit acts as the dialysate, and is passed through the dialysate compartment of the blood dialyser. Albumin-bound toxins in the plasma pass on to the membrane-impregnated albumin, a possible mechanism being that albumin, when attached to polymers, have a higher affinity for albumin-bound toxins[92]. These toxins are subsequently picked up by the albumin dialysate, which, in turn, is regenerated by haemofiltration/ haemodialysis, followed by passage through 2 sequential adsorbent columns (containing activated charcoal and anion exchange resin), which remove most of the water-soluble and albumin-bound toxins and thus cleanse it. Substances with a molecular weight of

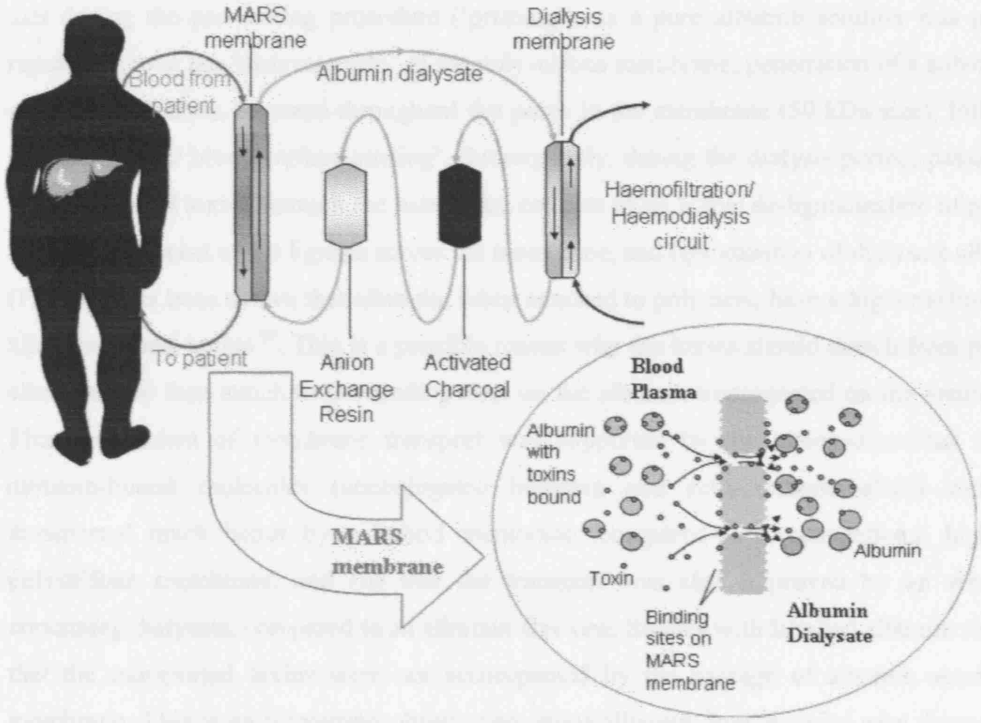


Fig 5: Schematic diagram of the MARS circuit, showing direction of flow of the blood and the dialysate (20% albumin). The albumin-bound toxins from the patient's blood pass on to the albumin in the dialysate, which is then cleansed sequentially by a haemodialysis/ haemofiltration module (removing water-soluble substances), and adsorber columns containing activated charcoal and anion-exchange resin (removing most of the albumin-bound substances). The dialysate is thus regenerated, and once more capable of taking up more toxins from the blood. *Inset*- Movement of toxins from plasma albumin to the albumin binding sites on the MARS membrane, and thence to the albumin in the dialysate.

more than 50 kDa such as essential hormones bound to carrier proteins, growth factors and albumin are not removed from the perfused plasma because of the pore size of the MARS membrane.

1.2.3.1 MARS: The early investigations

The initial *in vitro* studies by the Rostock group showed effective removal of unconjugated bilirubin, drugs with a high protein-binding ratio (sulfobromophthalein, theophylline), and a protein-bound toxin (phenol)[91] with MARS. Further studies demonstrated that it effectively removed strongly albumin bound toxins (unconjugated bilirubin, free fatty acids) from plasma and blood *in vivo* too[93].

Subsequent studies tried to determine how the hybrid membrane formed, how it worked, and what was the effect of MARS on toxins, as well as other physiologically important molecules in the body [90]. Working with Texas Red-labelled albumin, they showed that during the pre-coating procedure ('priming'), as a pure albumin solution was passed repeatedly over the 'dialysate side' of the polysulfone membrane, penetration of a substantial amount of albumin occurred throughout the pores in the membrane (50 kDa size), followed by an 'internal/ blood surface coating'. Subsequently, during the dialysis period, passage of albumin-bound toxins through the membrane consists of an active de-ligandization of plasma albumin, transport of the ligands across the membrane, and ligandization of dialysate albumin (Fig 5). It has been shown that albumin, when attached to polymers, have a higher affinity for albumin-bound toxins [92]. This is a possible reason why the toxins should detach from plasma albumin, and then attach to the binding sites on the albumin impregnated on the membrane. This mechanism of membrane transport was supported by the observation that tightly albumin-bound molecules (unconjugated bilirubin and sulfobromophthalein) were (i) transported much better by a hybrid membrane, compared to a conventional high-flux polysulfone membrane, and (ii) that the transport was also improved by an albumin-containing dialysate, compared to an albumin-free one. Studies with labelled albumin showed that the transported toxins were not accompanied by the passage of albumin across the membrane. This is an interesting observation, since albumin does seem to pass through the pores during 'priming'. A possible explanation is that the sieve coefficient for large molecules like albumin is higher in pure aqueous solutions (as during pre-coating/ priming) than in whole plasma (as during treatment) because no deposition of a mixed protein layer occurs [94].

The same study also evaluated the *in vivo* effects of MARS[90], and showed that there was an improvement of the amino acid profile, with relative clearance of the aromatic amino acids (which accumulate in hepatic insufficiency) and an improved ratio of branched chain

amino acids to aromatic amino acids following its use. There was no significant removal of physiologically important proteins (albumin, α-1-glycoprotein, α-1-antitrypsine, α-2-macroglobulin, transferrin) or hormone-binding proteins (thyroxine binding globulin), nor was there a significant change of hormone systems (assessed using the thyroid hormone profile). There was, however, a significant removal of all the albumin-bound toxins, which was most effective for fatty acids, followed by bile acids, tryptophan and bilirubin. This study thus attempted to demonstrate that MARS was relatively selective for albumin-bound toxins.

1.2.3.2 MARS: Clinical application

Acute-on-chronic liver failure

In the initial clinical trials MARS has predominantly been evaluated in the context of ACLF, even though most studies were small and uncontrolled. Improvement of hyperbilirubinaemia, HE, circulatory and renal functions have been observed[74, 95, 96]. The improvement of HE has been associated with a reduction of serum ammonia levels, decrease of intracranial pressures and increase of cerebral perfusion pressures[74, 75, 95, 96]. Circulatory changes following MARS treatment have been manifest in the form of increased mean arterial pressure and systemic vascular resistance and decreased cardiac output[72-74, 95]. One preliminary report described an acute reduction of portal pressure in four patients with ACLF after the first session of MARS, which increased before the second session but again underwent reduction, albeit less steep, after the second session[97].

The largest series of patients with ACLF (n=26), with intrahepatic cholestasis (bilirubin level > 20 mg/dL), treated with MARS has been reported from Rostock[98]. The series included 10 patients with a United Network Organ Sharing (UNOS) status 2b, all of whom survived, and 16 patients with a UNOS status 2a, of whom 7 survived.

The first randomized trial of MARS evaluated 13 ACLF patients with type-I hepatorenal syndrome who were treated with either MARS (n=8) or standard medical therapy including haemodiafiltration (n=5)[72]. The mortality rate was 100% in the group receiving haemodiafiltration at day 7 compared with 62.5% in the MARS group at day 7 and 75% at day 30, respectively ($P < 0.01$). Mean survival was longer in the MARS group, which was accompanied by a significant decrease in serum bilirubin and creatinine, and increase in serum sodium and prothrombin activity. MAP at the end of treatment was significantly greater in the MARS group. Although urine output did not increase significantly in the MARS group, 4 of the 8 patients showed an increase compared with none of the control group.

The most recent (and largest completed) randomized controlled trial, performed in two centres (Rostock and Essen), included 24 patients with ACLF with marked hyperbilirubinemia (serum bilirubin>20mg/dl [340μmol/L]) who were randomized to receive

standard medical therapy alone (n=12) or MARS in addition (n=12)[99]. The primary end point of bilirubin<15 mg/dL for 3 consecutive days was reached in 5 of 12 MARS patients and in 2 of 12 control patients. Compared to controls, bilirubin, bile acids and creatinine decreased and MAP and HE improved in the MARS group. Most importantly, albumin dialysis was associated with a significant improvement in 30-day survival (11/12, versus 6/11 in controls).

Acute liver failure

In the context of ALF, no controlled studies have been performed as yet, which is not surprising considering the difficult nature of this task. Outcome data are available from three centres- Rome, Helsinki and Gothenberg. Novelli et al[100] from Rome have treated 9 cases of fulminant hepatic failure. 3 patients survived without requiring transplanation. The remaining 6 were transplanted, of whom 4 survived, while 2 died due to sepsis. The authors have extended the series to 16, in whom they report improvement of serum bilirubin, INR and ammonia as well as neurological status (though outcome is not described)[101]. Isoniemi[102] (Helsinki) has reported 26 cases of ALF (13-toxic (including paracetamol), 1-pregnancy induced, 12-unknown aetiology) managed with MARS. 20 of the 26 patients (77%) survived, which is a strikingly high proportion. Native liver recovered in 11 cases, 8 of whom had a toxic aetiology. 10 patients were transplanted, of whom 9 survived. Haemodynamic and neurological improvements were noted following MARS therapy in most cases. While these results are quite encouraging, especially in those with a toxic aetiology, it is to be noted that at least in some of these cases MARS was started before the development of encephalopathy, and the improvement noted in the absence of matched controls is difficult to interpret. Felldin et al (Gothenberg) describe 10 patients of ALF treated with MARS, of whom 7 survived. Beneficial effect was most evident in those who received 5 or more sessions of treatment (4/5 survivors)[103, 104].

Among other studies, one has described improvement in encephalopathy, a reduction of cerebral oedema and intracranial pressure, with increase of cerebral perfusion pressure in three ALF patients treated with MARS[105]. A recent small randomised controlled study in patients with hyperacute liver failure found that a single session of MARS treatment (n=8) improved systemic haemodynamics (mean arterial pressure, systemic vascular resistance and cardiac output) compared to controls (n=5), who had only been mechanically cooled to match the MARS group[106]. A phase I trial from the US evaluated a slightly modified system using continuous albumin dialysis with continuous haemodiafiltration in 9 ALF patients (UNOS status-I: 5; status-IIA: 4)[89]. Of those with status-I, one recovered native hepatic function while 3 were bridged to transplantation.

There are also individual case reports of the use of MARS (n=4) [107, 108] or albumin dialysis (n=1)[109] to treat Wilson's disease with ALF (grade 3-4 encephalopathy, oligo-anuria,

coagulopathy), all of whom were successfully bridged to transplantation. MARS has also been used to treat ALF due to paracetamol overdose (n=3)[107, 110, 111] and mushroom intoxication (n=3)[107, 112, 113], where all survived without requiring transplantation, while a case of acute Budd-Chiari syndrome died in spite of MARS and transplantation[107]. A recent series of 5 MARS-treated paracetamol overdose patients report survival in 4, with one patient dying of brain oedema[114].

Other indications

Results with MARS in the treatment of primary graft dysfunction following liver transplantation are also limited. One study[115] reported six such patients (primary non-function: n=4, delayed non-function: n=2), where MARS treatment led to a recovery in five cases, eliminating the need for re-transplantation. The sixth patient died. Another study[107] reported two patients with liver failure following transplantation, one due to primary non-function and the other due to severe graft dysfunction, both of whom were bridged to re-transplantation with MARS. The former died following sepsis, while the latter survived. Data available from four other centres describe eight cases of primary graft dysfunction treated with MARS, of whom three recovered and four were successfully bridged to re-transplantation[116].

There have been reports of the use of MARS for the treatment of liver failure following hepatic resection (n=7)[107, 117-119], but only one such patient survived[107]. There have also been anecdotal reports of the use of MARS in progressive intra-hepatic cholestasis due to chronic graft-versus-host disease (n=1)[120], and in patients with heart failure, complicated by liver failure, awaiting heart transplantation[121].

Over the last couple of years, a new clinical indication for the use of MARS therapy is emerging. Intrahepatic cholestasis with intractable pruritus can be an extremely debilitating condition, severely impairing the patient's quality of life. Therapeutic options are limited for cases which fail to respond to currently available medications. In this setting, MARS appears to have a rapid, significant and reasonably well-sustained response[122-124]. Mullhaupt et al described rapid resolution of pruritus following a single session of MARS in a patient with primary biliary cirrhosis, but with recurrence of symptoms within five days[123]. Macia et al described a similar improvement with MARS in two patients with primary biliary cirrhosis and one patient with post-transplantation biliary stenosis, with the effect persisting for 3-8 weeks[125]. In one of these patients, out-patient follow-up with a MARS session every few months (after the intial therapy) was successful in achieving long-term improvement. Doria et al reported three patients with hepatitis C-virus related intractable pruritus, treated with seven sessions of MARS, where an improvement persisting for several months was noted[124]. Bellmann et al recently described seven patients with post-transplantation intractable pruritus

who were treated with three consecutive sessions of MARS[126]. Six patients responded, and the improvement was sustained for >3 months in three of them. The last two studies also demonstrated an associated reduction of serum bile acids, implicating this as a potential mechanism of the response. However, removal of plasma opioids such as met-enkephalins[127] may be responsible as well.

1.2.3.3 Safety profile

The safety of this device has been evaluated through its use in over 3000 patients worldwide. The MARS Registry, which is maintained by the University of Rostock, contains data on about 500 patients treated with this device[128,129]. In general, the treatment is well-tolerated and the only consistent adverse finding with the use of MARS is thrombocytopenia. Critical analysis of the data from the Registry, in patients with ACLF suggests that its use should be contraindicated in those with established disseminated intravascular coagulation (DIC) or in those patients with 'incipient' DIC characterised by progressive thrombocytopenia and coagulopathy.

1.2.3.4 Pathophysiological basis

The pathophysiological basis of the development of the clinical manifestations ACLF is only poorly understood. Similarly, very little is known about the mechanisms underlying the observed clinical improvements following MARS therapy. The improvement in HE may be due to the associated significant reduction of serum ammonia levels[74, 95, 96], as a result of direct removal by the MARS system. Another possible explanation might be a favourable alteration of the amino acids profile, with relative clearance of aromatic amino acids, as had been observed in the early *in vivo* studies[90]. A reduction of plasma nitrates and nitrites following MARS, probably due to direct removal by the system, may contribute to the improvement of the circulatory status. Whether production of nitric oxide is altered as well following therapy is as yet not known. This systemic haemodynamic improvement may, at least in part, be responsible for the associated improvement of renal function. An alteration in the profile of inflammatory mediators and cytokines, either by removal by the MARS circuit, or by altered production due to an 'improved' internal environment following blood purification by MARS, might conceivably have a role to play. The ability of MARS to reduce oxidative stress might also have an important role to play.

1.2.4 Other systems currently available

1.2.4.1 Prometheus

The fractionated plasma separation and adsorption (FPSA)[130] system, introduced in 1999, has been presented as albumin dialysis, but in reality utilises somewhat different principles- fractionation of the plasma with the subsequent detoxification of the native albumin by adsorption. It uses an albumin-permeable membrane with a cut-off of 250 kDa.

Albumin, and possibly other plasma proteins with their bound toxins cross the membrane and pass through special adsorbers (one or two columns in series in the secondary circuit, containing a neutral resin adsorber and an anion exchanger) that remove the toxins. The cleansed albumin is returned to the plasma. In the commercially produced Prometheus system (Fresenius Medical Care AG, Bad Homburg, Germany)[131] (Fig 6) the FPSA method is combined with high-flux haemodialysis (of the blood directly, as opposed to the MARS system, where haemo-dialysis/ filtration of the albumin dialysate is performed).

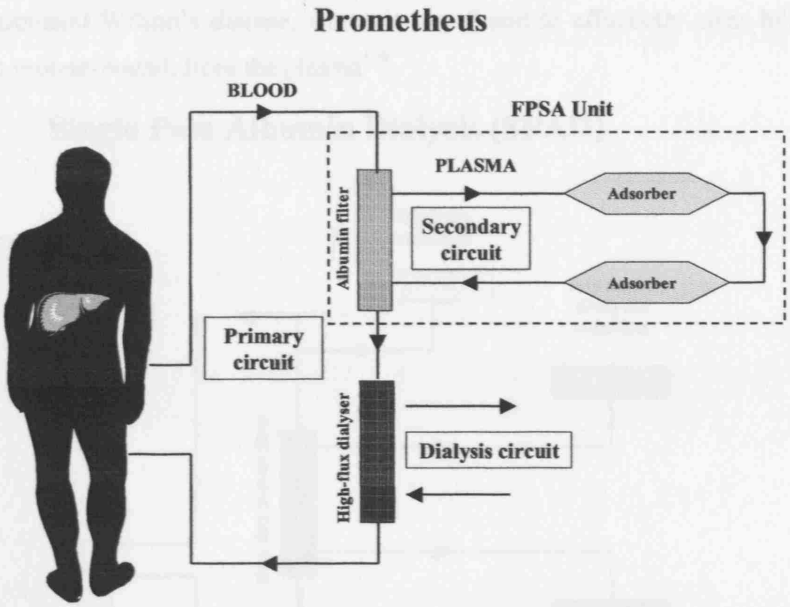


Fig 6: Schematic diagram of the Prometheus circuit, utilizing the principle of fractionated plasma separation and adsorption (FPSA). The plasma is fractionated across an albumin-permeable membrane, with the subsequent detoxification of the native albumin by adsorption of bound toxins across one or two adsorber columns (containing a neutral resin adsorber and an anion exchanger) in the secondary circuit. The cleansed albumin is returned to the plasma. The reconstituted blood subsequently undergoes high-flux haemodialysis before being returned to the patient.

The results of Prometheus treatment in 11 patients with ACLF and accompanying renal failure have recently been published[131]. Improvement of serum levels of conjugated bilirubin, bile acids, ammonia, cholinesterase, creatinine, urea and blood pH occurred. A drop in blood pressure in two patients, and uncontrolled bleeding in one patient were the adverse events noted. Another study compared alternating treatments with MARS and Prometheus in five patients with ACLF. Reduction ratios of both bilirubin and urea were more with Prometheus. Their safety profiles were found to be comparable[132]. Prospective controlled trials are planned for the future.

1.2.4.2 Single Pass Albumin Dialysis (SPAD)

The newly-developed SPAD system (Fig 7) dialyses blood/ plasma against a 4.4% solution of albumin, which is disposed of after a single pass. A standard renal replacement

therapy machine is used without any additional perfusion pump system, making the equipment required simpler. This fact, and the use of considerably more diluted albumin as the dialysate (4.4%, as opposed to 20% in case of MARS), offsets the cost of not recirculating the dialysate. Continuous veno-venous haemodiafiltration can be undertaken in conjunction as well. *In vitro* studies suggest that its detoxifying capacity is similar to, or even greater than (especially with regard to bilirubin and ammonia clearance) that of MARS[133]. However, would these results hold up *in vivo* too? As of now, the only clinical use reported has been in a case of fulminant Wilson's disease, where it was found to efficiently clear bilirubin and copper, both protein-bound, from the plasma[109].

Single Pass Albumin Dialysis (SPAD)

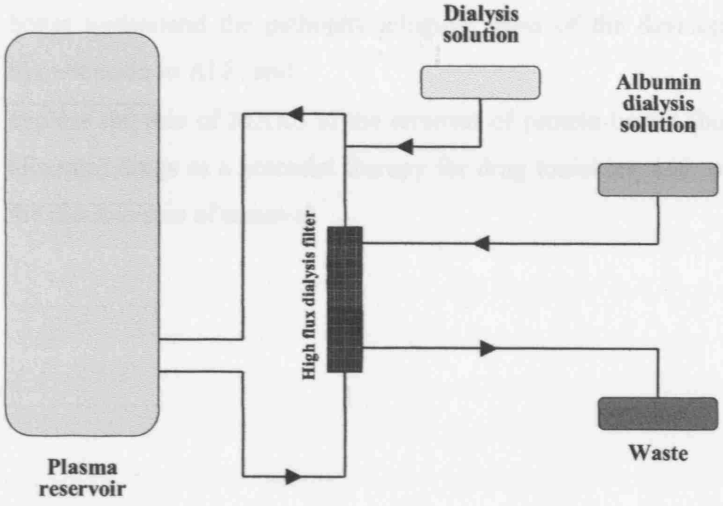


Fig 7: Schematic diagram of the single pass albumin dialysis (SPAD) system, as used in *in vitro* studies. Plasma from a reservoir is dialysed against a 4.4% solution of albumin, which is disposed of after a single pass. A standard renal replacement therapy machine is used to run the circuit.

1.2.5 Conclusion

Among the liver support systems currently available, albumin dialysis using MARS is the one which has been most extensively investigated. It consistently improves hyperbilirubinaemia and HE, and most importantly, early data in patients with ACLF suggest that survival may also be improved. However most of the studies conducted so far have been small, usually uncontrolled, and the exact mechanisms underlying the clinical effects are poorly understood. At present, a multi-centre randomised controlled trial with ACLF patients is being conducted in Europe, while another in HE patients is being carried out in the US, to gain a more definite perspective of the efficacy of MARS.

2. AIMS

The aims of the set of studies described here were to

- evaluate the clinical impact of albumin dialysis using the Molecular Adsorbents Recirculating System (MARS) in patients with severe alcoholic hepatitis leading to acute-on-chronic liver failure (ACLF);
- investigate the impact of MARS dialysis on portal, hepatic and systemic haemodynamics in ACLF;
- understand the pathophysiological basis of the clinical effects observed in ACLF patients treated with MARS;
- use a large animal model of acute liver failure (ALF) to study the effect of MARS on the cerebral changes of ALF, focusing on intracranial hypertension, and thereby better understand the pathophysiological basis of the development of intracranial hypertension in ALF; and
- explore the role of MARS in the removal of protein-bound (both albumin and non-albumin) drugs as a potential therapy for drug toxicities, and as a tool to understand the mechanisms of removal.

3. METHODS

3.1 Human studies

3.1.1 Monitoring and 'Standard medical therapy'

The patients were evaluated clinically and biochemically (including liver and renal function tests, coagulation profiles) both prior to and after each MARS treatment. The Child Pugh score[4], Model for End-stage Liver Disease (MELD) score[5], Maddrey's discriminant function (DF)[134], APACHE II[135] and SOFA[136] scores were calculated at the same time. These scoring systems have been validated to be good predictors of outcome in patients with liver disease and also in patients that are admitted to the intensive care unit with ACLF. The severity of HE was assessed using the West Haven criteria[137]. HRS was defined according to the Ascites Club criteria[27]. Patients were mechanically ventilated if they became hypoxemic. Mean arterial pressure (MAP), 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. Wedged pulmonary artery pressure and cardiac output were measured using a Swan-Ganz catheter (Edward Lifesciences, Irvine, CA) in 3 patients prior to and after the first MARS session. Intravascular volume was maintained using crystalloids, colloids or red cells as appropriate to maintain central venous pressure between 8 and 10cm H_2O. Renal support was instituted with continuous veno-venous haemofiltration (Hospal BSM 22c, France), with blood flow of 150ml/min and 1 litre cycle exchanges where appropriate. Terlipressin was not used for the treatment of HRS because it is not licensed for this indication in the UK. Noradrenaline was used to maintain MAP above 55 mmHg where necessary. Blood glucose was maintained between 5-7mmol/L. Tense ascites was treated with paracentesis and adequate volume replacement[138]. Concomitant infection and spontaneous bacterial peritonitis was treated with appropriate antibiotics and variceal bleeding was treated with endoscopic band ligation. Hepatic encephalopathy was treated with lactulose and bowel enemas[139].

3.1.2 MARS Treatment

The MARS system (Teraklin AG, Rostock, Germany) has been described in details previously and elsewhere[90, 96]. Briefly, it consists of 3 compartments - a blood circuit, an albumin circuit and - in our case – a continuous veno-venous haemofiltration compartment (Hospal BSM 22c, France).

3.1.2.1 ***Priming:*** All three compartments were primed with 0.9% saline and made air-free. To prime the blood compartment (approximate capacity 260 ml), 2 l of saline with 10000 IU heparin added was used, so that the inner walls of the tubing would be coated with a layer of heparin to prevent clotting within the extracorporeal circuit. If the patient's INR

was >1.4, no heparin was used during priming. 600 ml of 20% human albumin (Zenalb, BPL, UK) was used to replace the saline in the albumin circuit. The entire process of assembling and priming the circuit took 75-90 mins.

3.1.2.2 ***Operating MARS:*** The blood circuit uses a veno-venous access, and is driven by the blood roller pump of the haemofiltration monitor at 150 ml/min. The albumin circuit is driven by the roller pump of the MARS monitor at 150 ml/min. The volume of ultrafiltrate removed (at 1 l/hr) was replaced into the blood-circuit (at 1 l/hr using Hemovex 2, Hospal, France) proximal to the MARS membrane. Heparin was used as anticoagulant and administered at a dose of 0 to 1000 IU/h (guided by the patient's coagulation profile) to prevent clotting of the extracorporeal circuit.

3.1.2.3 ***Duration of treatment sessions:*** Each MARS session was continued for a duration of 6-8 hours, depending on the specific study protocol.

3.1.2.4 ***Technical problems encountered:***

a. Blood leak across the MARS membrane into the albumin circuit due to damaged membrane (two occasions)
b. Clotting within the extracorporeal blood circuit (four occasions)
c. Pressure rise within the circuit due to mal-positioned/ obstructed vascular access ('vascath' problems, encountered with same frequency as in performing any renal replacement therapy)

3.1.3 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. Patients were sedated for the procedure using midazolam (mean dose of 3 mg; Phoenix Pharma Ltd., Gloucester, UK). None of the patients in whom haemodynamic measurements were made were on beta- blockers.

3.1.3.1 ***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 catheterisation and every 5 minutes thereafter (Hewlett-Packard, Model 86S, HP, Palo Alto, CA, USA). The pulmonary artery was catheterised 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.

3.1.3.2 ***Hepatic haemodynamics:*** *(a) Hepatic venous pressure gradient-* 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. The coefficient of variation between HVPG measurements was 4% for the study group. The hepatic venous catheter was kept in place for 24 hours and thus HVPG was measured before, during and after extracorporeal therapy.

(b) Hepatic blood flow- Hepatic blood flow (HBF) was determined using a prime (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 by standard techniques. Plasma flow rate of the liver was calculated using formulae based on the method of indicator dilution and Fick's principle[140, 141] and was converted to blood flow using the haematocrit.

(c) Intrahepatic vascular resistance- Intrahepatic vascular resistance (IHVR) was calculated from HVPG and HBF:-

$$\text{IHVR } (dyne.s.cm^{-5}.10^{-3}) = 80 \times 10^{3} * \text{HVPG } (mm\ Hg) / \text{HBF } (ml/min)$$

3.2 Animal studies

3.2.1 Animal model of acute liver failure

A large animal model of acute liver failure induced by hepatic devascularisation was studied. Female Norwegian Landrace pigs weighing 23-30 (26.8±0.3, mean ± SEM) kg were used. Details regarding the animal room facilities, anesthesia and surgical preparation has previously been reported [142-146].

3.2.1.1 Animal room environment: The pigs were kept in the animal department for at least 2 days before the experiments, at a temperature of 21±1°C, a relative humidity of 55±10%, and with a 12:12-hr light/dark cycle. The animals were fed with Combi Fri chow (Felleskjøpet, Trondheim, Norway), but were fasted overnight prior to the experiment.

3.2.1.2 Premedication & anaesthesia: The pigs were premedicated with an intramuscular injection of ketamine (20mg/kg) and atropine (1mg). Anaesthesia was induced with an

intravenous bolus of 10mg/kg pentobarbital (Pentobarbital; Nycomed Pharma, Oslo, Norway) and 10mg/kg fentanyl (Leptanal; Janssen Pharmaceutica, Beerse, Belgium) and maintained during surgery with a central venous infusion of 4mg/kg /hr pentobarbital, 0.02mg/kg/hr fentanyl, and 0.3mg/kg/hr midazolam (Dormicum; Roche, Basel, Switzerland). Anaesthesia was stopped after the liver was devascularised. If there were clinical signs of light sedation, small doses of fentanyl and midazolam were given as a bolus. During MARS treatment, the animals were kept sedated by a continuous infusion of 0.04mg/kg/hr fentanyl, and 0.6mg/kg/hr midazolam, with additional bolus doses given when clinically indicated.

3.2.1.3 Animal preparation: The pigs underwent a tracheostomy, were intubated and ventilated (FiO_2=0.5) on a volume-controlled respirator (Servo 900, Elema-Schönander, Stockholm, Sweden). Tidal volume was adjusted by means of repeated arterial blood gas analyses to maintain $PaCO_2$ between 4.5 and 5.0 kPa. Core body temperature was maintained at 38.5±1°C with a heating pad and blankets. All animals received 500mL 0.9% NaCl containing 625mg of glucose as a preoperative load in order to prevent any preoperative dehydration. During the experiment, 0.9% NaCl was infused at a rate of 3ml/kg/hr. After the induction of hepatic devascularisation (ALF and ALF+MARS groups), 50% glucose and 20% human albumin (Octapharma, Hurdal, Norway) were continuously infused at rates of 0.6048ml/kg/hr and 0.66ml/kg/hr respectively. 2500 IU heparin was given intravenously to all pigs at the start of the experiment. Subsequently, heparin was given to keep the activated clotting time (ACT)>100 sec. In the ALF+MARS group, during MARS dialysis, additional heparin was given to keep ACT>180 sec.

3.2.1.4 Devascularisation of liver: A midline incision was made from the xiphoid to the pubis. The portal vein was dissected free of surrounding tissue and lymph nodes from the porta hepatis to the confluence of the splenic vein. The inferior vena cava immediately cranial to the renal veins was cleared and clamped with an exclusion clamp, and a 1.5 cm longitudinal incision was made. The portal vein was then clamped, transposed and anastomosed end-to-side to the inferior vena cava, using a continuous over-and-over polypropylene 5-0 suture. The total period of portal vein occlusion was between 11 and 15 min. During this time, and for a further 10 min, 1000 ml of 0.9% NaCl was infused to every animal in order to maintain the arterial pressure. Dissection of the structures in the hepatoduodenal ligament was performed carefully to ensure that the arterial blood supply to the liver was completely interrupted. The common bile duct was preserved, but the hepatic artery was tied by two polyglycolic 2 ligatures. Finally, the abdominal wound was closed in two layers with polyglycolic acid 2-0.

3.2.1.5 Catheter placement: A very thin catheter developed by Ten Have et al [140, 147] was introduced into the abdominal aorta. MARS was performed through an 11.5Fr dual lumen

catheter (Mahurkar, Tyco Healthcare, UK) in the inferior vena cava (positioned during the abdominal surgery). ALF and sham animals also received a vena caval catheter to be comparable. A 7Fr pigtail cathether (Cordis, Johnsen & Johnsen, Miami, FL) was inserted via the left carotid artery into the left ventricle of the heart for injection of microspheres. Position was confirmed by online pressure curves.

3.2.2 MARS dialysis

The MARS albumin circuit was set up as described earlier. However, as the porcine study was designed to specifically evaluate the role of albumin dialysis in ALF, haemo-filtration/dialysis was not performed (i.e. no additional removal of free water/ water-soluble toxins other than what might be removed by a closed-circuit recirculating albumin dialysis), and the renal part of the circuit was clamped off. Thus, this was a 'modified' MARS circuit. Albumin recirculation continued as usual (Fig 1). A blood pump (Stöckert Shiley roller pump, Stöckert Instruments, Munich, Germany) was used to run the blood circuit at 150ml/min, with the albumin dialysate circulated by the MARS pump also at 150ml/min. The same pump speed as used in adult humans was used for these lighter subjects (~27kg) to compensate for the shorter dialysis duration (usually~8hrs), following pilot observations that haemodynamically the animals tolerated these flow rates well.

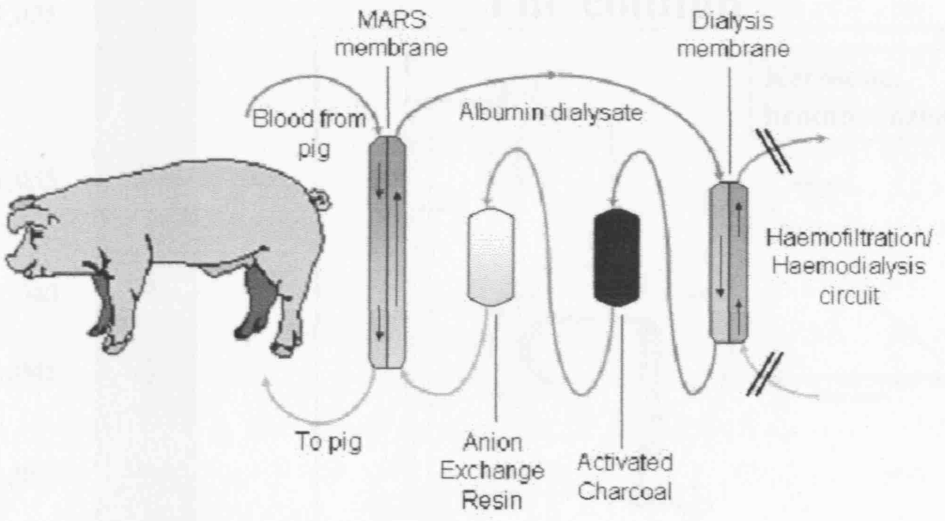


Fig 1: The modified MARS circuit used in the animal studies.

3.2.3 Measurements of cerebral changes

3.2.3.1 ICP monitoring: A burr hole was created over the right frontal region of the skull (2cm lateral, 1cm rostral from bregma and 1 cm ventral), the dura mater was incised and a stable drift-free ICP transducer for continuous measurements (Codman MicroSensor, Johnson

& Johnson, MA, USA) was inserted into the frontal cortex. The burr hole was then sealed with bone wax to ensure stable positioning of the catheter and to prevent pressure release. Monitoring of ICP has been described in detail elsewhere [142-145]. Calibrated transducers (Transpac 3, Abbott Critical Care Systems, Chicago, IL, USA) were used for continuous pressure measurements. The transducers were connected to an amplifier (Gould ES 2000, Valley View, Tex., USA). Pulsative signals were displayed on a monitor (Gould), digitalized, and stored electronically (Macintosh Quadra 950, Apple Computers, Cupertino, CA, USA). The sampling rate for all channels was 0.25 Hz. Average values from the last 5 minutes before each time-point were used for analysis of the continuous data. For graphic presentation and calculations the on-line recorded data were reduced to intervals of 1 min by the data acquisition program Labview version 3.1.1 (National Instruments, Austin, Tex., USA) and exported to an Excel spreadsheet (Microsoft Corp., Redmond, WA, USA). After the experiments were terminated, a craniotomy was performed and the brain was examined for any intracranial haemorrhage.

3.2.3.2 Measurement of brain water: This was performed by a simple gravimetric technique and is illustrated in Fig 2. This allows the percent gram water per gram tissue of samples less than 2 mm^3 to be determined within minutes to an accuracy of greater than 1% [148].

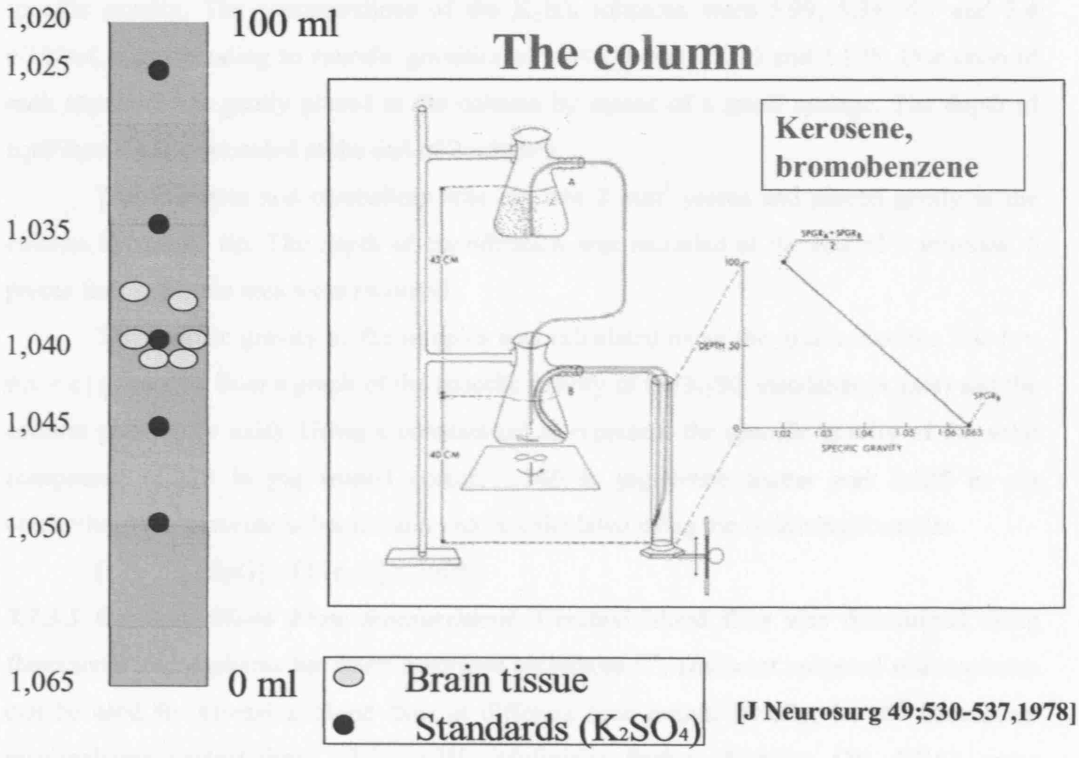


Fig 2: A gravimetric technique of the measurement of brain water.

Two mixtures of kerosene and bromobenzene were used in the preparation of the gradient. One litre of mixture A was prepared from 735.6 mls of kerosene and 264.4 mls bromobenzene to give a specific gravity of 0.9750. One litre of mixture B was prepared from 608.8 mls kerosene and 391.2 mls of bromobenzene to give a specific gravity of 1.0650. A flask containing 100mls of mixture B was placed 40 cm above an empty 100ml graduated cylinder. 100mls of mixture A was then placed 43cm above mixture B.

The fluid kinetics of this system are such that a linear gradient column is produced if the outflow from the constantly mixed Mixture B to the graduated cylinder is exactly twice the outflow from A to B. This was accomplished by using equal lengths of polyethylene outflow tubing of length 90 cm and internal diameter 0.76mm from flask B to the graduate and a single length of tubing from Flask A to Flask B. By this technique, the specific gravity at the very bottom of the graduated cylinder is equal to that of Mixture B (1.065), while the specific gravity at the top is equal to the arithmetic mean of the two solutions (1.020). A steady flow to the surface of the graduated cylinder was maintained by gradually lowering the cylinder in 2 mm increments as the fluid level increased. When a cylinder volume of 100ml was reached the tubes were clamped. The gradient was then permitted to stabilize for 15 minutes and calibrated with standards made up of potassium sulphate (K_2S0_4) of known specific gravity. The concentrations of the K_2S0_4 solutions were 5.99, 5.34, 4.7 and 3.4 g/100ml, corresponding to specific gravities of 1.045, 1.040, 1.030 and 1.025. One drop of each standard was gently placed in the column by means of a small syringe. The depth of equilibration was recorded at the end of 2 minutes.

The forebrain and cerebellum was cut into 2 mm^3 pieces and placed gently in the column by needle tip. The depth of equilibration was recorded at the end of 2 minutes. 6 pieces for each brain area were recorded.

The specific gravity of the samples was calculated using the gradient of the line ($y= mx + c$) generated from a graph of the specific gravity of the K_2S0_4 standards (x axis) and the column position (y axis). Using a constant (c) to represent the specific gravity of the solid component (1.238 in pig frontal cortex, 1.146 in pig white matter and 1.208 in pig cerebellum) the percentage brain water can be calculated using the following formula:

$$[c/(c-1)] / SpG] - [1/(c-1)] \times 100\%$$

3.2.3.3 Cerebral Blood Flow measurement: Cerebral blood flow was determined using fluorescent microspheres has been described elsewhere [149]. Different coloured microspheres can be used for assessing blood flow at different time points. Briefly, 1x 10^6 fluorescent microspheres (polystyrene, 15.5μm±2%; Molecular Probes, Eugene, OR, USA), were injected via the left ventricular catheter. Simultaneously a reference arterial sample was collected from the arterial cannula at 4ml/min x 2mins (Harvard withdrawal pump, Harvard

Apparatus, Millis, MA, USA) to allow the calculation of absolute blood flow. At the end of the experiment brain tissue samples (forebrain, cerebellum) were collected and stored at -80°C until analysis. Samples were weighed before digestion in ethanolic KOH (Sigma, Poole, Dorset, UK) in a 60°C oven over 24 hours, before vacuum filtration through an 8μm polycarbonate membrane (Kunstoff, Grafenhausen, Germany) [150]. The filtered microspheres were washed with 1% Triton- X100 (Sigma, Poole, Dorset, UK) and stored in the dark until measurement. Immediately prior to measurement, the microspheres were dissolved in diethylene glycol monoethyl ether acetate (Sigma, Poole, Dorset, UK) and analysed using a fluorescence spectrometer (LS50B, Perkin-Elmer, Norwalk, CT, USA) and an autosampler and diluter station using the common autosampler software (models AS90/91and DS6, respectively, FLWinLab, Perkin-Elmer). Values are expressed as ml/min/100g tissue (wet weight).

3.3 Sample analysis

3.3.1 Measurement of ammonia in plasma

A modified Bethelot assay (indophenol) was used to determine ammonia levels in plasma[151]. All chemicals were obtained from Sigma- Aldrich (Poole, Dorset, UK) and were of the highest laboratory grade. 50 μL of plasma was mixed with 150 μL of 5% trichloroacetic acid and spun in a microcentrifuge at 10,000 rpm at 4°C for 10 minutes. 50 μL of the supernatant was pipetted out onto a 96 well plate (Maxisorb, NUNC, Denmark). To this was added 50 μL of a phenol (3.5% w/v) nitroprusside (0.04% w/v) solution and 50 μL of a solution containing sodium hydroxide 1.8% w/v and 250 mM sodium hypochlorite.

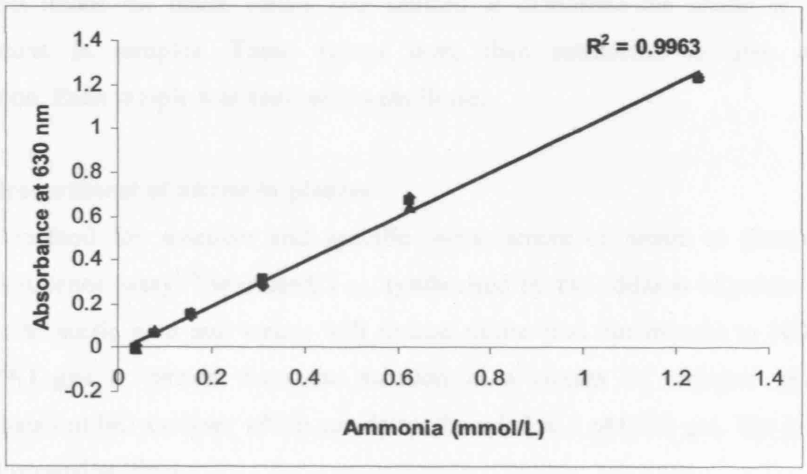


Fig 3: A typical ammonium chloride standard curve

The plate was then covered and placed in a 50 °C oven for 60 minutes. The blue colour formed by reaction of ammonia with phenol and hypochlorite can be measured

colorimetrically at 630nm. This was read in a 96 well plate reader (Tecan Sunrise, Salzburg, Austria). Each sample was analysed in duplicate and compared to an ammonium chloride standard curve (Fig 3).

3.3.2 Measurement of nitrate and nitrite in plasma

A method for simultaneous detection of nitrate and nitrite in plasma with a modified Greiss reaction, as described by Miranda et al[152] 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_20. Greiss Reagent B was prepared from sulfanilamide (2% w/v) in 5% hydrochloric acid. All three solutions were stored in the dark.

100 µ/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 Giovannoni et al[153]. For the measurement of nitrate, 50 µ/L of the filtrate was pipetted out into a 96 well plate (Maxisorb, NUNC, Denmark). To this were added 50 µ/L of vanadium (III) chloride solution, 50 µ/L of Greiss Reagent A, and 50 µ/L of Greiss Reagent B, 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 NOx concentrations in samples. These values were then subtracted to give the nitrate concentration. Each sample was analysed in duplicate.

3.3.3 Measurement of nitrite in plasma

A method for sensitive and specific measurement of nitrite in plasma using a chemiluminescence assay[154] was used. I_3-, 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 Fig 4.

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_20. 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 illustrated in Fig 4. The system was then allowed to reach steady baseline.

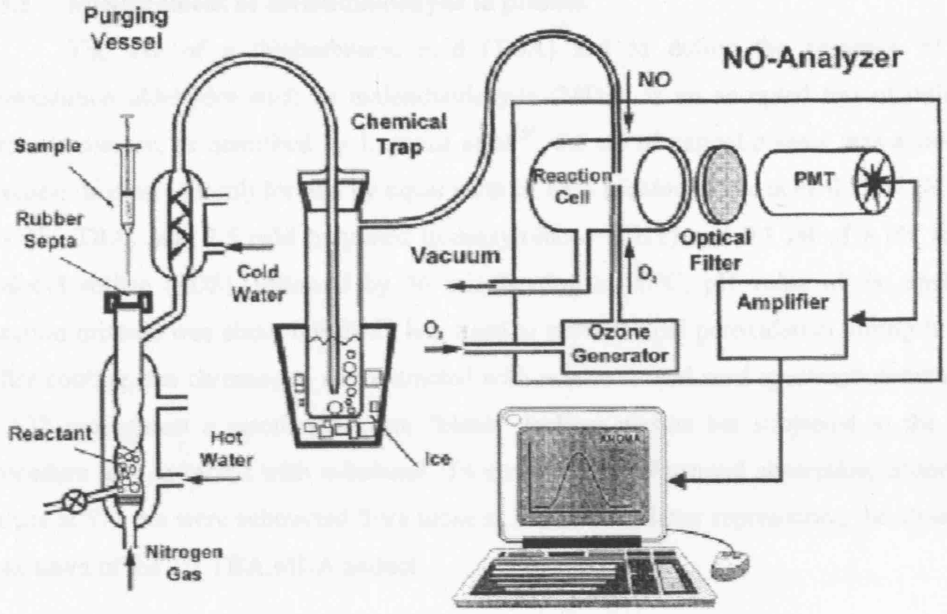


Fig 4: A schematic diagram of the chemiluminescence instrumental setup

100 µ/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[153]. 10 µL 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.

3.3.4 Measurement of cytokines/chemokines in plasma

Capture antibodies for TNFα, interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and soluble TNF receptors I and II (TNF-RI, TNF-RII) (BioSource International, Nivelles, Belgium) were coated at 1 µg/ml onto 96 well Maxisorb (NUNC, Denmark) ELISA plates. The plates were blocked with bovine serum albumin (Fraction V, Sigma, Poole, Dorset, UK) before the addition of 100 µl standard or sample together with biotin-conjugated detection antibody (100 µl; 0.4 µg/ml). After incubation at room temperature for 2h and washing, streptavidin was added before incubation with substrate (o-phenylenediamine HCl).

The reaction was stopped with 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. After a single batch analysis, the intra-assay coefficient of variation was 5.4%.

3.3.5 Measurement of malondialdehyde in plasma

The use of a thiobarbituric acid (TBA) test to define the presence of lipid peroxidation aldehydes such as malondialdehyde (MDA) is an accepted test of oxidative stress in disease, as described by Lapenna et al[155]. 0.5 ml of patient plasma 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 nm against a reaction mixture "blank" 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.

3.3.6 Measurement of oxygen-based free radicals in plasma

Plasma levels of oxygen-based free radical production were measured using a spin trap (α-phenyl N-tert-butyl nitrone, Sigma, Poole, Dorset, UK) in saline (0.125 mol/l, 1ml) added to venous blood (4ml). The spin-trap was then extracted into toluene and dried using a centrifugal concentrator (Eppendorf 5301, Cambridge, UK) before freezing in liquid nitrogen. Samples were reconstituted into deoxygenated toluene (100μL), placed into a quartz tube and measured using electron paramagnetic resonance (EPR) spectroscopy (Bruker EMX, Karlsruhe, Germany). The conditions were:- power 12.66mW, frequency 9.467GHz, Mod. Amp. 8G, Time const. 40.96ms, temperature 20°C. The separation between sets of peaks in the EPR spectrum (coupling constant) was used to identify the lipid radical species trapped. These were identified as carbonyl ($L^{\cdot}$) and alkoxyl ($LO^{\cdot}$) free radicals[156].

3.3.7 Measurement of F2-isoprostanes in plasma

Free 8-Isoprostane F2alpha was assayed with a commercial EIA kit (Cayman Chemical, Ann Arbor, MI) according to manufacturers instructions[157, 158]. Briefly, 200μl plasma was deproteinised with 600μl ethanol containing 8KBq ^{3}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 applied to a preconditioned C18 SPE cartridge

(Waters Corp, Milford, MA). The column was washed with water, dried with nitrogen and eluted with 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 ^{3}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.

3.3.8 Drug concentration analysis

Total and free plasma concentrations of midazolam and fentanyl were measured by high-pressure liquid chromatography (HPLC)-coupled mass-spectrometry.

HPLC grade methanol (J.T.Baker, Deventer, Holland), methylclonazepam (F. Hoffmann La-Roche AG, Switzerland), sulphuric acid and chloroform (Merck-Schuchardt, Germany) were used. Water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Standard stock solutions were prepared in methanol (15µM for fentanyl, 1.5mM for midazolam), and stored at -20°C. Plasma, albumin and normal saline standard samples were prepared by addition of stock solutions.

Before analysis samples underwent ultrafiltration: 150µl sample was added to Amicon Centrifree (Millipore Bedford, MA, USA) tubes and centrifuged (1500g, 12 min, 37°C). The ultrafiltrate was collected and stored for less than one week at -20 °C. For analysis, 100µl of sample was mixed with sulphuric acid (50µl 0.05M), internal standard (50µl, 1µM methylclonazepam) and chloroform (1.5ml). The sample was mixed and then centrifuged (1500g, 5 min, 20°C), 1ml of the organic phase transferred to a clean tube and evaporated to dryness under a nitrogen gas stream at 35°C. The residue was dissolved in 150µl mobile phase (40% methanol in 5mM aqueous formic acid). HPLC was performed on a model 2695 (Waters, Milford, MA, USA) separation system with a MS C18 3.5µm 2.1x10mm guard column (Waters); triple quadrupole tandem mass spectrometer, Quattro Micro (Micromass Inc., Manchester, UK); data system, MassLynx version 3.5 (Micromass). Ionisation mode was positive ion electrospray. Quantitative analysis was performed by multiple reaction monitoring (MRM). Injection volume was 3-8µl with an injection interval of 2.5 min. Autosampler temperature was 10°C; desolvation gas temperature 300°C; source temperature 120°C; desolvation gas flow 600L/h; cone gas flow, 30L/h; collision gas pressure 4 x 10^{-3}mBar (argon); ion energies were set at 1.0V for both quadrupoles.

4. STUDY 1- CLINICAL IMPACT OF EXTRACORPOREAL LIVER SUPPORT WITH MOLECULAR ADSORBENTS RECIRCULATING SYSTEM IN PATIENTS WITH SEVERE ACUTE ALCOHOLIC HEPATITIS

4.1 Introduction

Patients with chronic alcoholic liver disease developing acute alcoholic hepatitis (AH) with decompensated liver function and multi-organ failure (acute-on-chronic liver failure, ACLF)] have hospital mortality rates of 60-100%[1, 4, 5, 134-136, 159]. While glucocortiocids[160-163] and pentoxifylline[164] can be tried in earlier stages, options are limited once end-organ failure develops. The availability of an effective form of liver support to give additional time for liver regeneration and spontaneous recovery to occur would represent a major advance. Of the various devices developed, one type (bio-artificial) includes living liver cells, aiming to provide all the functions of the normal liver. The other approach is based on detoxification using membranes and adsorbents that can remove the putative toxins associated with liver failure. Such liver support devices have been rarely used in patients with AH, and the main thrust of activity has been in developing extracorporeal support for patients with acute liver failure (ALF)[165-168]. The one used in the present study was the Molecular Adsorbents Recirculating System (MARS), which utilises the principles of albumin dialysis. The aims of the current study were to evaluate the feasibility, safety and efficacy of MARS in patients with severe AH in the setting of an Intensive Care Unit in the UK.

4.2 Patients and Methods

Studies were undertaken with written informed consent from each subject or next-of-kin, with the approval of the local research ethics committee, and in accordance with the Declaration of Helsinki (1989) of the World Medical Association. Eight patients were enrolled. They were monitored and given standard medical therapy as described before. Patients were followed for 3 months after inclusion into the study.

4.2.1 Inclusion criteria

(i) Patients were included with severe AH, with history of alcohol abuse, and clinical and laboratory stigmata of acute AH, supported by histological evidence. (ii) All had acute deterioration in liver function over 2-4 weeks due to a defined precipitant (Table 1) leading to severe, progressive clinical deterioration despite supportive care (over 72 hours) with (a) increasing jaundice (bilirubin>150μmol/L), and (b) either hepatic encephalopathy (HE) (≥Grade 2)[137] or renal failure or both. (iii) In addition, the Sepsis-related Organ Failure Assessment (SOFA)[136] score had to be ≥9.

4.2.2 Exclusion criteria

Patients were excluded if they were <18 or >75 years old, adequate consent could not be obtained, was already enrolled in another study protocol, had known hepatic/extrahepatic malignancy, was pregnant, had co-existing HIV infection or severe cardiorespiratory disease. Concomitant or previous corticosteroid therapy was *not* an exclusion criterion.

4.2.3 MARS Treatment

The MARS system was set up and run as described earlier. The number of treatments received by each patient was guided by their response. Patients were treated on alternate days, each for a duration of 8 hours. Treatment with MARS was stopped if the patient showed evidence of on-going clinical improvement over 3 days after stopping treatment, had a maximum of 12 treatments over a 3-week period or if they deteriorated haemodynamically and required>1μg/Kg/min of noradrenaline to maintain an MAP>50mmHg.

4.2.4 Statistics

Results were expressed as median (range). Significance of differences between values before MARS therapy and at the end of a course of treatment was tested using Wilcoxon matched pairs test. Linear correlation was used where applicable. $P<0.05$ was taken as statistically significant. Softwares used were Microsoft Excel 2000 (Microsoft Corp., Redmond, WA) and GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

4.3 Results

4.3.1 Patients

All 8 patients were jaundiced and encephalopathic at the time MARS was initiated. In addition, 5 patients had severe form (Type 1) of hepatorenal failure (HRS) and 2 others had the milder form (Type 2 HRS)[27]. None of the patients were mechanically ventilated and two required inotropic support. Transjugular liver biopsy was consistent with a diagnosis of severe AH and cirrhosis in all patients (Table 1). All except Case no. 4 were drinking up until the time of presentation with the current illness. Their biochemical profile is given in Table 2.

4.3.2 Survival

Five of the 8 patients treated with MARS were discharged from the hospital and four of them (50%) were alive with good liver function, not requiring further hospital admission, 3 months after inclusion into the study. Of the 5 patients with Type 1 HRS, 2 patients died within 30 days and 1 other within 90 days. One patient was successfully bridged to orthotopic liver transplantation (OLT), 21 days after initiating MARS therapy and the fifth patient is alive, at 3 months of follow up. Of the remainder, 1 patient died from multiorgan failure, and the other 2 are alive at 3 months of follow up (Table 1). The predicted mortality at 3 months using the MELD score was a median of 76% (range 76-83). This was consistent with similar predicted mortality using Maddrey's DF, SOFA and APACHE II scores (Table 3). Patients

were treated with 4.5 (3-12) sessions of MARS each with duration of 8 (1-24) hours. The differences in post-MARS prognostic scores compared to pre-treatment values were significant (Table 3).

4.3.3 Liver function

The most striking and significant change in the liver biochemistry was noted in the serum bilirubin levels, which dropped from 385 (225-708) to 197 (164-336) (μmol/L) (p= 0.008). Fig 1a (Case no. 2) shows a representative course of serum bilirubin over time in the 4 patients who survived to leave hospital. There was a sharp reduction in bilirubin level following the first few treatments that then plateaued and did not rise to the baseline level even after stopping further MARS therapy. Fig 1b (Case no. 6) shows a representative course of serum bilirubin in one of the non-survivors, with only a marginal reduction immediately following treatment. The subsequent rise did not respond well to therapy, and this patient died from multiorgan failure/septic shock. An improvement of hepatic synthetic function was observed, as demonstrated by a significant improvement of prothrombin time [INR reducing from 2.9 (1.6-3.3) to 1.7(1.3-3.4), p=0.04]. There was no change of serum albumin.

4.3.4 Hepatic encephalopathy

Only 5 of the 8 patients showed evidence of overt HE at the time of hospital admission but all 8 were encephalopathic at the time MARS was initiated [grade 2 (2-3)]. All the eight patients regained consciousness after a median of 2 (range 1-3) sessions of MARS therapy. Though some patients showed a subsequent deterioration, often associated with sepsis, overall the grade of HE improved significantly post-treatment (to 1 (0-4), p=0.05) (Fig 2).

4.3.5 Renal function

Renal function improved in all patients, as demonstrated by a significant reduction of serum creatinine from 162 (51-312) to 108 (34-231) μmol/L; p=0.02. Three of the 5 patients with Type 1 HRS remained anuric (Patient 1, 6 and 7) but there was normalisation of serum creatinine with improvement of urine output in the other two patients. Serum creatinine (and urine output) was normalized in both the patients with Type 2 HRS by the end of treatment.

Table 1: Clinical characteristics of patients treated with MARS

Patient	Sex	Age (yrs)	Etiology	Type 1 HRS	HE (grade)	Precipitating event	Other Complications	Pre-MARS hospital days	No of MARS	Follow up from start of MARS (upto 3 months)	Cause of death
01	m	46	alcohol	+	3	UGI bleed	sepsis	9	9	Died, 36 days	septic shock
02	m	50	alcohol	+	2	UGI bleed	SBP	10	3	Alive	-
03	m	38	alcohol		2	SBP	sepsis, SBP, UGI bleed	3	3	Alive	-
04	m	44	alcohol	+	2	UGI bleed	UGI bleed	3	5	21 days, OLT	-
05	m	44	alcohol + HepC		2	SBP	sepsis	4	6	Alive	-
06	m	61	alcohol	+	3	pneumonia	sepsis	9	4	Died, 12 days	septic shock
07	m	54	alcohol	+	2	binge drinking	sepsis	6	4	Died, 9 days	septic shock
08	m	45	alcohol		3	SBP	sepsis	3	12	Died, 38 days	multiorgan failure

HRS – Hepatorenal syndrome, HE – Hepatic encephalopathy, SBP – Spontaneous bacterial peritonitis, UGI – Upper gastrointestinal, OLT – Orthotopic liver transplantation

Table 2: Biochemical characteristics of patients before start of and after end of treatment with MARS

Patient	Bilirubin (μmol/L)		ALT (IU/L)		Prothrombin time (sec)		INR		Albumin (g/L)		Creatinine (μmol/L)		Urea (μmol/L)	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
01	374	164	42	20	24.9	15	2.17	1.3	14	19	283	231	17.5	17.8
02	396	172	63	29	26.4	15.2	2.3	1.32	34*	24	156	140	14.6	16.6
03	259	196	29	33	33.8	17.8	2.97	1.55	30	28	60†	72	12.6	12.7
04	270	197	17	13	34.9	40.4	2.9	3.4	32	33	169	75	12.9	3.2
05	425	198	17	19	32	33	2.9	2.7	39*	30	115	34	24.5	2.5
06	225	202	64	62	17.6	15	1.55	1.31	19	22	168	146	14.5	22.5
07	708	180	90	87	33.5	20	3.01	1.77	37*	18	312	214	50.3	34.4
08	632	336	52	44	38.3	27.3	3.3	2.7	29	22	51	50	3.9	5.1

ALT- alanine aminotransferase

INR-International Normalised Ratio

*- received prior intravenous albumin administration

†- received prior haemofiltration

Table 3: Change of prognostic scores of patients with MARS treatment

Patient	Child Pugh Pre	Child Pugh post	MELD pre (predicted 3-month-mortality)	MELD post (predicted 3-month-mortality)	Maddrey's DF pre	Maddrey's DF post	SOFA pre	SOFA post	APACHE II pre	APACHE II post
01	14	11	32 (83%)	21 (76%)	76.6	18.8	15	14	12	9
02	13	10	27 (76%)	15 (27%)	84.8	21.4	11	9	11	8
03	13	10	21 (76%)	12 (27%)	108.1	33.5	14	11	7	2
04	12	12	25 (76%)	9 (4%)	192	96.3	13	12	11	4
05	11	11	21 (76%)	15 (27%)	112.3	93.5	10	9	9	2
06	13	11	21 (76%)	17 (27%)	39	21	16	13	10	9
07	11	14	39 (83%)	24 (76%)	135.7	42.7	13	15	14	19
08	13	13	22 (76%)	17 (27%)	153.3	85.4	10	9	11	10
Median (range)	**13 (11-14)**	**11 (10-14)**	**24 (21-39)**	**16 (9-24)**	**110 (39-192)**	**38 (19-96)**	**13 (10-16)**	**12 (9-15)**	**11 (7-14)**	**9 (2-19)**
P-value		**NS**		**0.008**		**0.008**		**0.08**		**0.08**

MELD- Mayo End-stage Liver Disease, DF-discriminant function, SOFA- Sepsis-related Organ Failure Assessment, NS- not significant
Pre values refer to those obtained prior to starting MARS therapy, Post values refer to those at the end of the course of treatment with MARS

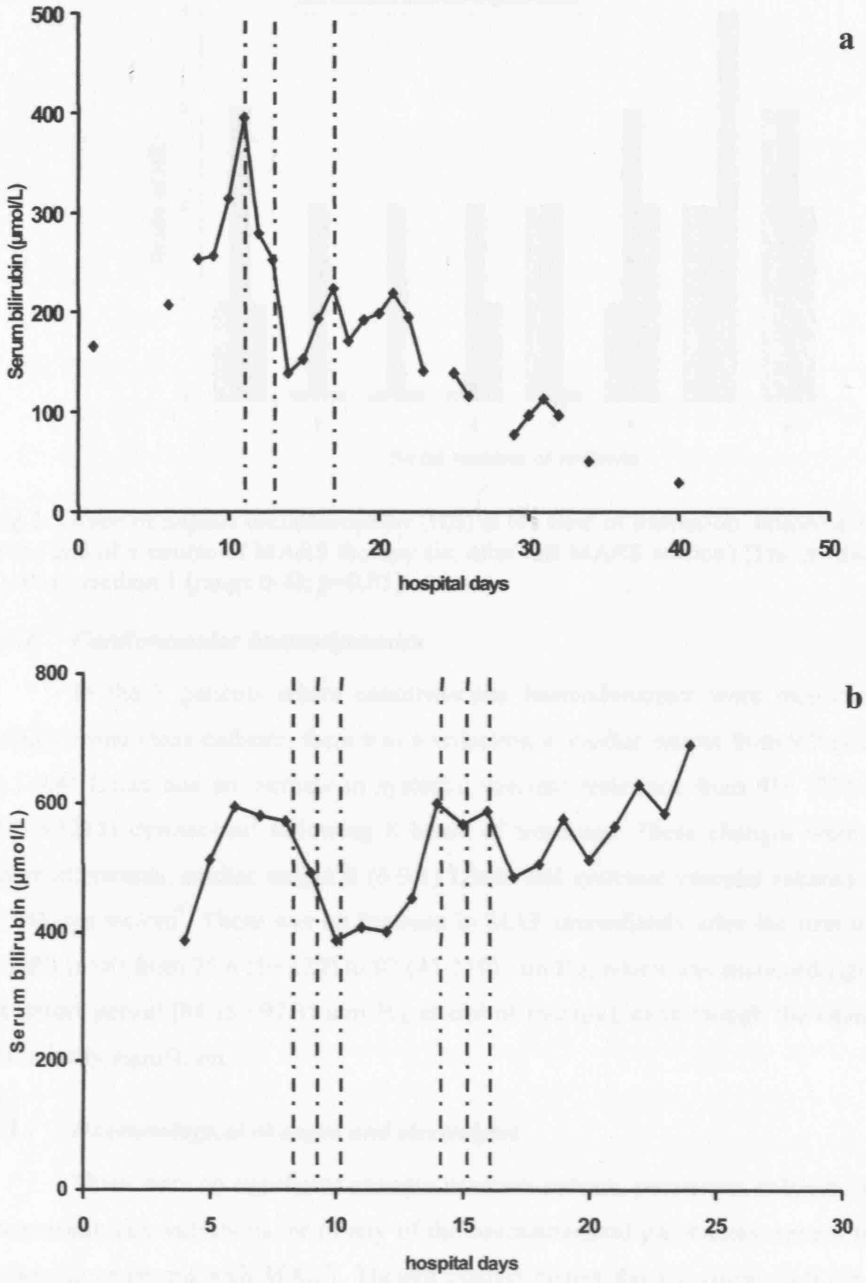


Fig 1: Changes in bilirubin concentration. The dashed lines represent the treatments with MARS. **(a)** This shows the representative course of serum bilirubin (from patient 2) over time in the 5 patients who survived to leave hospital **(b)** This shows a representative course of serum bilirubin (from patient 6) over time in one of the patients who died in hopsital

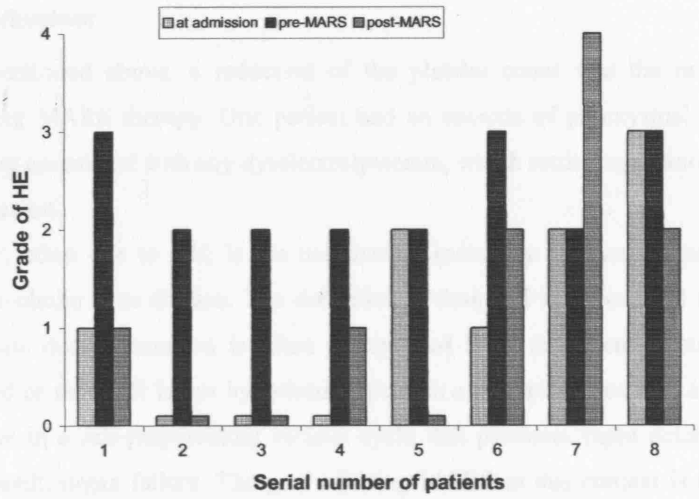


Fig 2: Grade of hepatic encephalopathy (HE) at the time of admission, immediately before and at the end of a course of MARS therapy (ie, after last MARS session) [Pre: median 2 (range 2-3), Post: median 1 (range 0-4); p=0.05]

4.3.6 Cardiovascular haemodynamics

In the 3 patients where cardiovascular haemodynamics were monitored invasively using a Swan-Ganz catheter, there was a reduction in cardiac output from 9.7 (7.2-11.4) to 7.8 (6.1-9.4) L/min and an increase in systemic vascular resistance from 914 (774-918) to 1178 (1010-1211) dyn.sec/cm^5 following 8 hours of treatment. These changes were sustained 24 hours afterwards, cardiac output 8 (6-9.1) L/min and systemic vascular resistance 1101 (974-1194) dyn.sec/cm^5. There was an increase in MAP immediately after the first treatment with MARS (n=8) from 75.6 (50-120) to 82 (45-110) mm Hg, which was sustained right through the treatment period [84 (50-97.3) mm Hg at end of therapy], even though the changes were not statistically significant.

4.3.7 Haematological changes and electrolytes

There were no significant changes in serum sodium, potassium, calcium, phosphate and magnesium concentrations, or in any of the haematological parameters, except platelet count, following treatment with MARS. Though platelet counts did fall (from 88.5 (38-195) to 63.5 (22-157) x 10^9/L, p=0.008), this was not clinically significant.

4.3.8 Complications

As mentioned above, a reduction of the platelet count was the only complication observed during MARS therapy. One patient had an episode of paroxysmal supraventricular tachycardia, not associated with any dyselectrolytaemia, which settled spontaneously.

4.4 Discussion

ACLF, often due to AH, is the commonest indication for hospitalisation of patients with severe alcoholic liver disease. The definition is designed to encompass those patients in whom the acute decompensation is often precipitated by a complication such as a gastro-intestinal bleed or sepsis. It is our hypothesis that such a precipitant leads to a series of events that culminates in a self-perpetuating vicious cycle that produces rapid deterioration in liver function and multi-organ failure. The goal of using MARS in this context is to try and return the patient to the clinical condition that they were in prior to the acute deterioration, or, in cases eligible for OLT, to support the patient's condition until a donor organ becomes available.

The most striking effect of MARS, as in other reports, is on the serum bilirubin level[72, 90, 96, 98]. Serum bilirubin concentration is an important independent predictor of mortality in AH patients. Its course after treatment is also an important predictor of likely outcome. In the patients who survive, the initial reduction after the first few treatments is sustained whereas in the patients who die, the reduction in bilirubin is only apparent immediately after the individual treatments. Whether more prolonged courses of treatment would have improved chances of survival in those not responding satisfactorily remains to be determined. The proportional reduction of serum bilirubin after each treatment correlates well with the pre-treatment bilirubin/albumin ratio, rather than the serum bilirubin level itself (Fig 3). In patients with AH, a bilirubin/albumin ratio<5 is associated with minimal further effect on the removal of bilirubin suggesting that this ratio may be a useful predictor of when MARS treatment should be stopped. These results are in keeping with a recent study showing that serum bilirubin reduction with MARS correlates with pre-dialysis molar ratio of bilirubin and albumin[169]. The improvement in prognostic scores using APACHE II, MELD, Maddrey's DF and SOFA scores was associated with improvement in survival. Although the lack of a suitable control group makes firm conclusions difficult, the results suggest that the use of MARS in these patients resulted in an apparent reduction in mortality. This statement is supported by the marked difference between the predicted mortality (>75%) and the observed mortality (50%).

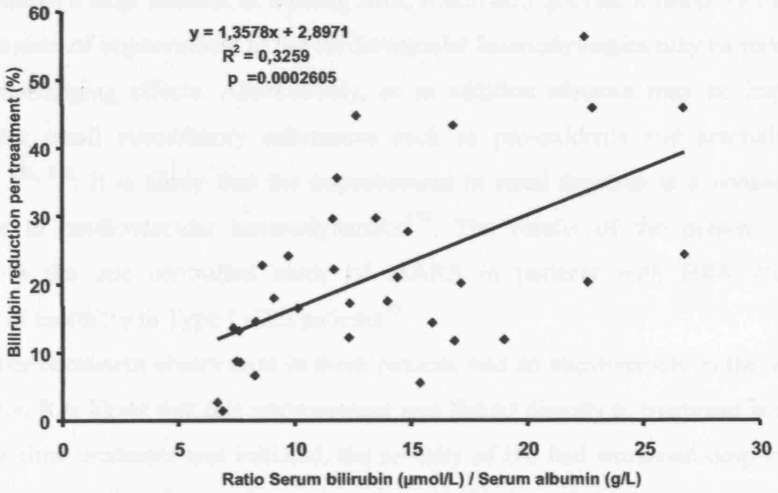


Fig 3: Percentage of bilirubin reduction per treatment against bilirubin level

This is the first study of the effect of MARS in AH patients with multi-organ failure. The improvement of synthetic function (reduced INR and prothrombin time) suggests that removal of albumin-bound toxins may improve liver function and possibly liver injury. Although, the protocol did allow for the concomitant use of corticosteroids in the patients with AH, all the patients had one or more absolute contraindications such as sepsis, renal failure and upper gastrointestinal bleeding[161, 162]. It would be interesting in future to compare the efficacy of MARS with other medical treatments such as steroids and pentoxifylline in patients with AH. A body of opinion is growing that OLT might be a suitable option in patients with severe AH, who might not fare as badly as was previously thought[170]. This is an area where MARS would become relevant as a bridge to OLT (as was done in Case no. 4). As has already been mentioned, terlipressin was not used for the treatment of HRS because it is not licensed for this indication in the UK. Whether the two modalities of therapy have additive effects in HRS is a question for future studies to answer.

Treatment with MARS was also associated with an improved cardiovascular status in the patients as evidenced by an increased MAP (though not achieving statistical significance), reduced cardiac output and increased systemic vascular resistance. The increase in MAP following treatment with MARS has also been described in previous studies[72, 90, 96, 98]. The exact mechanism of this improvement is not clear but is likely to be related to the function of the albumin[85, 171, 172]. Previous studies have suggested that nitric oxide may be important in the vasodilatation and the vascular hyporesponsiveness that is characteristic of cirrhosis[9, 173].

Albumin possesses a large number of binding sites, which may provide a sump for nitric oxide and the mechanism of improvement in the cardiovascular haemodynamics may be related to the nitric oxide scavenging effects. Alternatively, or in addition albumin may be important in removing other small vasodilatory substances such as pro-oxidants and arachidonic acid metabolites[85, 171, 172]. It is likely that the improvement in renal function is a consequence of improvements in cardiovascular haemodynamics[174]. The results of the present study are consistent with the one controlled study of MARS in patients with HRS showing an improvement of mortality in Type I HRS patients[72].

Another consistent observation in these patients was an improvement in the severity of encephalopathy. It is likely that this improvement was linked directly to treatment with MARS because at the time treatment was initiated, the severity of HE had worsened despite standard medical measures with lactulose and bowel enemas. Reduction of plasma ammonia levels and improved cerebral perfusion may be the underlying mechanisms. In a recent communication, MARS treatment was followed by a reduction in ammonia and also an increase in cerebral perfusion[75]. It is likely that the increase in cerebral perfusion results from improvements in cardiovascular haemodynamics[175].

To date, the extra-corporeal circuit of MARS has included a haemodialysis component for the removal of water-soluble toxins[72, 90, 96, 98]. In the present study, this was replaced by continuous veno-venous haemofiltration because of the observation that in critically ill patients with liver disease, the use of continuous forms of renal replacement therapy is preferred for its improved cardiovascular tolerance compared with intermittent dialysis[176]. The results show that it is possible to substitute the haemodialysis module by haemofiltration, without affecting its safety profile. Overall, our evaluation suggested that the MARS technique was safe to use, even among patients with advanced liver failure. A reduction of platelet counts was observed, as often occurs in any extracorporeal therapy, but this could be detected and corrected easily, and did not pose a problem clinically. One patient had an episode of paroxysmal supraventricular tachycardia that settled spontaneously. This episode was not associated with any dyselectrolytaemia, and may well have occurred coincidentally. No other significant adverse effects, such as dyselectrolytaemia or disseminated intravascular coagulation were observed.

Thus, the results of this study suggest that AH patients with deteriorating liver function manifested by increasing serum bilirubin, HE and/or renal impairment would probably benefit from treatment with MARS particularly if there were a precipitating factor that can be controlled.

5. STUDY 2- ALBUMIN DIALYSIS REDUCES PORTAL PRESSURE ACUTELY IN PATIENTS WITH SEVERE ALCOHOLIC HEPATITIS

5.1 Introduction

Portal hypertension is a major cause of mortality and morbidity in patients with advanced cirrhosis. About 30% patients with cirrhosis bleed from oesophageal varices within 2 years of diagnosis and up to 30% will die from its effects[177]. The most important factors determining this early mortality are the inability to control bleeding and severity of the underlying liver disease. Despite advances in the endoscopic and pharmacologic therapies, bleeding remains uncontrolled in about 10-20% and recurs within five days in another 10-15% of patients[178]. Moreover, the occurrence of variceal bleeding in patients with advanced cirrhosis and associated organ failure is almost universally fatal.

Alcoholic hepatits (AH) often precipitates decompensation in patients of alcoholic cirrhosis and leads to associated organ failure. It has also been suggested that inflammation contributes to the worsening of portal hypertension in these patients[179]. Recent studies suggest that the inflammatory mediators of AH, especially pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, are especially important, and modulation of these factors may result in an amelioration of portal pressure[180, 181]. 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. Thus a therapeutic modality of value in liver failure which can acutely reduce portal pressure in addition could be potentially life-saving, in conjunction with endoscopic therapy, especially in the setting of uncontrolled/ poorly controlled acute variceal bleeding.

Albumin dialysis using the Molecular Adsorbents Recirculating System (MARS; Teraklin AG, Rostock, Germany) has been used mainly in the treatment of liver failure[99], and we have previously shown its potential benefit in patients with severe acute AH. 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[97]. A noteworthy point is the fact that all four had evidence of hepatic inflammation (AH-3, non-alcoholic steatohepatitis-1). This raises the exciting possibility that MARS may contribute to

the management of variceal bleeding in patients with AH-related acute decompensation of alcoholic cirrhosis, in which setting the outcome is poor with the currently available therapies.

Thus the aims of the present study were to evaluate the acute effects of albumin dialysis, using MARS, on portal and hepatic 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.

5.2 Methods

With approval of the institutional Ethics Committee, patients were included following informed written consent (next-of-kin assent in encephalopathic patients) at the University College London Hospitals.

5.2.1 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) 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>85μmol/L), and (b) either hyperbilirubinaemia>300μM and/or encephalopathy (≥grade 2) [137] and/or renal failure. (iii) Clinically significant portal hypertension (hepatic venous pressure gradient (HVPG) >12mmHg). *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 cardiorespiratory disease. Concomitant or previous corticosteroid therapy was *not* an exclusion criterion.

5.2.2 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 haemofiltraion alone. HVPG changes at 6 and 24 hours were the primary end-points.

5.2.3 Monitoring

This has been described earlier. The patients were evaluated clinically and biochemically. The Child Pugh score[4] and Maddrey's discriminant function[134] were calculated.

These scoring systems have been validated to be good predictors of outcome in patients with liver disease and also in patients that are admitted to the intensive care unit with ACLF. The severity of encephalopathy was assessed using the West Haven criteria[137]. Mean arterial pressure (MAP), 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_2O. Blood glucose was maintained between 5-7mmol/L with infusion of 50% dextrose.

5.2.4 Extracorporeal therapy

MARS: The MARS system has been described in detail elsewhere[91,90]. Heparin was used as required to prevent clotting in the extracorporeal circuit. The blood and albumin circuit were run at 150 ml/min, with 1 L/hour fluid exchange.

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.

5.2.5 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. Patients were sedated for the procedure using midazolam (median dose of 3 [range 2-5] mg; Phoenix Pharma Ltd., Gloucester, UK).

Hepatic haemodynamics

Hepatic venous pressure gradient: 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. The coefficient of variation between HVPG measurements was 4% for the

study group. The hepatic venous catheter was kept in place for 24 hours and thus HVPG was measured before, during and after extracorporeal therapy.

Hepatic blood flow (HBF): A prime (12 mg) followed by a continuous infusion (1 mg/min) of indocyanine green (ICG) (Akorn Inc., Buffalo Grove, IL, USA) was used to determine HBF. 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 by standard techniques. Plasma flow rate of the liver was calculated using formulae based on the method of indicator dilution and Fick's principle[140, 141] and was converted to blood flow using the haematocrit.

Intrahepatic vascular resistance (IHVR): This was calculated from HVPG and HBF:-

$$\text{IHVR } (dyne.s.cm^{-5}.10^{-3}) = 80 \times 10^{3} * \text{HVPG } (mm\ Hg) / \text{HBF } (ml/min)$$

Cardiovascular haemodynamics

Heart rate, oxygen saturation and ECG were recorded continuously and the MAP (1/3 [systolic – diastolic] + diastolic pressure) was measured prior to catheterisation and every 5 minutes thereafter (Hewlett-Packard, Model 86S, HP, Palo Alto, CA, USA). In three MARS patients and all haemofiltration patients the pulmonary artery was catheterised 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.

5.2.6 Statistics

Results were expressed as mean ± standard deviation. Significance of difference within a group was tested by paired t-test or Wilcoxon's matched-pair test. $P<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Patients

Baseline characteristics of patients are described in Table 1. All patients included had histologically proven severe acute AH on a background of alcoholic cirrhosis. Leukocytosis and raised C-reactive protein levels re-emphasised the inflammatory nature of the condition (Table 2). Patients in the two groups were comparable with respect to severity of liver disease and AH, as evidenced by similar Child-Pugh and Maddrey's scores (Table 1). Hyperbilirubinaemia improved significantly with MARS, but not with haemofiltration. Haemoglobin as well as inflammatory markers (leukocytosis, C-reactive protein) remained unchanged in both groups after 24 hours (Table 2). Although, the protocol did allow for the

concomitant use of corticosteroids in the patients with AH, all the patients had one or more absolute contraindications such as recent sepsis, renal failure and recent upper gastrointestinal bleeding[161,162].

Table 1: Baseline characteristic of patients in the MARS (n=8) and haemofiltration (n=3) groups. Data are presented as mean ± standard deviation.

	MARS group (n=8)	Haemofiltration group (n=3)
Age (years)	45.8±4.9	47.0±7.9
Sex	M:F= 7:1	M:F= 2:1
Precipitant (in addition to alcoholic hepatitis)	Infection- 3 Variceal bleed- 2	Infection- 1
Hepatic encephalopathy	5	3
Renal dysfunction	4	1
Ascites	7	3
Child-Pugh's score	11.6±2.0	11.5±2.1
Maddrey's discriminant function	72.1±26.3	73.0±32.5

Table 2: Laboratory parameters before and after (at 24 hours) a 6-hour session of extracorporeal treatment in the MARS (n=8) and haemofiltration (n=3) groups. Data are presented as mean ± standard deviation.

	MARS group (n=8)		Haemofiltration group (n=3)	
	Baseline	24 hours	Baseline	24 hours
Serum Bilirubin (μM)	483.0±158.6	320.6±128.2***	316.3±131.4	351.7±144.3*
Haemoglobin (g/dl)	10.3±2.2	9.0±1.1	11.9±2.5	11.8±2.1
Leukocyte count (x 10^9/l)	18.7±6.7	16.4±4.5	11.8±7.1	10.5±7.6
C-reactive protein (mg/l)	50.7±28.8	69.6±40.1	35.5±10.6	36.1±15.5
Platelet count (x 10^9/l)	84.5±60.9	73.4±55.5	68.0±16.1	75.0±21.4
INR	1.78±0.33	1.75±0.46	1.91±0.73	2.1±0.92

INR- international normalized ratio
P-value vs baseline: * $p<0.05$; *** $p<0.001$

5.3.2 Hepatic haemodynamics

All patients included had a mean HVPG of 17.0±2.8 mmHg (n=11). With MARS therapy, HVPG reduced significantly (p=0.003) at 6 hours (end of the session), and remained at this level at 24 hours (Table 3). There was a >20% reduction from baseline levels in 7/8 patients, both at 6 and 24 hours (Fig 1A). Six of the patients reached a level of ≤12mmHg at 6 hours. In two patients with repeated measurements during the session, reduction in HVPG was apparent within the first two hours (Fig 1B). Indocyanine green extraction was <10% in five patients, making the estimation of HBF unreliable. In the remaining three, HBF improved over 6 hours (p=0.0001), also sustained at 24 hours. IHVR, which was ~10 times normal in these

three, also improved similarly (p=0.047) (Table 3). These parameters remained unaltered in those treated with haemofiltration (Table 3).

5.3.3 Cardiovascular haemodynamics

Mean arterial pressure (MAP) increased over 6 hours and was sustained at 24 hours (p=0.02). CO (p=0.03) showed a similar rapid sustained improvement. None of these parameters improved with haemofiltration over the 24 hour study period (Table 3).

Table 3: Hepatic and cardiovascular haemodynamic parameters before and after a 6-hour session of extracorporeal treatment in the MARS (n=8) and haemofiltration (n=3) groups. Data are presented as mean ± standard deviation.

	MARS group (n=8)			Haemofiltration group (n=3)	
	Baseline	6 hours	24 hours	Baseline	24 hours
HVPG (mm Hg)	17.4±3.3	11.5±1.9**	11.9±2.0***	16.0±1.0	15.7±2.9
HBF (ml/min)††	378±178	482±178***	448±161*	340±88	318±171
IHVR (dyne.s.cm^{-5}.10^{-3})††	4344±1266	1970±374*	2517±810*	3966±1145	4695±2183
MAP (mm Hg)	68.0±12.2	78.8±15.1	77.3±10.1*	75.7±13.6	70.0±7.0
CO (l/min)†	9.7±3.6	7.8±1.7*	7.0±1.6**	10.3±1.4	9.5±2.3

HVPG- hepatic venous pressure gradient HBF- hepatic blood flow
IHVR- intrahepatic vascular resistance MAP- mean arterial pressure
CO- cardiac output
† n=4 and †† n=3 in MARS group
P-value vs baseline: * p<0.05; ** p<0.01; *** p<0.001

5.3.4 Safety profile

No patient developed any adverse events in either group. Platelet counts reduced with MARS, albeit not significantly, while coagulation profile remained unchanged (Table 2).

5.3.5 Outcome

There were six in-hospital deaths in the MARS group and two in the haemofiltration group, though it is important to note that this study was neither designed nor powered adequately to look at survival. The three survivors were all alive at three months. In the MARS group, one death occurred due to variceal haemorrhage (three weeks after completion of the study protocol) while five occurred due to multi-organ failure (four with associated sepsis). In the haemofiltration group, both deaths occurred due to multi-organ failure (one with associated sepsis).

5.4 Discussion

The results of our study demonstrates an acute reduction in portal pressure with albumin dialysis using MARS, that was sustained for up to 18 hours after stopping the treatment. Although the exact mechanism of this portal hypotensive effect of MARS is unclear, the reduction in portal pressure is the result of effects on both the forward and also the backward components of portal hypertension. Our observation may have important clinical implications in the management of variceal bleeding in patients with severe acute AH and also provides a potentially exciting tool to further investigate the pathophysiological basis of portal hypertension.

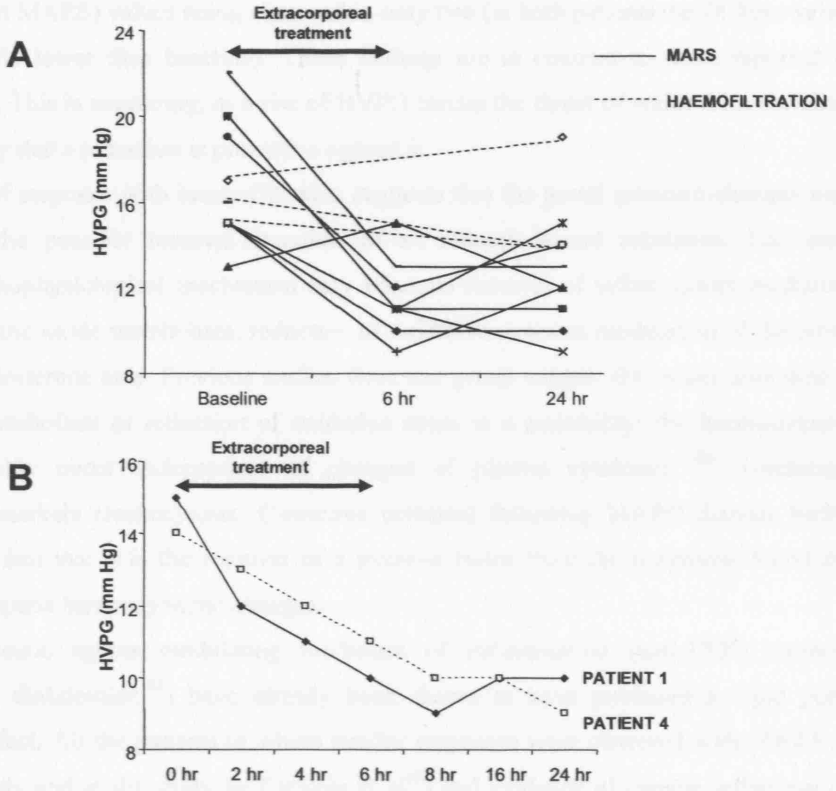


Fig 1: (A) Hepatic venous pressure gradients (HVPG) before and after a 6-hour session of extracorporeal treatment in the MARS (n=8) and haemofiltration (n=3) groups. 6-hour values were available only in the MARS group. All but one MARS-treated patient showed a significant (>20%) reduction of HVPG (both at 6 and 24 hours), while none of the haemofiltered patients did. **(B)** Two patients with repeated HVPG measurements during MARS therapy, demonstrating rapid reduction (starting within the first 2 hours) which was sustained up to 24 hours.

The most important finding of the present study from the clinical perspective was 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. An increase in portal pressure (indicated by HVPG) to values greater than 12mmHg underlies the development of varices and a reduction to values ≤12mmHg[182] or by >20% of the baseline[183] is known to prevent rebleeding[177]. In the present study, within 6 hours HVPG fell by ≥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 marked response. This reduction was sustained for at least 18 hours after stopping treatment. Interestingly, no rebound increase of HVPG prior to the next MARS session was observed in the majority of patients, with a rise >20% of the 6-hour (immediate post MARS) values being observed in only two (in both patients the 24-hour values remaining >20% lower than baseline). These findings are in contrast to those reported by Catalina et al[97]. This is reassuring, as a rise of HVPG carries the threat of variceal haemorrhage in the same way that a reduction is protective against it.

Lack of response 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 nitric oxide metabolism, reduction of oxidative stress or modulation of the renin-angiotensin-aldosterone axis. Previous studies from our group suggest that while alteration of nitric oxide metabolism or reduction of oxidative stress is a possibility, the haemodynamic changes probably occur independent of changes of plasma cytokines [184]. Unchanged inflammatory markers (leukocytosis, C-reactive proteins) following MARS dialysis further emphasise the fact that it is the removal of a putative factor from the peripheral blood that mediates the hepatic haemodynamic changes.

Therapeutic agents modulating mediators of inflammation (anti-TNFα antibody [infliximab][180], thalidomide[181]) have already been shown to have produced a rapid portal hypotensive effect. All the patients in whom similar responses were observed with MARS (in the present study and in the study by Catalina et al[97]) had evidence of hepatic inflammation. This leads us to postulate that portal hypertension in such a pro-inflammatory setting is perhaps more labile and rapidly amenable to therapeutic interventions. By the same logic, one must be cautious about extending the findings of this study to cirrhosis without any hepatic inflammation, where portal hypertension may be more difficult to ameliorate.

The reduction of CO along with the increase of MAP appeared to be part of the general amelioration of the hyperdynamic circulation of liver failure, as has been reported by other

studies with MARS[72-74, 95]. Alteration of nitric oxide metabolism[9] may be a possible mechanism. Haemofiltration alone did not cause any improvement of systemic haemodynamics. However, it should be noted that the baseline MAP was substantially lower in the MARS group than in the haemofiltration group, and this might possibly explain the improvement observed with MARS, as data from the International MARS Registry suggests that the beneficial effect of MARS on MAP is only evident in those having a substantially low MAP to start off with[128].

Portal hypertension is believed to have an underlying 'forward' component, due to increased splanchnic blood flow, and a 'backward' component, due to increased IHVR[185]. Associated reduction of CO suggests that the forward component of portal hypertension was improved, while reduction of the IHVR and increase of HBF suggests that the backward component was affected as well. The reduction of the backward component of portal hypertension is potentially a more exciting finding from the pathophysiological perspective. IHVR has a 'fixed' component due to fibrosis and altered hepatic architecture, and a 'variable' component that may be influenced by several factors. Activation of hepatic stellate cells is believed to be important[186, 187], as is the intra-hepatic deficiency of nitric oxide, perhaps due to decreased intra-hepatic endothelial nitric oxide synthase activity[188, 189]. The hepatic inflammation of AH would make these factors more prominent, and it is therefore possible that either or both of these factors are influenced by albumin dialysis. The rapid pressure changes observed (sometimes evident within one hour) make it unlikely that apoptosis of stellate cells or remodeling of extracellular matrix following MARS is important. However, de-activation of stellate cells may be a potential mechanism (though data is lacking that such a thing can happen once the cells are activated), as is alteration of intra-hepatic nitric oxide metabolism following albumin dialysis. Irrespective of the exact underlying mechanism, the observed increase of HBF, which contrasts with the reduction brought about by portal pressure reducing drugs such as beta-blockers[190], should be beneficial in the setting of a severely damaged liver.

One therapy which has been used to rapidly lower portal pressure in the setting of uncontrolled variceal haemorrhage is the transjugular intrahepatic portasystemic stent shunt (TIPSS) [191-193]. However, the shunting of portal venous blood that is essential to decompresses the portal vasculature also often leads to new or worsened hepatic encephalopathy in 25-50% of cases [193]. Indeed, in some patients acute TIPSS insertion for uncontrolled variceal bleeding results in death from cerebral herniation[39]. Thus the use of TIPSS in patients with liver failure and associated end-organ dysfunction is not without its difficulties even among those patients where successful control of bleeding is achieved with TIPSS[194-197]. In such a setting, the use of

albumin dialysis would not only help treat the encephalopathy and/or liver failure, but as evident from these data, also lower portal pressure.

There was no accompanying worsening of coagulation, although an insignificant reduction in platelet count was observed. This has been frequently reported with MARS therapy[128,129], is similar to what has been observed following most forms of renal replacement therapy, and does not pose a problem under most clinical settings. However, one needs to be extremely cautious from this point of view when treating a patient with acute variceal bleeding. Future improvement in the biocompatibility of the membrane used in albumin dialysis will therefore be useful.

In conclusion, this study suggests that MARS has a rapid, clinically significant portal pressure lowering effect. Our results suggest that albumin dialysis may be a useful adjunct to existing therapies in the context of variceal bleeding in patients with severe acute AH and associated organ failure. In these patients, the effects of MARS on portal pressure may provide added value in terms of greater ability to control the bleeding and also counteract its deleterious metabolic consequences. The mechanism of how MARS achieves this effect on intrahepatic resistance remains unclear and should be the focus of future studies.

6. STUDY 3- PATHOPHYSIOLOGICAL EFFECTS OF ALBUMIN DIALYSIS IN ACUTE-ON-CHRONIC LIVER FAILURE: A RANDOMISED CONTROLLED STUDY

6.1 Introduction

The pathophysiological basis of acute-on-chronic liver failure (ACLF) is uncertain but current hypotheses suggest that systemic inflammatory response may underlie the transition of a patient from a stable cirrhotic state to developing progressive liver injury and end-organ failure. Cytokines are believed to be important in the pathogenesis of decompensated alcoholic liver disease[198]. A predominantly pro-inflammatory cytokine profile might cause hepatic inflammation and necrosis. Mitochondria are a target for tumour necrosis factor (TNF) initiated death signals, releasing reactive oxygen species, leading to apoptosis and cell death[199, 200]. Inflammation and oxidative stress also induce production of nitric oxide (NO), which appears to cause the circulatory[9, 11, 12, 15, 17, 19, 20] and renal[25, 28, 30] disturbances of liver failure. There is increasing evidence that the mediators of inflammation (pro-inflammatory cytokines, NO, oxidative stress) could modulate[41, 201] the effect of hyperammonaemia[33-35] in precipitating encephalopathy.

Several studies with the Molecular Adsorbents Recirculating System (MARS), including two small randomised controlled trials[72 99], in patients with acute decompensation of cirrhosis, have demonstrated improvement of serum bilirubin[72, 90, 96, 98, 202], hepatic encephalopathy[74, 95, 96], systemic haemodynamics[72-74, 95], renal function[74, 95, 96], and short-term survival[74, 95, 96, 107]. The hypothesis is that accumulation of non-dialysable 'toxins' in liver failure contributes to the pathogenesis of end-organ dysfunction. Their removal would interrupt the progressive clinical worsening, allowing recovery from the acute episode. However, which toxins are important remain undefined.

In an uncontrolled study[202], we demonstrated an apparently improved survival among severe alcoholic hepatitis patients following MARS therapy. The present study was designed as a follow-up to determine whether and how MARS affects the cytokine profile, markers of oxidative stress, NO and ammonia.

6.2 Methods

This prospective randomised controlled study was conducted on eighteen patients at University College London Hospital, with informed, written consent from all patients with encephalopathy less than grade 2, or assent from next-of-kin for all other patients, with approval of the local research ethics committee in accordance with the Declaration of Helsinki (1989).

6.2.1 ***Selection criteria:*** *Inclusion-* Patients included had alcoholic liver disease with ACLF, defined as acute deterioration in liver function over 2-4 weeks with a defined inflammation-related precipitant (infection or alcoholic hepatitis) leading to severe progressive clinical deterioration despite supportive care (over 48 hours) with increasing jaundice (serum bilirubin>100μmol/L) and either encephalopathy (≥Grade 2) or hepatorenal syndrome (HRS) in a patient with clinical, radiological, biochemical and histological evidence of cirrhosis. Variceal bleeding or infection (if present) was controlled for at least 48 hours before inclusion.

Exclusion- Age <18 or >75 years, lack of consent/assent, prior enrolment in another study, known hepatic/extrahepatic malignancy, uncontrolled infection or upper gastro-intestinal bleeding over the previous 48 hours, pregnancy, prior treatment with terlipressin for HRS, co-existing HIV infection or severe cardiorespiratory disease.

Timing of randomisation- Following initial evaluation, patients received standard medical therapy (SMT) and the precipitating factors treated appropriately. If there was no improvement over 48 hours, they were randomised for the study.

6.2.2 ***Study design:*** Randomisation was performed by opening sealed pre-numbered envelopes. One group received SMT alone (SMT group), while the other received MARS treatment plus SMT (MARS group). All patients were monitored over 7 days from inclusion.

Sample size- A documented biochemical result of MARS treatment is reduction of serum bilirubin[72, 99, 202], which we used as a surrogate marker for its pathophysiologic effects. Sample size was calculated assuming a reduction in bilirubin of 40% after 4 MARS treatments[72, 99, 202]. With an α= 0.05 and β=0.2, 17 patients were required to show a difference between MARS-treated and non-MARS-treated patients. Twenty patients with ACLF were evaluated for study, of which two had non-alcoholic aetiologies. Eighteen were finally included.

6.2.3 ***MARS treatment:*** The MARS treatement (Teraklin AG, Rostock, Germany) was performed as described previously[91,90]. Patients were treated for 4 sessions of 8 hours, over the seven-day study period.

6.2.4 ***Monitoring:*** The patients were evaluated clinically and biochemically before and after each MARS treatment as has been described before.

6.2.5 ***Sampling:*** Blood samples were collected at baseline and on day-7 from all patients. In the MARS group, samples were also collected before and after each MARS treatment. Following each MARS treatment, albumin dialysate samples were taken from the different segments of the circuit (segment-1: between blood and haemofilter columns; segment-2: between haemofilter and charcoal columns; segment-3: between charcoal and resin columns;

segment-4: between resin and blood columns). A complete set of pre-/post-MARS samples was available for 20 sessions. Venous plasma samples from 8 healthy volunteers were used to obtain control values for the various laboratory measurements.

Samples were analyzed for:

- cytokines (control: interleukin (IL)-6; IL-8; IL-10; and TNF-α- undetectable; TNF-R1- 1.59±0.13 ng/ml; TNF-R2- 1.88±0.25 ng/ml (mean±SD)),
- nitrite and nitrate (control: 40 (30-50)μM for nitrate and 2 (1-3.5)μM for nitrite [median (range)],
- ammonia (control: 20 (9-30)μM),
- malondyaldehyde (control: 1.8 (1.2-2.5)μM), and
- oxygen-based free radicals (measured in the last 8 patients (MARS-4, SMT-4)).

6.2.6 ***Statistics:*** Results were expressed as median (range). Significance of difference within a group was tested by paired t-test or Wilcoxon's matched-pair test, and between groups by ANOVA-single factor or Mann-Whitney test as appropriate. Linear regression was used to determine relationship between variables. A Kaplan-Meier-style plot was used to study the time course of change in encephalopathy between the two groups, and a logrank test was used to test significance between them. $P<0.05$ was considered statistically significant.

6.3 Results

The demographic profile on inclusion is described in Table-1.

Table 1: Baseline demographic profile of the 18 patients included in the study.

	MARS group (n=9)	**SMT group (n=9)**
Age [years]	45 (34-52)	44 (33-64)
Sex (male:female)	7:2	6:3
Aetiology	Ethanol- 6	Ethanol- 9
	Ethanol + Hepatitis C virus- 3	
Precipitant: Infection	4	6
Alcoholic hepatitis	5	3
Hepatic encephalopathy: Grade 1	1	2
Grade 2	4	4
Grade 3-4	4	3
Hepatorenal syndrome	Type I: 3, Type II: 2	Type I: 2, Type II: 3
Continuous veno-venous haemofiltration requirement	3	3
Child-Pugh class (A:B:C)	0:0:9	0:0:9

6.3.1 Clinical and biochemical changes

Clinical, biochemical and prognostic parameter changes over the 7-day period are described in Table-2. Table-3 describes changes of encephalopathy and MAP over 20 individual sessions of MARS in the 9 MARS-treated patients.

Table 2: Change of clinical, biochemical and prognostic parameters, and plasma values of cytokines, malondialdehyde, NOx (nitrate and nitrite) and ammonia over 7 days in the MARS and SMT group of patients.

	MARS group (n=9)		**SMT group (n=9)**	
	Baseline	*Day 7*	*Baseline*	*Day 7*
Encephalopathy grade	2.5 (2-4)	1 (0-4)**	2 (2-4)	2 (0-4)
MAP (mm Hg)	85 (60-130)	75 (55-105)	77 (57-100)	80.5 (66-97)
Serum bilirubin (μmol/L)	396 (281-708)	182 (140-348)***	232 (115-416)	280 (102-544)
Serum creatinine (μmol/L)	88 (62-267)	86.5 (33-270)	113 (35-203)	62.5 (28-97)
Prothrombin time (sec)	22.1 (14-33.5)	18.3 (14.5-33)	20.4 (16.5-31.3)	20.1 (13.7-28.2)
INR	2 (1.2-3)	1.7 (1.3-2.7)	1.8 (1.5-2.8)	1.7 (1.2-2.5)
Serum albumin (g/L)	23 (16-37)	27 (12-33)	27 (19-36)	26 (22-39)
Child-Pugh score	13 (11-14)	11 (10-14)**	12 (10-13)	12 (8-14)
MELD score	16.5 (13.1-31.2)	14.1 (4.8-41.9)**	19.4 (4.3-25.2)	14.5 (2.9-18.4)*
TNF-α (pg/ml)	30 (3-92)	24 (3-132)	20 (3-193)	4 (3-334)
TNF-R1 (ng/ml)	8.7 (2.9-27.4)	10 (3.3-27.4)	11.3 (2-20.9)	7.1 (2.5-24)
TNF-R2 (ng/ml)	21.5 (8.8-47.9)	24.4 (10.7-34.3)	21.1 (8.8-29.3)	14.4 (9.1-42.1)
IL-6 (pg/ml)	3 (3-43)	57 (3-315)	10 (3-119)	9 (3-276)
IL-8 (pg/ml)	3 (3-61)	27.5 (3-165)	12.5 (3-135)	6 (3-103)
IL-10 (pg/ml)	3 (3-74)	16 (3-208)	3 (3-253)	3 (3-188)
Malondialdehyde (μM)	6.4 (5.3-13.5)	6.3 (4.1-10.2)	6.3 (2.9-9.2)	6.7 (4.2-14.4)
Nitrate (μM)	88.3 (25.9-248.9)	73 (15.4-89.4)*	92 (49-247.3)	61.2 (22.4-325.7)
Nitrite (μM)	8 (2.3-17.5)	2.8 (0.2-17)*	3.9 (0-33.5)	4.3 (0-12.8)
NH_3 (arterial) (μM)	109 (26-172)	80 (39-169)	74 (39-184)	100 (26-139)

p-value (compared to baseline): *<0.05, **<0.01, ***<0.001
MAP- mean arterial pressure INR- international normalised ratio NH_3- ammonia
MELD- Model for End-Stage Liver Disease
TNF- tumour necrosis factor TNF-R- TNF-receptor IL-Interleukin

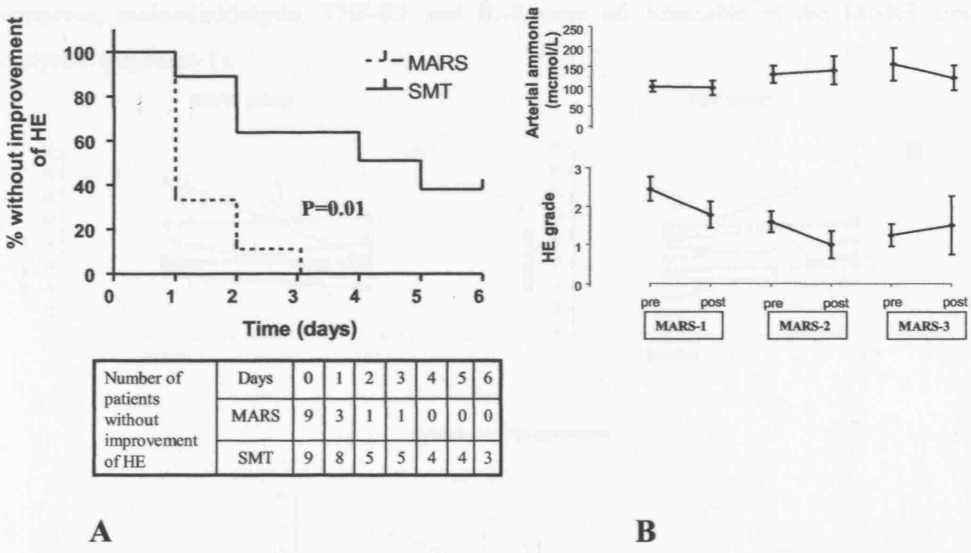
Table 3: Change of hepatic encephalopathy, mean arterial pressure and plasma values of NOx (nitrate and nitrite), ammonia, malondialdehyde and cytokines over 20 individual sessions of MARS treatment.

	Pre-MARS	Post-MARS
Encephalopathy grade	2 (1-4)	1 (0-4)*
MAP (mm Hg)	84 (60-120)	84.5 (55-105)
TNF-α (pg/ml)	3 (3-144)	3 (3-114)
TNF-R1 (ng/ml)	8.9 (2.9-27.4)	7.9 (3.3-27.4)
TNF-R2 (ng/ml)	24.8 (8.8-57.6)	24.9 (10.1-57.6)
IL-6 (pg/ml)	5 (3-168)	3 (3-165)
IL-8 (pg/ml)	31 (3-286)	30 (3-165)
IL-10 (pg/ml)	6 (3-208)	6 (3-313)
Malondialdehyde (μM)	6.2 (4.1-13.5)	6 (4.1-10.2)
Nitrate (μM)	79.5 (25.9-248.9)	73.3 (14.9-173.9)*
Nitrite (μM)	5.9 (0-17.5)	4.3 (0-17)
NH_3 (arterial) (μM)	112 (26-258)	100 (39-258)

*$p<0.05$ compared to pre-MARS values
MAP- mean arterial pressure TNF- tumour necrosis factor TNF-R- TNF-receptor
IL-Interleukin NH_3- ammonia

6.3.1.1 Haemodynamics: MAP did not change significantly in either group. Two patients in each group received inotropes. However, inotrope requirement did not change with MARS therapy. Even excluding these four patients from the analysis, no significant change of MAP was seen in either group.

6.3.1.2 Encephalopathy: Encephalopathy improved significantly in the MARS group over individual MARS sessions as well as over the 7-day study period, but not in the SMT group. Fig 1A depicts the percentage of patients without improvement of encephalopathy (defined as reduction by ≥1 grade) over the 7-day period in both groups. Taking day 0 to be the start of the study, in the MARS group only 11.1% had not shown an improvement by day 2, while in the SMT group 38.1% had not improved even at the end of the study period ($p=0.01$). The rapid improvement of encephalopathy in the MARS group was not matched by a corresponding improvement of arterial ammonia (Fig 1B). Two patients in the MARS group showed a subsequent worsening of encephalopathy by one grade, related to infection in one and variceal bleeding in the other.



Number of patients without improvement of HE	Days	0	1	2	3	4	5	6
	MARS	9	3	1	1	0	0	0
	SMT	9	8	5	5	4	4	3

A **B**

Fig 1: (A) A Kaplan-Meier style plot showing percentage of patients without improvement of hepatic encephalopathy (defined as reduction by ≥1 grade) over the 7-day study period in MARS and SMT groups, with a logrank test used to show the difference between the two groups. The actual number of patients without improvement of encephalopathy on each individual day is shown in the table. **(B)** A comparison of the change of encephalopathy with the change of arterial ammonia over the first three MARS sessions in the patients in the MARS group. The rapid improvement of encephalopathy in the MARS group was not matched by a corresponding improvement of arterial ammonia.

6.3.1.3 Renal function: No significant change of renal function (serum creatinine, urine output) was observed in either group. Of the five patients with HRS in the MARS group, three (type I-2, type II-1) did not survive (one death within the 7-day study period). In the SMT group, there were five HRS patients as well, of whom three (type I-1, type II-2) died while in hospital (none in the study period).

6.3.1.4 Biochemistry: A significant improvement of serum bilirubin over 7 days was seen in the MARS group, but not in the SMT group. Serum albumin and prothrombin time remained unchanged.

6.3.1.5 Prognostic markers: Child-Pugh score improved significantly only in the MARS group, while the MELD score improved significantly in both groups over 7 days.

6.3.2 Pathophysiological changes

The associated changes of plasma cytokines, malondialdehyde, nitrate and nitrite (NOx) and ammonia (arterial) over the 7-day study period are described in Table-2. Table-3 describes changes of plasma cytokines, malondialdehyde (Fig 2), NOx (Fig 3) and ammonia (arterial) observed over 20 individual MARS sessions in the 9 MARS-treated patients. NOx,

ammonia, malondialdehyde, TNF-R1 and IL-8 were all detectable in the MARS albumin dialysate (segment-1).

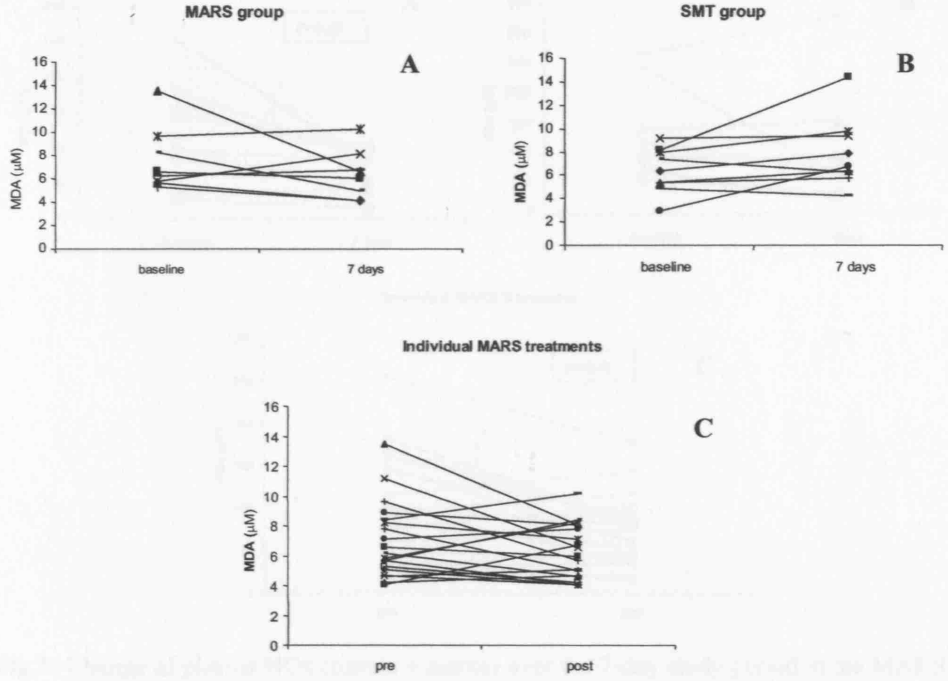


Fig 2: Change of plasma malondialdehyde (MDA) over the 7-day study period in the MARS **(A)** and SMT **(B)** groups. Change of plasma malondialdehyde over 20 individual MARS treatment sessions in the patients in the MARS group is also shown **(C)**.

6.3.2.1 Cytokines: Plasma levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) and their receptors (TNF-R1 and R2) were elevated in most patients in both groups, along with elevated anti-inflammatory cytokines (IL-10). However, there were no significant changes in either group, either over 7 days or over individual MARS sessions. TNF-R1 and IL-8 were detectable in the MARS albumin dialysate (segment-1). TNF-R1 was removed across the haemofilter column (between segments 1 and 2) while IL-8 was removed across the charcoal column (between segments 2 and 3) (Fig 4).

6.3.2.2 TBARS assay for malondialdehyde: Malondialdehyde level was elevated four-fold in both groups of patients at baseline. The reduction of plasma malondialdehyde (relative to baseline) in the MARS group [15.9 (-39.7 to 51.1) %] was significantly greater than in the SMT group [-18.9 (-131.0 to 15.1) %] ($p<0.05$) (Fig-2). Malondialdehyde was detectable in the MARS albumin dialysate (segment-1), but did not appear to be removed by any particular part of the circuit.

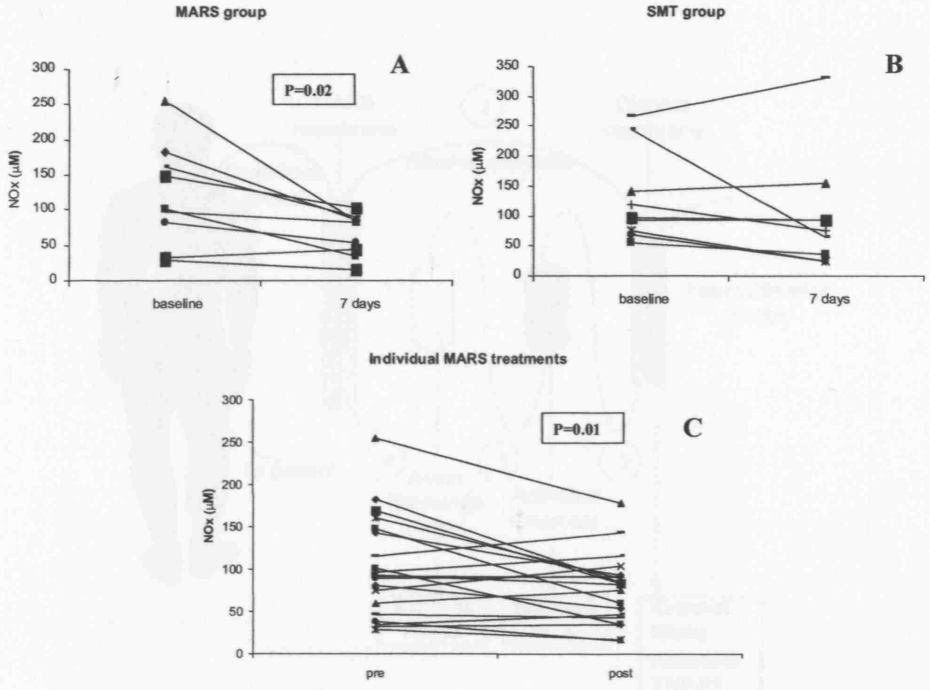


Fig 3: Change of plasma NOx (nitrate + nitrite) over the 7-day study period in the MARS **(A)** and SMT **(B)** groups. Change of plasma NOx over 20 individual MARS treatment sessions in the patients in the MARS group is also shown **(C)**.

6.3.2.3 Oxygen-based free radical production: Levels of spin-trapped oxygen-based free radicals derived from venous blood decreased in all four MARS-treated patients over 7 days [median -66.2%], but increased in all four SMT-treated patients [47.6%] where spin traps measurements were performed, though due to the limited sample numbers, statistical significance was not attained (Fig 5). Analysis of the electron paramagnetic resonance spectra suggests that the radicals generated were alkoxyl (identified by coupling constants aN-13.9 Gauss, aβH -2.2 Gauss) and carbonyl (aN-14.1 Gauss; aβH -4.0 Gauss) radicals. These assignments, which agree with previous studies[203], suggest that these radicals were derived from decomposition of lipid hydroperoxides in the extracellular compartment.

6.3.2.4 Nitrate/nitrite (NO_3^-/NO_2^-): Plasma nitrate and nitrite, which were elevated two- to three-fold in both groups, reduced significantly in the MARS group over 7 days, but not in the SMT group (Fig 3). A significant reduction in nitrate was also observed over individual MARS treatments. Within the MARS circuit nitrite was removed across the haemofilter column (between segments 1 and 2) and nitrate across the anion exchange resin column (between segments 3 and 4) (Fig 4).

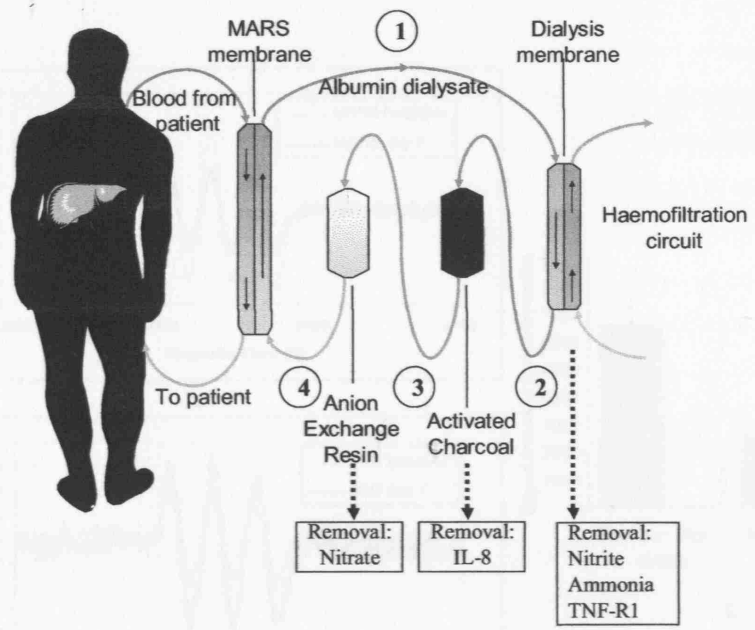


Fig 4: A schematic diagram of the MARS circuit showing the various segments (1-4) and filter columns, along with sites of removal of NOx, nitrite, ammonia, TNF-R1 and interleukin-8 from the albumin dialysate.

6.3.2.5 Ammonia: Plasma ammonia was elevated four- to five-fold and did not change significantly in either group. No difference in concentration was detected either over 7 days or over individual MARS sessions even though ammonia was detected in the dialysate of the MARS circuit (segment-1), from which it was found to be removed across the haemofilter column (between segments 1 and 2) (Fig 4).

6.3.3 Survival

There were 5 in-hospital deaths in both groups, while 4 patients from each group could be discharged (all alive after 3 months follow up). In the MARS group, two patients died due to variceal bleeding and three due to multi-organ failure (two with associated sepsis). In the SMT group, one patient died due to variceal bleeding and four due to multi-organ failure (two with associated sepsis). There was one death within the 7-day study period in the MARS group (due to multi-organ failure on day 7; the data for day 6 for this patient (prior to the onset of the final acute deterioration) has been used in the analysis.) and none in the SMT group. Neither clinical, nor biochemical or pathophysiological parameters over the 7-day study period were

significantly different between those who ultimately survived and those who did not in either group (data not shown).

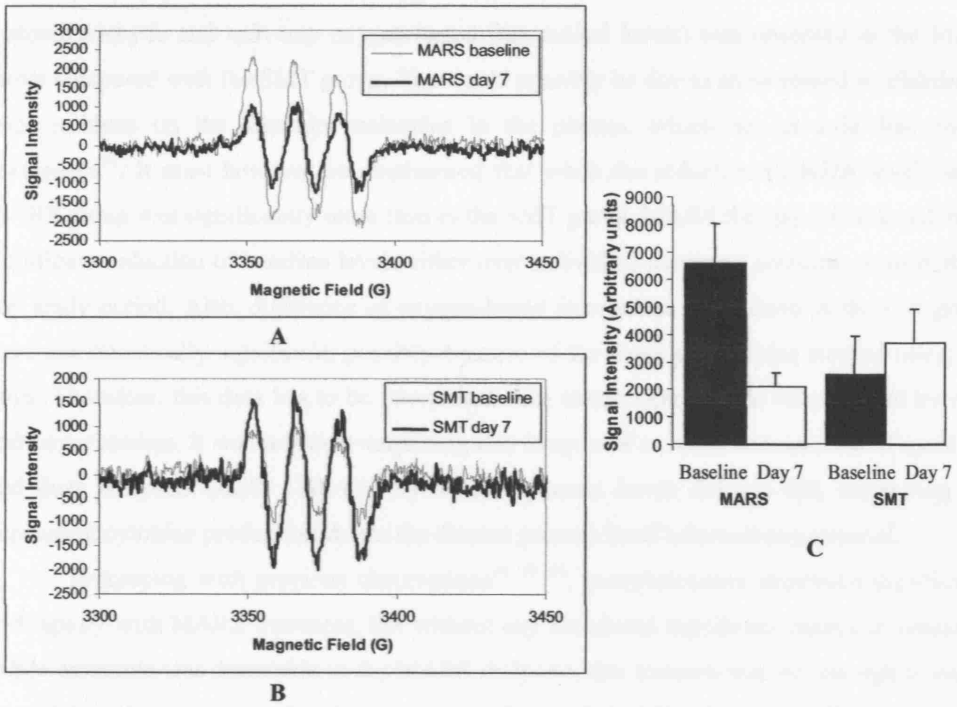


Fig 5: PBN (α-phenyl N-tert-butyl nitrone) spin trap samples extracted from venous blood from a MARS-treated patient **(A)** and a SMT patient **(B)** at baseline and on day 7. Over the 7 days, a decrease in signal intensity in the MARS group, and an increase in signal intensity in the SMT group can be seen. Change in observed signal intensity over 7 days in the MARS-treated (n=4) and SMT (n=4) group are shown **(C).**

6.4 Discussion

Although several investigators have described the clinical effects of MARS[74, 95, 96, 99, 107], this study is the first to investigate the pathophysiological alterations produced in a relatively homogeneous group of patients with ACLF due to inflammation-related precipitants in a randomised controlled manner. It is important to note that this study was not designed to look at mortality but at the pathophysiologic basis of MARS treatment. The main findings were improvement of encephalopathy without improving blood pressure or renal function, which was accompanied by a significant reduction of oxidative stress and NOx, without significant change of cytokine profile or plasma ammonia.

The primary hypothesis tested in the present study related to whether MARS treatment could alter the cytokine profile and/or reduce oxidative stress in patients of ACLF due to inflammation-related precipitants. No significant changes in the cytokine levels were detected over the 7-day period. However, a significant reduction of oxidative stress (quantified by malondialdehyde and spin-trap oxygen-based free radical levels) was observed in the MARS group compared with the SMT group. This could possibly be due to an increased availability of thiol residues on the albumin molecules in the plasma, which act as avid free radical scavengers[86]. It must however be emphasised that while the reduction of MDA levels in the MARS group was significantly more than in the SMT group, MARS therapy failed to achieve a significant reduction of baseline levels either over individual treatment sessions, or over the 7-day study period. Also, difference of oxygen-based free radical production in the two groups were not statistically significant, possibly because of the small sample size studied using spin traps. Therefore, this data has to be interpreted with caution and should be validated by other and larger studies. It was however surprising that in spite of an observed removal of cytokines and their receptors (IL-8, TNF-R1) by MARS, plasma levels did not fall, suggesting that concurrent cytokine production due to the disease process itself balanced any removal.

In keeping with previous observations[74, 95, 96], encephalopathy improved significantly and rapidly with MARS treatment, but without any associated significant change in ammonia. While ammonia was detectable in the MARS dialysate, this removal was not enough to reduce arterial levels, suggesting that improvement of encephalopathy due to MARS treatment is probably independent of improvement of hyperammonaemia. Inflammation is also believed to be important in the pathogenesis of encephalopathy[41]. A reduction of pro-inflammatory cytokines could have been responsible for improvement of encephalopathy, but this was not observed. However, a significant reduction was noted in the markers of oxidative stress, which is increasingly thought to be important in the pathogenesis of encephalopathy[204-207] through its effects upon mitochondria, oxidation of membrane phospholipids and various enzymes involved in energy metabolism[207]. Another potential underlying mechanism may be the reduction of plasma NOx levels[207]. The role of NO in encephalopathy is unclear, but evidence is emerging that nitrosation of critical proteins may contribute by altering their function or causing injury by formation of nitrotyrosine[208]. Thus, one might hypothesize that in the presence of hyperammonaemia, the mediators of inflammation may contribute to its adverse neuropsychological effects[201]. It is also important to note that in the group of patients studied, encephalopathy was not present as a distinct entity, but rather as a component of ACLF. Thus, it is difficult to look at the change of encephalopathy in isolation without regarding the entire

clinical picture as a whole. The same principle applies to any underlying pathophysiological basis, which one would expect to differ in ACLF from that of 'pure' hepatic encephalopathy.

It was surprising to find no change in MAP following MARS therapy, in contrast to some previous studies[72-74, 95, 202] (which also demonstrated MARS-related improvement of systemic vascular resistance and cardiac output). However, most of these studies were uncontrolled. The effects of haemofiltration, inadequate resuscitation and hypothermia also need to be considered as these can affect MAP. It must be pointed out that most of the patients in the present study were not substantially hypotensive at baseline, which might explain the lack of subsequent rise of MAP post-MARS. At least one other study has reported no change of MAP following MARS in a group of patients who were haemodynamically stable pre-treatment[209]. The substantial reduction of plasma NOx in the MARS group, both as an immediate effect of treatment, and over the 7-day study period, demonstrates a possible mechanism of improving systemic haemodynamics. A reduction of plasma NOx indicates either direct removal by MARS, or reduction of NO synthesis (with reduced production of its metabolites), or a combination of both. Fairly high levels of NOx in the dialysate indicate direct removal. Whether there is an additional reduction of NO synthesis by altering factors favouring its production in ACLF, e.g. by removing endotoxins[12, 13], needs further study. The biological significance of removal of NOx by MARS is unclear, but nitrite (NO_2^-) has been implicated in the formation of nitrotyrosine residues under oxidative stress conditions[210].

It is difficult to explain the lack of any significant change in MAP despite a reduction of NOx. To clarify this apparent discrepancy, the alterations in the vasoconstrictor neurohormonal profile in three other patients of alcohol-related ACLF being treated with MARS were investigated[11, 211]. Plasma renin activity, angiotensin II, noradrenaline and endothelin-1 were substantially elevated in the patients prior to MARS, and were reduced markedly at the end of treatment and sustained 12 hours after MARS (Fig 6, unpublished data). It is possible that the lack of improvement in MAP observed in the present study despite a reduction in NOx may be because of concurrent removal of the vasoconstrictor neurohormones. However, these data need to be confirmed by other studies designed for this purpose.

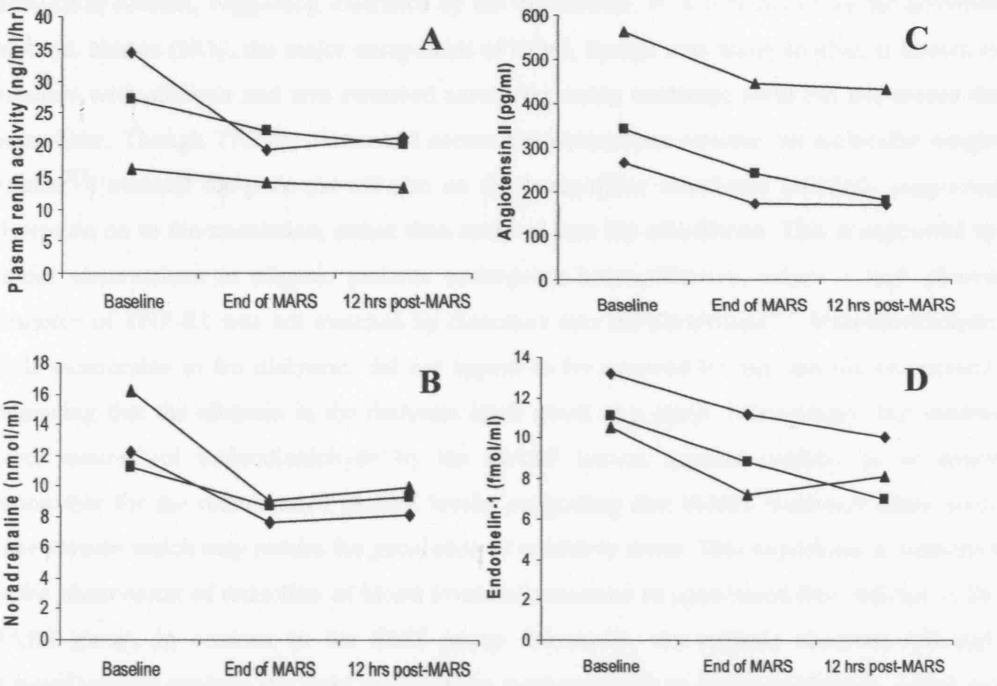


Fig 6: Vasoconstrictor neurohumoral profile of three patients treated with MARS, at baseline, end of treatment and 12 hours after end of treatment. Normal values: PRA: 1.7 ± 0.8, Angiotensin II: 3.2 ± 0.3, Noradrenaline: 1.4 ± 0.4 [211], Endothelin-1: 1.2 ± 0.2 [11]

The lack of improvement of renal function following MARS therapy was also unexpected, and apparently contradicts some previous studies that have reported improvement in patients with HRS following MARS treatment. While most reports did not have a control arm[74, 95, 96], the one study which did[72] had a small sample size (n=13). One more death in the MARS arm would have meant that the significance of the observation would have been lost. In addition, it should also be noted that rather than looking at 'pure' HRS, the present study included ACLF patients who had renal dysfunction as a component of their clinical picture. Thus, any observed clinical change, or the lack of it, and the underlying pathophysiological basis is not necessarily comparable with other such studies. An apparent reduction of serum creatinine was observed over 7 days in the SMT group, which was not statistically significant, but which probably accounted for the significant improvement of MELD score in this group (while the significant reduction of serum bilirubin probably achieved the same in the MARS group).

The roles of the different components of the MARS circuit in the removal of 'toxins' was also examined. Ammonia and nitrite (highly water-soluble) are removed across the

haemofilter column, suggesting clearance by the ultrafiltrate. IL-8 is removed by the activated charcoal. Nitrate (NO_3^-, the major component of NOx), though also water soluble, is known to associate with albumin and was removed across the anion exchange resin but not across the haemofilter. Though TNF-R1 decreased across the haemofilter column, its molecular weight (55kDa[212]) exceeds the pore cut-off size on the haemofilter membrane (30kDa), suggesting adsorption on to the membrane, rather than removal into the ultrafiltrate. This is supported by similar observations in oliguric patients undergoing haemofiltration, where a high plasma clearance of TNF-R1 was not matched by clearance into the ultrafiltrate[213]. Malondialdehyde, while measurable in the dialysate, did not appear to be removed by any specific component, suggesting that the albumin in the dialysate itself acted as a sump. Interestingly, this modest direct removal of malondialdehyde by the MARS system appears unlikely to be solely responsible for the reduction of plasma levels, suggesting that MARS treatment alters some other process which may reduce the generation of oxidative stress. This hypothesis is supported by the observation of reduction of blood levels of measured oxygen-based free radicals in the MARS group, in contrast to the SMT group. Moreover, the radicals observed (alkoxyl, carbonyl) would produce the lipid peroxidation products, such as malondialdehyde, which are measured by the TBARS assay.

Finally, it must be re-emphasised that while the present study looks at encephalopathy, renal dysfunction and circulatory disturbances in liver disease, it does not look at them in isolation, but rather as part of the overall clinical picture of ACLF. This might explain the difference in clinical changes observed in this study, as well as the underlying pathophysiological basis, from other studies performed in the past, which did not evaluate ACLF as a whole.

The main clinical change with MARS therapy observed in this study was an improvement of encephalopathy. The inability to reproduce some of the other clinical benefits previously reported (such as improvement of blood pressure or renal function) suggests that results of treatment may be determined by the population of patients being studied, timing of intervention, precipitating factor of ACLF and the baseline haemodynamics. For instance, data from the International MARS Registry indicates that haemodynamic improvements with MARS therapy is predominantly seen in patients with a lower baseline MAP than was seen in the present study[128]. The findings from our earlier uncontrolled study showing improvement of systemic haemodynamics and renal function following MARS dialysis in patients with ACLF could not be reproduced in this randomised study. This might represent an effect of selection bias in our earlier study, as also perhaps in some of the other non-randomised studies. Given the

variability in the observed clinical responses, further studies need to be performed to obtain definitive data before the widespread and unrestricted application of MARS in clinical practice can be recommended. These results also show that the clinical changes observed with MARS treatment in patients with ACLF precipitated by inflammation, are independent of changes in plasma cytokine levels, although significant amounts of cytokines were removed in the circuit. The predominant pathophysiologic effect of MARS is on the other mediators of inflammation like NOx and possibly oxidative stress, which are reduced with MARS treatment, possibly due to a combination of removal and reduced production. An important observation is the significant improvement of encephalopathy with MARS, perhaps due to reduced inflammatory mediators rather than changes in ammonia, highlighting the importance of factors other than ammonia in its pathogenesis. Whether altered nitric oxide and oxidative stress are pathophysiologically important, or just associations, need to be clarified in future studies.

7. STUDY 4- REDUCTION IN WHITE MATTER OEDEMA ATTENUATES INTRACRANIAL HYPERTENSION IN PIGS WITH ACUTE LIVER FAILURE FOLLOWING ALBUMIN DIALYSIS

7.1 Introduction

Raised intracranial pressure (ICP), in conjunction with cerebral oedema, is a common complication of acute liver failure (ALF) contributing significantly to mortality and morbidity[214-218]. The underlying pathophysiological mechanisms are not entirely clear, however hyperammonaemia is a consistent finding and believed to be important[35]. This is emphasized by studies where ammonia-lowering strategies prevented brain edema and severe encephalopathy in rats with ALF[219, 220]. It has also been suggested that increased cerebral blood flow (CBF)[221] may be coupled with intracranial hypertension in ALF. However, whether increased CBF is a cause of raised ICP (temporally preceding it), an effect, or just an association remains unclear. Recent studies suggest that other factors such as inflammation and subliminal infection may contribute[41, 201] too.

Recent studies have investigated the role of Molecular Adsorbents Recirculating System (MARS) in liver failure[74, 95, 96]. One consistent observation with its use has been an improvement of hepatic encephalopathy (HE)[74, 75, 95, 96]. However, most of the data pertains to patients with acute decompensation of chronic liver disease, and the effect of MARS therapy on cerebral status in ALF remains unclear.

A porcine model of ALF induced by hepatic devascularisation has been developed and validated[142-145], and shows the characteristic rise of ICP with concomitant increase in blood ammonia, along with a hyperdynamic vasodilated circulation and worsening liver function. The present study utilises this model to better understand the pathophysiological basis of intracranial hypertension, and investigate the impact of albumin dialysis on development of raised ICP in ALF, focusing on arterial ammonia, cerebral oedema (measuring brain water), CBF and plasma inflammatory mediators- factors of pathophysiological importance in ALF.

7.2 Methods

The study was performed at the Surgical Research Laboratory, University of Tromsø, Norway, with approval of the Norwegian Experimental Animal Board, between September and November 2002. Twenty-four female Norwegian Landrace pigs from three different litters, weighing 23-30 (26.8±0.3, mean±SEM) kg were used.

7.2.1 Study design

Sample size was determined using a power calculation for multiple groups[222]. Based on previous ICP data from this model[145], and limited evidence from MARS-treated patients[95], we

anticipated mean difference between groups of 30% with SD 16%. Designating α=0.05 and β=0.9, a minimum population size of 7 subjects per group would be required. Allowing for experimental failure, 8 animals were studied in each experimental arm.

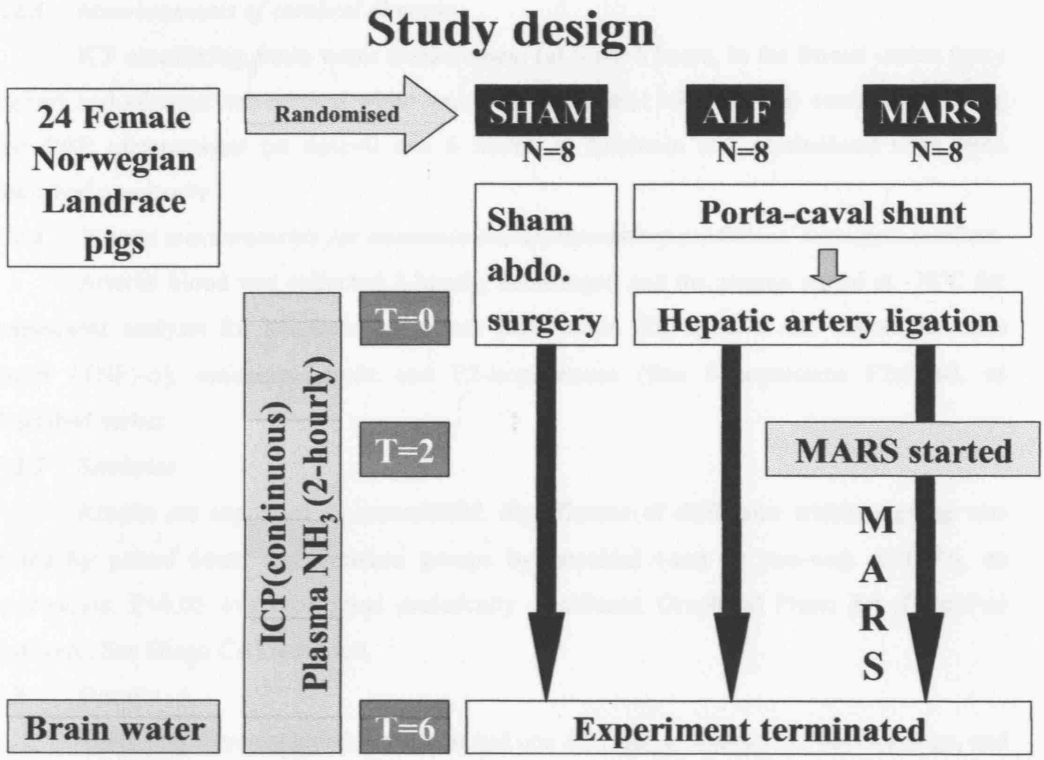


Fig 1: An outline of the study design.

The pigs were randomised by opening sealed, pre-numbered envelopes into one of three groups: 'sham', 'ALF' or 'ALF+MARS' (Fig 1). The randomisation sequence was generated by a colleague not participating in the study and concealed until groups were assigned by one of the principal investigators (LMY). In the sham group, 'sham' abdominal surgery was performed without interfering with hepatic blood supply. In the other two groups, hepatic devascularisation was performed by an end-to-side porta-caval anastomosis followed by hepatic artery ligation. Details of the surgery, including 'sham' operation, are described elsewhere[144, 145]. Time=0 hours was defined as time of hepatic artery ligation (ALF, ALF+MARS), or completion of surgery just prior to closing abdominal wall (sham). All pigs were monitored over the following 6 hours. In the ALF+MARS group, MARS dialysis was instituted from time=2-6 hours (4-hour session). Experiments were terminated with overdose of pentobarbital and potassium chloride at time=6 hours.

7.2.2 The animal model

Details regarding animal room facilities, anaesthesia, surgical preparation, surgery, catheter placement and MARS dialysis have been described previously[142-145, 223].

7.2.3 Measurements of cerebral changes

ICP monitoring, brain water measurement (at time=6 hours, in the frontal cortex (grey matter) and adjacent sub-cortical white matter of the frontal lobe, and the cerebellar cortex), and CBF measurement (at time=0 and 6 hours, in forebrain and cerebellum) have been described previously.

7.2.4 Plasma measurements for ammonia and inflammatory mediators/ surrogate markers

Arterial blood was collected 2-hourly, centrifuged and the plasma stored at -70°C for subsequent analysis for ammonia, cytokines [Interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)-α], malondialdehyde and F2-isoprostanes (free 8-Isoprostane F2alpha), as described earlier.

7.2.5 Statistics

Results are expressed as mean±SEM. Significance of difference within a group was tested by paired t-test, and between groups by unpaired t-test or two-way ANOVA, as appropriate. $P<0.05$ was considered statistically significant. GraphPad Prism 4.0 (GraphPad Software, San Diego CA) was used.

7.3 Results

Three pigs were excluded: one sham and one ALF due to intracranial haemorrhage, and one ALF due to technical problems with ICP monitoring. 21 pigs (sham-7, ALF-6, ALF+MARS-8) were ultimately included.

Following onset of liver devascularisation, the ALF group (7.0±0.8 to 15.5±1.7 mmHg) developed a significant increase in ICP over 6 hours (121.4±22.1%, $p=0.005$) compared to sham (8.4±0.9 to 9.7±1.2 mmHg, a 15.3±13.1% increase [$p>0.05$]) (ALF vs sham: $p<0.05$). This rise was attenuated in the ALF+MARS group (9.1±1.0 to 14.5±1.6 mmHg, a 58.9±16.0% increase [$p<0.001$]) (ALF+MARS versus ALF: $p<0.05$), though normalisation was not achieved (ALF+MARS versus shams: $p<0.05$) (Fig 2). Similarly, the absolute increase of ICP over 6hrs was significantly greater in ALF (8.5±1.5 mmHg) compared to sham (1.3±0.6 mmHg) ($p<0.01$). This rise was attenuated with MARS (5.4±1.0 mmHg, $p<0.05$ versus ALF), though it was still significantly greater than in sham ($p<0.01$). Finally, the area under the curve for relative change in ICP (mmHg) versus time over the duration of MARS dialysis (time=2-6hrs) was significantly lower with MARS (6.5±0.9 mmHg.hr) compared to the ALF group (11.5±2.4 mmHg.hr) ($p<0.05$).

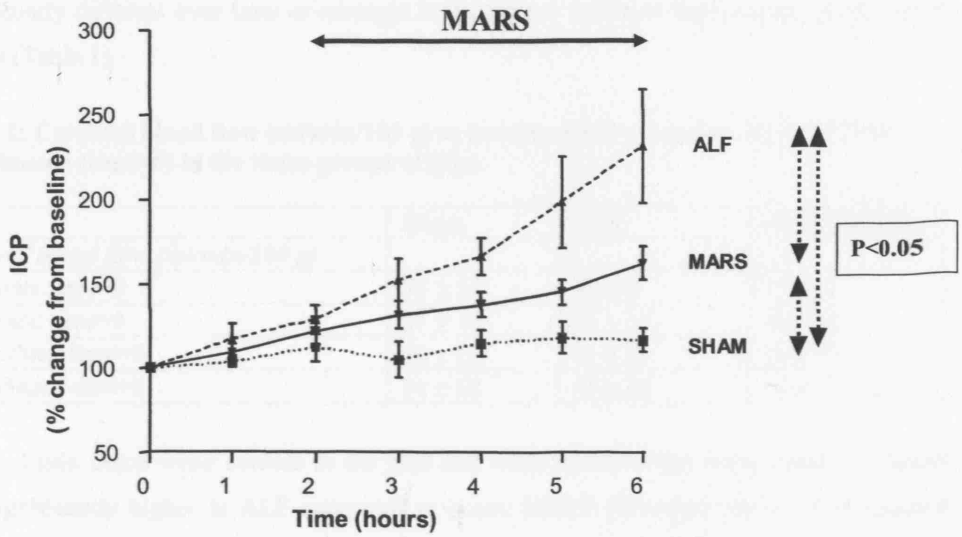


Fig 2: Rise of intracranial pressure (expressed as percentage change from baseline) over the duration of the study period in the three groups.

Elevation of arterial ammonia occurred over 6 hours in ALF and was significantly higher than in sham ($p<0.0001$). The ALF+MARS group also developed hyperammonemia which was not significantly different from the ALF group but significantly higher than in sham ($p<0.0001$) (Fig 3).

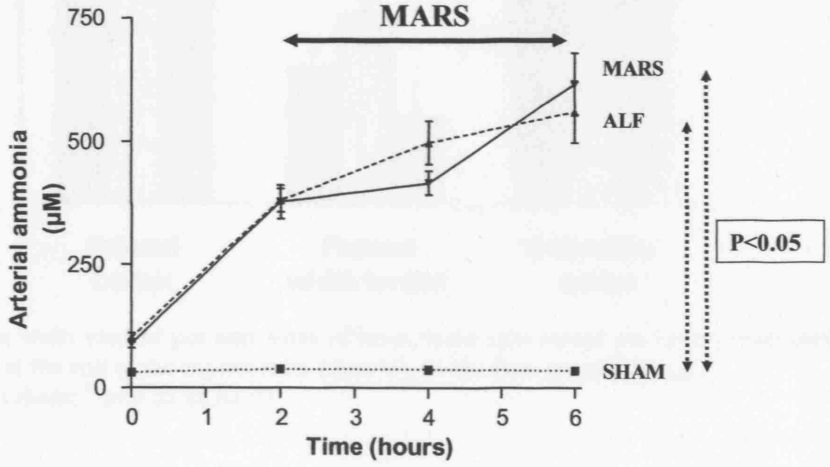


Fig 3: Rise in arterial ammonia over the duration of the study period in the three groups.

CBF, measured at time=0 and 6 hours in the forebrain and the cerebellum, was not significantly different over time or amongst brain regions within or between any of the three groups (Table 1).

Table 1: Cerebral blood flow (ml/min/100 g) at baseline (time=0) and at the end of the experiments (time=6) in the three groups of pigs.

	Sham	ALF	ALF+MARS
Cerebral blood flow (ml/min/100 g)			
Forebrain: time=0	59 ± 14	38 ± 8	37 ± 7
Forebrain: time=6	31 ± 16	33 ± 13	49 ± 19
Cerebellum: time=0	48 ± 19	41 ± 11	29 ± 9
Cerebellum: time=6	34 ± 14	35 ± 16	35 ± 18

Brain tissue water content in the grey and white matter of the frontal lobe at 6 hours was significantly higher in ALF compared to sham. MARS prevented rise of ALF-induced water content in the white matter (p=0.04), but not in the grey matter. Cerebellar water contents were not significantly different between the three groups (Fig 4).

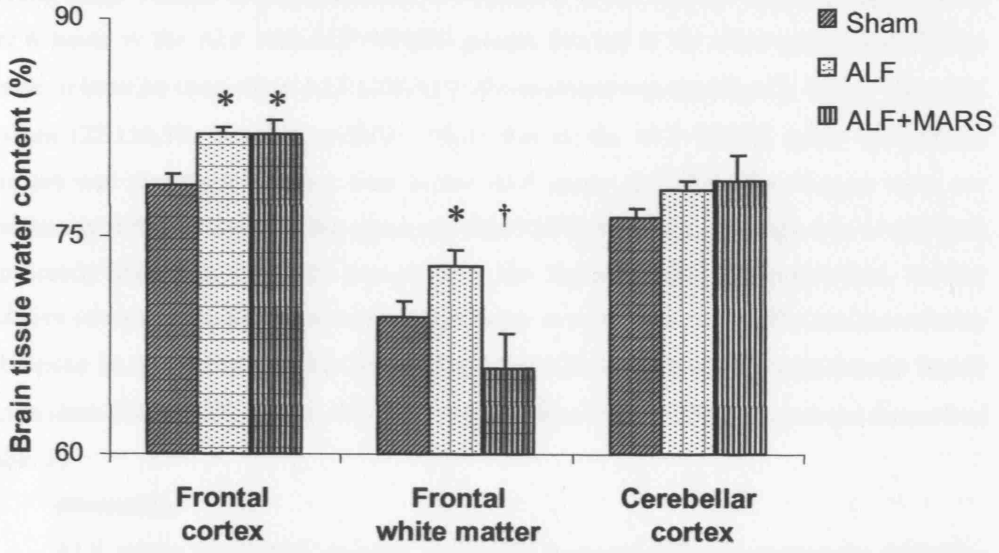


Fig 4: Brain water content per unit mass of brain tissue (gm water/ gm tissue, expressed as a percentage) at the end of the experiments (time=6), in the three groups of pigs.
(* $p<0.05$ vs sham; † $p<0.05$ vs ALF)

Table 2: Plasma levels of cytokines, malondialdehyde (MDA) and F2-isoprostanes in the three groups of pigs at baseline (time=0) and at the end of the experiments (time=6).

	Sham		ALF		ALF+MARS	
	Time= 0 hrs	Time= 6 hrs	Time= 0 hrs	Time= 6 hrs	Time= 0 hrs	Time= 6 hrs
IL-6 (μM)	BDL	BDL	BDL	BDL	BDL	BDL
IL-8 (μM)	BDL	BDL	BDL	BDL	BDL	BDL
TNF-α (μM)	BDL	BDL	BDL	BDL	BDL	BDL
MDA (μM)	1.4 ± 0.2	1.1 ± 0.2	1.6 ± 0.4	2.9 ± 0.3**,†	1.8 ± 0.2	2.4 ± 0.1**,†
F2-isoprostanes (pg/ml)	100.1 ± 17.0	76.0 ± 13.0*	89.6 ± 8.7	105.5 ± 15.4	83.9 ± 10.0	96.6 ± 10.8

BDL- below detectable limits (all samples < 5 pg/ml)
* $p < 0.05$ vs baseline (time=0 hrs) values
** $p < 0.01$ vs baseline (time=0 hrs) values
† $p < 0.001$ vs sham group time=6 hrs values

None of the inflammatory mediators (or surrogate markers) studied (cytokines: IL-6, IL-8, TNF-α), increased significantly over time in any of the groups, or differed significantly between them. Plasma malondialdehyde, an oxidative stress marker, increased significantly after 6 hours in the ALF and ALF+MARS groups, but not in the sham group. The change relative to baseline (time=0) in ALF (120.1±42.0% increase) was significantly higher compared to sham (22.1±9.3% decrease, $p<0.01$), while that in the ALF+MARS group (35.2±8.4% increase) was significantly lower than in the ALF group ($p<0.05$). The changes were not significantly different between the sham and ALF+MARS groups, although 6-hr levels were significantly higher in the latter compared to the former. Plasma F2-isoprostane, another oxidative stress marker, did not increase significantly in any of the groups. The increase relative to baseline in ALF (22.1±16.4%) and ALF+MARS (21.7±15.0%) were significantly higher than in sham (20.0±7.2% decrease) ($p<0.05$) but not significantly different amongst themselves (Table 2).

7.4 Discussion

ALF results in cerebral oedema, leading to increased ICP and consequent morbidity and mortality[214-217]. However, the underlying mechanisms have not been fully elucidated. A consistent clinical response observed with MARS therapy has been improved cerebral status[74, 75, 95, 96]. The present study investigates the pathophysiological factors responsible for raised ICP in ALF, using MARS dialysis as an experimental tool. A porcine liver-devascularised ALF model previously demonstrated to have a characteristic increase in ICP[142-145], and large enough to study an extracorporeal device in, was used. The rise of ICP and increase in brain water

without significant change in CBF and inflammatory markers suggest a significant initiating role of ammonia in the pathogenesis of increased ICP in ALF. The attenuation of ICP increase with MARS was not due to reduced arterial ammonia and was independent of changes in CBF or peripheral inflammatory markers suggesting that other undefined mechanisms are at play. Our data also demonstrate asymmetric distribution of brain water, possibly implicating different types of astrocytes or other cell types in the pathogenesis of brain oedema in ALF. Normalization of brain water in the white matter by MARS suggests this region as a possible new therapeutic target.

A noteworthy point is the variation in baseline ICP between the three groups, with the difference between ALF (lowest) and ALF+MARS groups (highest) tending towards statistical significance ($p=0.09$). The probable reason is inter-litter variation, with uneven distribution of animals from the three litters used for the study between the three groups, in spite of the randomisation protocol used, leading to this phenomenon. This confounded the analysis of the ICP data (using actual values) between the groups. Consequently, we also analysed the data using (a) percentage changes, (b) differences in absolute changes (delta ICP) and (c) area under the curve for relative change of ICP versus time. All techniques used indicate a rise of ICP in ALF compared to sham, and attenuation with MARS dialysis. Moreover, while analysis of absolute ICP values is important to interpret the clinical relevance of data, the present study was designed to allow early institution of albumin dialysis to attenuate rise of ICP. Thus, the absolute ICP values cannot be directly translated to the clinical setting of ALF, but the change of ICP can be used to study underlying pathophysiology.

Hyperammonaemia has traditionally been implicated in the pathogenesis of HE and raised ICP in ALF[33-35], and this is supported in our model where intracranial hypertension was accompanied by rising arterial ammonia in ALF (as opposed to sham). Similar arterial levels have previously been shown to be associated with high brain ammonia extraction[224] and brain stem herniation in ALF patients[35]. However, even though MARS attenuated the rise of ICP, hyperammonaemia persisted, suggesting other factors are important in the development of intracranial hypertension. Removal of these putative factors can potentially result in a beneficial lowering of ICP. The presence of extensive intracranial arteriovenous anastomoses[225, 226] in pigs meant that further analyses for brain ammonia flux/ extraction (using arterial and reverse jugular venous samples) would be difficult to interpret.

Cytotoxic brain oedema is commonly observed in ALF and thought to be the underlying mechanism of intracranial hypertension. Most studies investigating this phenomenon concentrate on brain water changes in the frontal cortex (grey matter) in different

animal models[219, 220, 227]. This study explored the possibility of regional cerebral differences by measuring brain water in the frontal cortex (grey matter), adjacent sub-cortical white matter, and the cerebellar cortex. In addition to finding the consistently observed increase in brain water in the grey matter in the ALF group, a significant increase was also noted in white matter but not in cerebellum, emphasizing that cerebral oedema in ALF is not entirely confined to grey matter. This may provide novel insights into the pathogenesis. It is known that astrocyte swelling (possibly due to accumulation of the ammonia-detoxification product glutamine) in grey matter causes brain oedema in ALF[217, 228]. However astrocytes also occur in white matter, though of a different type (fibrous astrocytes, compared to protoplasmic astrocytes in grey matter). This suggests that astrocytes in "general" are prone to swell and/or that other cells in white matter (e.g. oligodendrocytes) contribute to brain oedema in ALF. The additional observation of normalization of white matter water content with albumin dialysis, without improving grey matter oedema, implies that white matter water content may be the more labile component, more amenable to therapeutic interventions. Anecdotal reports in rat models of ALF demonstrating regional differences of brain water between forebrain (predominantly grey matter) and hindbrain (predominantly white matter)[229-231] support our findings.

Wide variations of CBF have been reported in the setting of ALF[232-235], perhaps due to variations of techniques used, and lack of longitudinal observations which would define a clearer temporal resolution. The pig is a difficult model to study CBF in, due to presence of intracranial arteriovenous anastomoses[225, 226], making the Kety-Schmidt technique[236] unreliable. This problem was circumvented using a technique based on microsphere trapping at the capillary level[237-240]. In our model, no significant change of CBF in the forebrain or the cerebellum (hindbrain) accompanied either the rise in ICP with ALF, or the decrease with MARS dialysis. All values observed remained within normal limits for pigs. This suggests that development of intracranial hypertension occur independent of altered CBF in this porcine model of ALF. Whether rise of CBF may develop secondary to, or as an association of, increased ICP remains to be investigated. Experimental evidence from hyperammonaemic rats suggest that raised ICP and cerebral oedema temporally precede the increase of CBF[227].

We also investigated the role of mediators of inflammation (or surrogate markers) in relation to these cerebral effects. Takada et al[42] showed in a pig model of ALF that animals administered lipopolysaccharide and amatoxin intraportally developed greater intracranial hypertension than animals given amatoxin alone. ALF patients who had higher systemic inflammatory response syndrome scores or were overtly infected were more likely to develop intracranial hypertension[43, 44]. Malondialdehyde levels showed statistically significant changes,

but remained within the normal range and were unlikely to be of pathophysiological relevance. F2-isoprostanes did not increase significantly in ALF, however the change from baseline was significantly higher compared to sham mainly because of a significant reduction of levels in sham (perhaps due to recovery from surgical stress). None of the pro-inflammatory cytokines studied increased significantly in any of the groups studied, suggesting that the cerebral changes over six hours in our model developed independent of any inflammation, including oxidative stress.

The cerebral effects of albumin dialysis observed in the present study were evident with a four-hour session started two hours after the induction of ALF, implying that early institution of therapy in ALF patients might bring about a rapid clinical response. Haemodialysis frequently worsens intracranial hypertension in ALF, and therefore a closed albumin dialysis without additional haemo-dialysis/filtration may be a worthwhile option. There was no significant change of arterial ammonia levels, even though ammonia was detectable in the MARS dialysate (data not shown). This is in keeping with our previous observation in patients with acute-on-chronic liver failure of a marked improvement of HE despite unchanged arterial ammonia (even though ammonia was being removed by the circuit)[184]. Moreover, the present study duration was too short to evaluate changes due to altered ammonia metabolism following MARS (e.g. increased ureagenesis), and a devascularised liver model would not have been appropriate to investigate such changes.

Recent evidence suggests that intracranial hypertension of ALF is accompanied by increasing CBF[221], which was not found in our model. A literature review shows that most of the data demonstrating raised CBF was associated with ICP levels above 20mmHg, which importantly was only observed in two pigs (both 21mmHg) in our study. In addition, most clinical data available on intracranial hypertension in ALF pertains to patients studied late in the course of their disease (advanced ALF) with additional complications such as inflammation/infection. It is becoming increasingly evident that duration of hyperammonaemia[241] or factors in addition to hyperammonaemia[41, 201] may contribute to the brain changes of ALF. Inflammation (including oxidative stress) and/or increasing CBF, perhaps late in the course of the disease, might lead to a further worsening of ICP.

From the present study, we conclude that while hyperammonaemia is probably essential for the initial development of cerebral oedema and intracranial hypertension in ALF, the interplay of CBF and inflammation is not. Regional differences exist for brain oedema, with white matter oedema being more amenable to therapeutic interventions. Factors in addition to hyperammonaemia also appear to be important. Identifying these putative factors, and

understanding the intracerebral effects they might have, would greatly improve our knowledge of the pathogenesis of intracranial hypertension in ALF.

8. STUDY 5- ALBUMIN DIALYSIS: A NEW THERAPEUTIC STRATEGY FOR INTOXICATION FROM PROTEIN-BOUND DRUGS

8.1 Treatment of phenytoin toxicity by the Molecular Adsorbents Recirculating System (MARS)

8.1.1 Introduction

Phenytoin is one of the most commonly used anti-epileptic drugs. However, it has a narrow therapeutic range, and a total serum level >80 μmol/l is associated with clinically relevant toxicity in many patients. Usually, it is metabolised by hepatic enzymes and excreted via the kidneys, but the hepatic enzymes are readily saturated, and, in the presence of renal failure, toxic accumulation of phenytoin can occur [242]. However, as it is 90% albumin-bound it is not removed by haemodialysis [243], though studies have suggested that uraemic toxins may increase free drug levels by displacing phenytoin from albumin [244], and haemodialysis using a high flux cellulose membrane, by removing these toxins, can actually reduce free phenytoin levels [245]. Treatment involving multiple oral dosing with activated charcoal is usually used, and in severe cases, charcoal haemoperfusion has been employed with some success [246]. However, haemoperfusion is not easily available in many parts of the world. Therefore, a system which could remove phenytoin from the patient's blood, and which is safe and relatively easily available, would be invaluable. The following report describes the use of the Molecular Adsorbents Recirculating System (MARS), a blood purification system based on the principle of albumin dialysis, as such a system.

8.1.2 Patient and Methods

A 45-year old man with status epilepticus was admitted in the Intensive Therapy Unit of the University College London Hospitals, London, in January 2001. He received high doses of intravenous phenytoin, and concomitantly developed rhabdomyolysis, leading to acute renal failure. This contributed to drug accumulation (serum phenytoin: total 79μmol/l, free 17.6μmol/l, with a plasma albumin level of 27g/l). Subsequently phenytoin was replaced by phenobarbitone, and despite continuous veno-venous haemofiltration (CVVHF) for anuria (haemodialysis not being feasible due to haemodynamic instability), the phenytoin level reduced very slowly to 40μmol/l (total) over the following eight days in spite of receiving enteral activated charcoal treatment. He remained unconscious even after withdrawal of sedation. Hepatic enzymes increased (alanine aminotransferase 367 IU/l, alkaline phosphatase

1493 IU/l) indicating hepatic toxicity, and he developed tachyarrhythmias (with episodic ventricular tachycardia) interspersed with bradyarrhythmias, requiring temporary cardiac pacing. No cause, other than phenytoin toxicity, could be recognized which could explain the hepatic toxicity or the arrhythmias.

Following informed consent from next-of-kin, he was treated with MARS, as described previously. Fig 1 gives a schematic diagram of the MARS circuit.

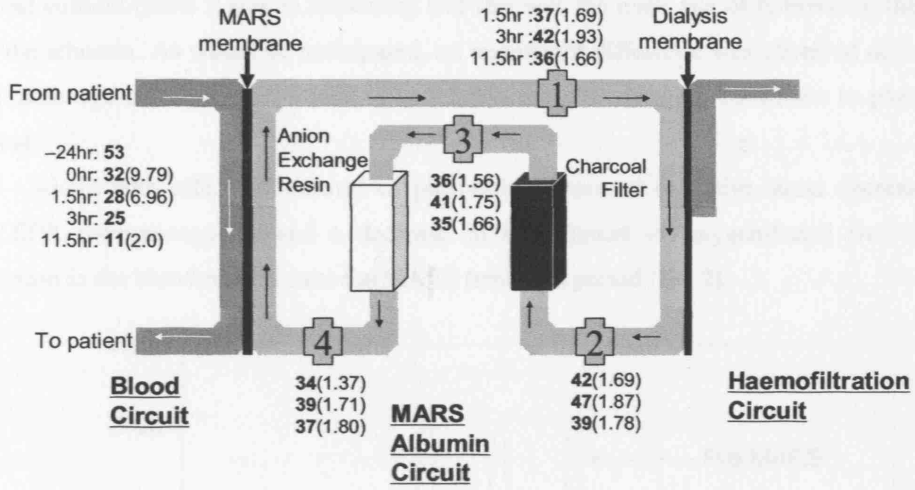


Fig 1: Scheme of the MARS system showing the direction of flow and the **total** (and free) phenytoin levels (μmol/l) at varying time points in the samples collected from the ports during treatment, in addition to the **total** (free) phenytoin levels in the patients serum prior to, and during MARS treatment.

In addition to the routine clinical investigations, total and free phenytoin levels were measured in the patient's serum and in the albumin dialysate by fluorescence polarization immunoassay (FPIA) and ultrafiltration. Samples were collected from the four segments of the MARS circuit (see Fig 1) just before, during (at 1.5 hours and 3 hours) and at the end of the MARS treatment session.

Blood levels of oxygen-based free radicals were measured using a spin trap and electron paramagnetic resonance (EPR) spectroscopy, as described earlier.

8.1.3 Results

MARS treatment was performed over a single session, which lasted for 11.5 hours. During treatment both the total and free serum phenytoin levels fell linearly and rapidly (from 32 to 11μmol/l and 9.8 to 2.0μmol/l respectively, correlation coefficients r^2=0.9932 & 0.9403, Fig 1). The next day the patient regained consciousness. Electrocardiogram normalized over a

further 24 hours, and hepatic enzymes gradually decreased over the following five days, after which he was successfully discharged from the intensive care unit.

Analysis of the albumin dialysate showed an increase in levels of phenytoin, clearly indicating its removal from the blood. The measured total phenytoin apparently increased across the haemofiltration component, ports 1 and 2, probably reflecting the removal of water. There was a consistent, significant reduction in both the total and free phenytoin across the charcoal column (ports 2 and 3) indicating that this was the main site of removal of the drug from the albumin. As would be anticipated, no significant difference was observed across the anion exchange column (ports 3 and 4), indicating that this does not contribute to phenytoin removal.

Along with effective removal of phenytoin, measured oxidative stress decreased as well. EPR spectroscopy showed a decrease in the amount of oxygen-based free radical production in the blood of 65% over the MARS treatment period (Fig 2).

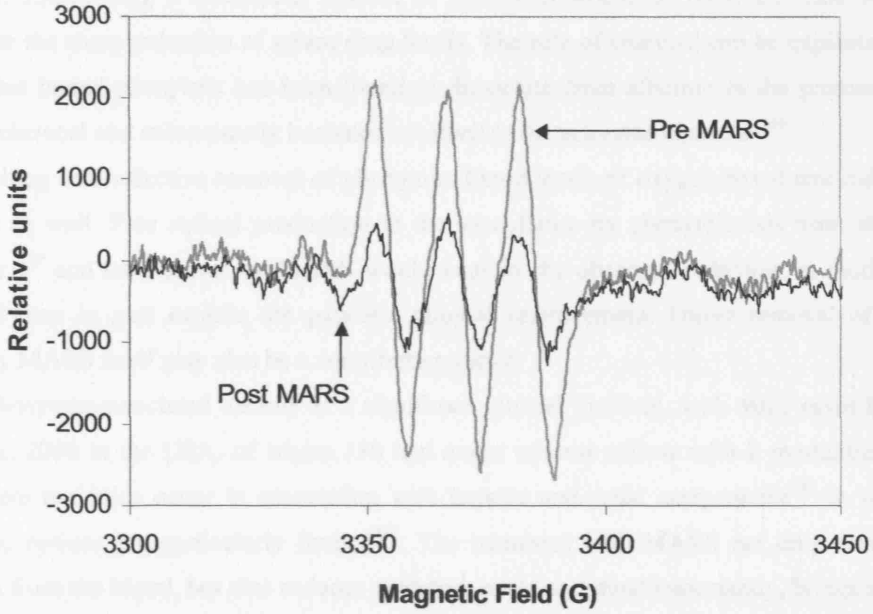


Fig 2: EPR spectra showing the alpha-phenyl-tert-butylnitrone spin trap signal extracted into toluene from the blood samples collected immediately prior to and post MARS treatment. A 65% drop in signal, representative of free radical production, is observed between samples. Measurement conditions:- temperature 22°C; Microwave frequency, 9.470 GHz; microwave power, 12.66 mW; time constant, 40.96 ms; and modulation amplitude 8G.

8.1.4 Discussion

The patient described in this report had a very high free serum phenytoin level, which was definitely in the toxic range (>8μmol/l [247]), compared to only a moderately high total drug

level. This can be attributed to this patient's low plasma albumin level (27g/l), which is a known factor causing such discrepancies between the two [248]. The high free phenytoin level can be considered particularly significant, as this is regarded to be more reliable as a marker for drug toxicity monitoring [249]. The presence of phenytoin in the albumin dialysate clearly indicated its removal from the blood. However, it must be emphasised that phenytoin levels in the MARS "closed" circuit cannot be directly compared to plasma levels, which relate to body volume. As the concentration of albumin in the circuit (200g/l) is considerably higher than that in the patient's blood (27g/l in this case), there is substantially increased binding capability, which facilitates the removal of albumin bound substances from the blood. There was a consistent, significant reduction of phenytoin levels across the charcoal column, indicating that this was the main site of removal of the drug from the albumin. The concentration gradient was not large, and only a small amount of the drug would be removed during each circulation of the dialysate. However, after approximately 170 circulations in 11.5 hours (600mL dialysate flowing at 150ml/min), a substantial amount of phenytoin would be removed. This would account for the sharp reduction of serum drug levels. The role of charcoal can be explained by the fact that bound phenytoin has been found to dissociate from albumin in the presence of activated charcoal and subsequently becomes adsorbed to the activated charcoal [246].

Along with effective removal of phenytoin, blood levels of oxygen-based free radicals decreased as well. Free radical production in the vasculature by phenytoin has been shown previously [250] and its removal by MARS would explain the observed reduction in oxidative stress and may in part explain the patient's clinical improvement. Direct removal of free radicals by MARS itself may also be a contributing factor.

Phenytoin-associated toxicity is a significant clinical problem, with 4021 cases being reported in 2000 in the USA, of whom 138 had major adverse effects with 2 mortalities [251]. Most severe toxicities occur in association with hepatic and renal compromise[242] in which therapeutic options are particularly limited[246]. The treatment with MARS not only removes phenytoin from the blood, but also reduces oxidative stress and most importantly, brings about a rapid clinical improvement. Moreover, the present study suggests that MARS might have applications in the treatment of other albumin-bound drug toxicities.

8.2 Removal of protein-bound drugs from plasma by extracorporeal albumin dialysis: Study in an animal model

8.2.1 Introduction

The Molecular Adsorbents Recirculating System (MARS) is an extracorporeal device that uses the principle of albumin dialysis to remove albumin-bound toxins, and is currently being explored as a liver support system [90, 91, 93]. However, the mechanism of action that makes extracorporeal albumin dialysis useful in liver failure means that this has the potential to remove a wide range of drugs with high albumin binding from the blood. In the case described above, we demonstrated the efficient removal by MARS of an anti-epileptic drug with 90% albumin-binding in a patient of phenytoin toxicity. If this applies to other protein-bound substances, the clinical implications would be two-fold. Firstly, MARS could be of considerable value in overdose/ toxicity with protein-binding substances, which otherwise cannot be efficiently removed from the body. Secondly, when MARS is being used for liver support, it could inadvertently remove drugs (e.g. antibiotics and anti-epileptics), thus lowering their plasma concentration and thereby efficacy, unless the drug dosing is adjusted accordingly.

The effects of MARS therapy on a drug, which is strongly albumin-bound in the plasma may well be different from those on a compound that is protein-bound but not significantly albumin-associated (which one would not expect to be significantly removed). The opportunity to investigate this was provided by a study designed primarily to evaluate the effect of MARS in a porcine model of acute liver failure, where a combination of midazolam (97% bound to plasma protein, mainly albumin [252]) and fentanyl (80-85% bound to plasma protein, mainly alpha-1-acid glycoprotein [252]) were used to anaesthetise the pigs. The present paper describes changes in the plasma concentrations of the two drugs during the MARS dialysis, and also examines the underlying mechanisms of removal.

8.2.2 Materials and methods

8.2.2.1 The animal model

The present study was performed at the Surgical Research Laboratory, University of Tromsø, Norway, with the approval of the Norwegian Experimental Animal Board. Seven Norwegian female Landrace pigs, weighing 25-30 (27.1±0.5, mean±SEM) kg were included. Details regarding animal room facilities, anaesthesia, surgical preparation, surgery, catheter placement and MARS dialysis have been described previously[142-145].

Acute liver failure was induced by an end-to-side porta-caval shunt, along with ligation of the hepatic arteries. The details of the surgery have been described elsewhere [144, 145]. MARS was started 2 hours after the hepatic devascularisation and continued for 4 hours (i.e. till 6

hours post-devascularisation), after which the experiments were terminated with an overdose of pentobarbital and potassium chloride.

8.2.2.2 Anaesthesia

The pigs were premedicated with an intramuscular injection of ketamine (20mg/kg) and atropine (1mg). Anaesthesia was induced with an intravenous bolus of 10mg/kg pentobarbital (Pentobarbital; Nycomed Pharma, Oslo, Norway) and 10mg/kg fentanyl (Leptanal; Janssen Pharmaceutica, Beerse, Belgium) and maintained during surgery with a central venous infusion of 4mg/kg/hr pentobarbital, 0.02mg/kg/hr fentanyl, and 0.3mg/kg/hr midazolam (Dormicum; Roche, Basel, Switzerland). Anaesthesia was stopped after the liver was devascularised. If there were clinical signs of light sedation, small doses of fentanyl and midazolam were given as a bolus. During MARS treatment, the animals were kept sedated by a continuous infusion of 0.04mg/kg/hr fentanyl, and 0.6mg/kg/hr midazolam, with additional bolus doses given when clinically indicated.

8.2.2.3 Use of MARS

The Molecular Adsorbents Recirculating System (MARS) (Teraklin AG, Rostock, Germany) (Fig 3) was set up and run as described earlier, with the renal part of the circuit clamped off, dialysing blood against 20% human albumin (a concentration (200 g/l) 5-7 times that in the plasma).

8.2.2.4 Sampling and drug concentration analysis

A central venous blood sample was collected at the start of MARS treatment. Four hours after the start, samples were taken from all the different segments of the MARS circuit. Blood samples were collected from the inflow and the outflow segments of the MARS filter. Samples of albumin dialysate were collected from the different segments of the dialysate circuit (segment 1: between blood and haemofilter column; segment 2: between haemofilter and charcoal column; segment 3: between charcoal and anion exchange resin column; segment 4: between resin and blood column) (Fig 3). Total and free concentrations of midazolam and fentanyl were measured by high-pressure liquid chromatography (HPLC)-coupled mass-spectrometry, as described earlier.

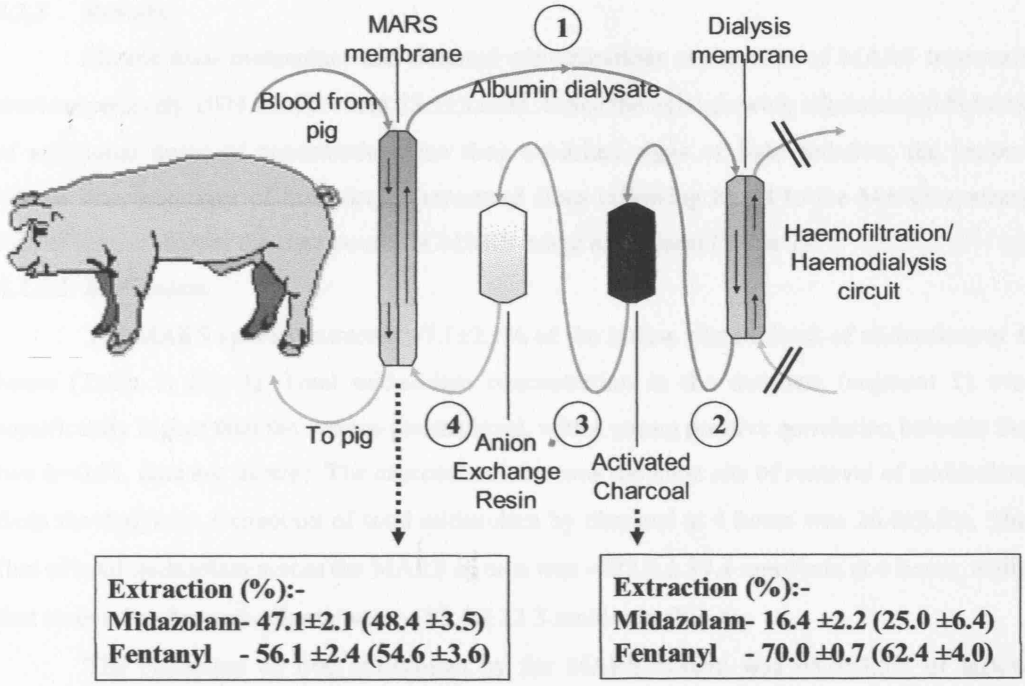


Fig 3: A schematic diagram of the MARS circuit (with the different segments marked), showing the extraction of midazolam and fentanyl at 4 hours of treatment from the plasma (in the blood filter column) and from the dialysate (by activated charcoal) (figures in parenthese are extractions of the free drug)

8.2.2.5 Statistical analysis

Results are expressed as mean±standard error of mean. Student's t-test was used to analyse significance of difference between means, and linear regression was used where applicable to determine relationship between variables.

Drug extraction across a filter column was calculated as:

Extraction (%) = 100 x (Inflow concentration – Outflow concentration)/ Inflow concentration

Drug flux across a filter column was calculated as:

Flux (nmol/min) = Flow rate x (Outflow concentration - Inflow concentration)

Plasma flow rate through the blood column of the MARS circuit was calculated as follows:

Plasma flow rate (L/min) = Blood flow rate x (1-Haematocrit)

= 0.15 x (1-Haematocrit)

$P < 0.05$ was taken to be statistically significant.

8.2.3 Results

Plasma total midazolam and fentanyl concentrations at the start of MARS treatment were respectively 1074.6±190.4 and 23.1±3.0nM. Since the animals were administered boluses of additional doses of anaesthetic when they exhibited signs of light sedation, the venous plasma concentrations of both drugs (measured from inflowing blood to the MARS system) further increased over the time course of MARS usage at 4 hours) (Table 1).

8.2.3.1 Midazolam

The MARS system extracted 47.1±2.1% of the inflow plasma level of midazolam at 4 hours (Table 1, Fig 3). Total midazolam concentration in the dialysate (segment 1) was significantly higher than the venous plasma level, with a strong positive correlation between the two (r=0.91, data not shown). The charcoal column was the main site of removal of midazolam from the dialysate. Extraction of total midazolam by charcoal at 4 hours was 16.4±2.2%. The flux of total midazolam across the MARS system was –222.6 ± 37.4 nmol/min at 4 hours, while that across the charcoal column was –185.4 ± 23.3 nmol/min (Fig 4).

The extraction of free midazolam by the MARS system was 48.4±3.5% of inflow plasma level at 4 hours (Table 1). Dialysate free drug levels (segment 1) were significantly lower than plasma free levels. The free drug extraction across the charcoal column was 25±6.4%.

There was no significant removal of either the total or free drug across the anion exchange resin.

Table 1: Concentrations of total and free midazolam and fentanyl (nM) in the plasma in the inflow and outflow of MARS, and in the albumin dialysate from the different segments of the circuit (segment 1: between blood and haemofilter column; segment 2: between haemofilter and charcoal column; segment 3: between charcoal and anion exchange resin column; segment 4: between resin and blood column). Results are expressed as mean±SEM. Paired t-test was used to test significance of differences between means. $P<0.05$ was regarded as statistically significant.

	Midazolam		**Fentanyl**	
	Total	***Free***	***Total***	***Free***
Inflow plasma	3879.4 ± 653.5	267.5 ± 46.9	81.4 ± 14.4	10.5 ± 1.8
Outflow plasma	2052.3 ± 362.7	140.8 ± 30.1	35.6 ± 6.3	4.5 ± 0.7
Dialysate: Seg 1	8146.6 ± 1344.2	135.7 ± 25.9	57.1 ± 8.6	6.8 ± 1.7
Dialysate: Seg 2	8229.6 ± 1318.0		53.8 ± 7.4	
Dialysate: Seg 3	6993.3 ± 1258.3	97.1 ± 16.7	16.1 ± 2.3	2.4 ± 0.4
Dialysate: Seg 4	6788.3 ± 1218.5	-	16.4 ± 2.9	-
P-values				
Inflow vs Outflow	0.0007	0.0009	0.001	0.002
Inflow vs Seg 1	0.001	0.002	0.01	0.05
Seg 2 vs Seg 3	0.0001	0.02	0.0002	0.01

8.2.3.2 Fentanyl

The MARS system extracted 56.1±2.4% of fentanyl from the inflow plasma at 4 hours (Table 1, Fig 3). Total fentanyl concentration in the dialysate (segment 1) was significantly lower than the venous plasma level, and there was a strong positive correlation between the two (r=0.90 at 4 hours, data not shown). As with midazolam, the charcoal column was the main site of removal of fentanyl from the dialysate, but with a higher extraction (3-4 times greater than that for midazolam). Extraction at 4 hours was 70±0.7%. The flux of total fentanyl across the MARS system was –5.6±1.1nmol/min at 4 hours, and across the charcoal column was –5.6±0.8nmol/min (Fig 4).

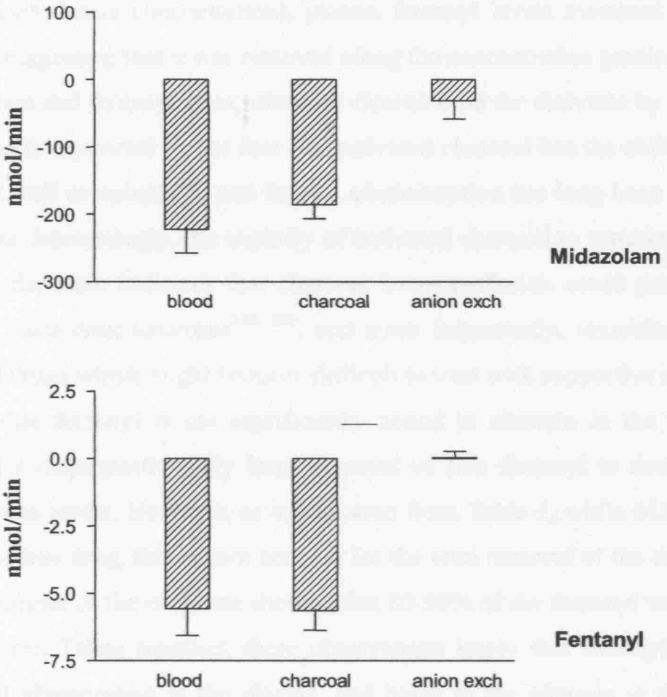


Fig 4: The flux of total midazolam and total fentanyl in blood across the MARS circuit, and dialysate across the charcoal and anion exchange resin column, at 4 hours are shown.

The extraction of free fentanyl by the MARS system from the inflow plasma was 54.6±3.6% at 4 hours (Table 1). Dialysate free drug levels (segment 1) were significantly lower than plasma free levels. The free drug extraction by the charcoal column was 62.4 ± 4 %.

There was no significant removal of total or free drug across the anion exchange resin.

8.2.4 Discussion

The present study investigated the role of albumin dialysis, using MARS, in the removal of two protein-bound drugs, one of which predominantly binds to albumin (midazolam) and one of which does not (fentanyl). Efficient clearance of midazolam (97% albumin-bound[252]) was not unexpected, and the strong affinity of midazolam for albumin would explain the higher drug concentration observed in the dialysate (200g/L albumin) compared with that in the venous plasma (~30g/L albumin). The fact that fentanyl (80-85% bound to alpha-1-acid glycoprotein[252]) was removed with equal efficacy was of considerable interest, and implies that MARS (or albumin dialysis) may have the capacity to remove drugs from binding sites on other plasma proteins. While midazolam was removed against a concentration gradient (dialysate concentration>plasma concentration), plasma fentanyl levels remained higher than those in the dialysate suggesting that it was removed along the concentration gradient.

Both midazolam and fentanyl were primarily cleared from the dialysate by the charcoal filter. This observation is supported by the fact that activated charcoal has the ability to adsorb benzodiazepines[253] as well as opioids[254], and its oral administration has long been used for the treatment of overdoses. Interestingly, the capacity of activated charcoal to remove these drugs from albumin in the dialysate indicates that charcoal haemoperfusion could possibly be an effective therapy for such drug toxicities[255, 256], and more importantly, toxicities with other similar protein-bound drugs which might be more difficult to treat with supportive care alone.

Considering that fentanyl is not significantly bound to albumin in the plasma, one might have expected a disproportionately large removal of free fentanyl to account for the reduction in total plasma levels. However, as can be seen from Table-1, while MARS resulted in a 55% extraction of free drug, this cannot account for the total removal of the drug from the plasma. Moreover, analysis of the dialysate showed that 80-90% of the fentanyl was present in the albumin-bound form. Taken together, these observations imply that fentanyl is removed from the alpha-1-acid glycoprotein in the plasma, and binds to the albumin in the dialysate. Fentanyl is not generally bound to albumin in the plasma, but there are studies showing that it has the capacity to bind to it and also to erythrocytes[257, 258]. If a fairly constant proportion of the total fentanyl concentration is present in the non-protein bound free form, it is conceivable that the binding sites on the MARS membrane take up some of the free drug. This in turn would reduce the proportion of free drug present in the plasma leading to release of a proportion of the protein-bound fentanyl. As the membrane-associated albumin gives up its load of fentanyl to the albumin in the dialysate the cycle is repeated and an effective concentration gradient established. Thus, without actually stripping off fentanyl from alpha-1-acid glycoprotein in the

plasma, the effect would be the net removal of fentanyl from the plasma. These data support the hypothesis that only the free drug is actually being removed by the MARS membrane, with constant re-equilibration of the free and bound components in the plasma, without any actual 'stripping' of the drug from its binding protein by the membrane.

The main site of removal of both drugs from the dialysate was the column of activated charcoal. The calculated flux data suggest that this removal by charcoal was almost entirely responsible for fentanyl removal from the blood by MARS. In case of midazolam, a higher flux across MARS compared to that across the charcoal column suggests that in addition to charcoal, the membrane/ albumin dialysate contributes to an extent in the removal of this highly albumin-bound drug from the blood.

The present study was not designed to look at the kinetics of drug removal, as the pigs had repeated bolus doses of anaesthetics to maintain a stable depth of anaesthesia. The dosage of anaesthetics required during MARS was actually higher than that given during the surgery, most likely due to removal of the anaesthetic agents. Moreover, the drugs were not administered for long enough to achieve steady state. Therefore, the drug removal data have been represented as extraction or removal rates at different points of time (2 and 4 hours into the MARS session). In spite of this weakness, this study indicates that better understanding is required of drug removal in order to make the necessary modifications to the dosage of drugs administered concurrently with MARS treatment. During the use of MARS for liver support, the clinician needs to be aware that it might remove some of the other medications the patient is receiving for therapeutic purposes. In addition to the groups of drugs mentioned above, a large variety of antibiotics are protein-bound (including many which are albumin-bound), as are anti-epileptic drugs. Furthermore, the present study shows that many more drugs (not necessarily bound to albumin) can be removed which may result in sub-therapeutic blood levels. Awareness of this possible side effect should lead to better adjustment of the dosage as well as timing of administration of these drugs.

The results of this study have other clinical implications as well. Both benzodiazepines and opioids are drugs that are commonly used clinically, and can lead to problems related to toxicity or overdosage. In the US alone, in 2001 there were 146 deaths due to benzodiazepine and 173 deaths due to opioid overdose[259]. However, the availability of effective specific antagonists (flumazenil for benzodiazepines and naloxone for opioids) means that in-hospital deaths related to either drug are quite rare, and the role of MARS in the therapy of these specific toxicities is limited. Moreover, removal of the parent drug (e.g. midazolam) might not necessarily mean that active metabolites (e.g. alpha-hydroxymidazolam glucuronide) are also

adequately removed. Rather, one should consider these two drugs as representative of albumin- and other protein-bound drugs that may be effectively removed by albumin dialysis. In the setting of a severe drug toxicity not responding adequately to specific pharmacological antagonists, this might prove to be an effective way of rapidly reducing circulating levels. We have already shown that MARS can effectively clear phenytoin from the plasma in cases of toxicity. Overdosage with other protein-bound drugs, such as verapamil and diltiazem (90 and 80%plasma protein-bound respectively[252]), which have no effective pharmacological antagonists, are other clinical settings where MARS might be beneficial.

Finally this study provides the first reliable data describing the mechanism by which extracorporeal albumin dialysis using MARS may act as a therapeutic tool in protein-bound substance toxicity. The results indicate how the system can be modified to improve its efficacy for specific substances. For example, in the case of the drugs described, an improved system could be designed by replacing the anion exchange resin with an additional activated charcoal column. Such future advances might lead to more efficient systems being developed.

In conclusion, extracorporeal albumin dialysis using MARS can lead to the efficient removal of both albumin-bound and non-albumin-bound substances from the blood. The mechanism of removal is probably by uptake of the free drug from the plasma, with constant re-equilibration of the free and bound components. The results of this study provide the rationale for exploring the use of extracorporeal albumin dialysis for the treatment of intoxication with protein-bound toxins in appropriately designed trials.

9. CONCLUSION

In spite of advances in medical care, liver failure continues to carry a bleak outlook. Orthotopic liver transplantation is the most promising therapeutic intervention available. However, most patients of acute-on-chronic liver failure are not eligible for an urgent transplant, and a significant proportion of acute liver failure patients continue to die while waiting for a donor organ to become available. Thus there continues to remain the requirement for an effective liver support system. Safety, cost and efficacy-related issues have meant that bioartificial devices have not become widely accepted. Artificial systems are the only ones currently in use, based upon the principle of albumin dialysis, and of these the Molecular Adsorbents Recirculating System (MARS) is the one which is being investigated most thoroughly. The series of studies which have been described here were designed to systematically investigate the role that MARS may have in the therapy of liver disease, and to explore how best it may be used as a tool to study the pathophysiology of liver failure.

The initial studies confirmed the safety and feasibility of MARS therapy, when performed in the appropriate clinical setting. The primary concern regarding the safety of therapy appears to be with respect to coagulopathies, and even though such problems were not encountered in any of the studies described here, the current consensus is to withhold its use in the presence of significant thrombocytopaenia ($< 50 \times 10^9/l$) and/ or coagulopathy (INR > 2.3), which might suggest the presence of 'incipient' disseminated intravascular coagulopathy (DIC).

As regards the efficacy of MARS therapy, improvement of liver biochemistry (especially serum bilirubin) and hepatic encephalopathy appear to be the most consistently observed findings. However, our studies suggest that the beneficial effect on systemic haemodynamics or renal function may not be as significant as was previously reported by other studies. A novel observation was the rapid, clinically significant and sustained portal pressure lowering effect of MARS in these patients. This is potentially of great importance, as it suggests that albumin dialysis may be a useful adjunct to existing therapies in the context of variceal bleeding in patients with advanced cirrhosis and associated organ failure. In these patients, the effects of MARS on portal pressure may provide added value in terms of greater ability to control the bleeding and also counteract its deleterious metabolic consequences. None of the studies described here were adequately powered to evaluate an impact on survival. However the data does suggest that there was an apparent reduction in mortality in the group of patients studied. At present, a multi-centre randomised controlled trial with acute-on-chronic liver failure patients is being conducted in Europe, while another in patients of hepatic

encephalopathy is being carried out in the US, to gain a more definite perspective of the efficacy of MARS.

The main focus of our studies was to investigate the pathophysiological mechanisms underlying the effects of albumin dialysis, and thus broaden our understanding of the pathophysiological basis of liver failure itself. In inflammation-related acute-on-chronic liver failure patients, albumin dialysis results in improvement of encephalopathy, accompanied by a reduction in oxidative stress, without significant changes in arterial ammonia or cytokines. The improvement in encephalopathy without any significant change in ammonia highlights the importance of other mediators in its pathogenesis. While cytokines are believed to contribute to the inflammatory basis of encephalopathy, our studies suggest that other factors such as oxidative stress have a role to play as well.

The consistently observed cerebral effects of albumin dialysis prompted us to further investigate this in a porcine model of acute liver failure. Attenuation of the rise of intracranial pressure was observed with MARS, as was a reduction of cerebral oedema (in the sub-cortical white matter), independent of any alterations of arterial ammonia, cerebral blood flow, cytokines or oxidative stress. The implications provide a novel insight into the pathophysiological basis of cerebral changes in acute liver failure. While hyperammonaemia is probably essential for the initial development of cerebral oedema and intracranial hypertension in acute liver failure, the interplay of cerebral blood flow and inflammation is not. Regional differences exist for brain oedema, with white matter oedema being more amenable to therapeutic interventions. Factors in addition to hyperammonaemia also appear to be important. Identifying these putative factors, and understanding the intracerebral effects they might have, would greatly improve our knowledge of the pathogenesis of intracranial hypertension in acute liver failure.

Finally, the effect of albumin dialysis on protein-bound drugs was studied as well. MARS was found to be efficient in the removal of both albumin-bound and non-albumin-bound substances from the blood. The mechanism of removal is probably by uptake of the free drug from the plasma, with constant re-equilibration of the free and bound components. The results suggest that it may have an important role in the management of intoxication with non-dialysable protein-bound substances, and provides the rationale for setting up appropriately designed trials.

Perhaps the most frequently asked question in relation to albumin dialysis is- does it work? Even after the completion of a series of carefully designed studies, we still do not have a final answer. What is certain is that albumin dialysis does bring about measurable biochemical

and pathophysiological changes, which translate to improvement of clinical parameters. However, the ultimate success or failure of a liver support device can only be measured by its impact on survival. So, does albumin dialysis save lives? At this point in time, the only honest answer to this question can be- we do not know. From the preliminary data, it certainly appears to do so, but only the conclusion of the ongoing randomized controlled trials will definitively settle the issue.

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ABBREVIATIONS

MARS- Molecular Adsorbents Recirculating System

ALF- acute liver failure

ACLF- acute-on-chronic liver failure

UGI- upper gastrointestinal

HE- hepatic encephalopathy

APACHE- Acute Physiology and Chronic Health Evaluation

SOFA- Sequential Organ Failure Assessment (or Sepsis-related Organ Failure Assessment)

HRS- hepatorenal syndrome

MELD score- Model for End-Stage Liver Disease (or Mayo End-Stage Liver Disease) score

PaO2 - arterial oxygen tension

FIO2 - fractional inspired oxygen

MAP - mean arterial pressure

NO- nitric oxide

NOS- NO synthase

eNOS- endothelial NOS

L-NMMA- L-N^G-monomethyl-arginine

GFR- glomerular filtration rate

iNOS- induced NOS

TNF- tumour necrosis factor

TIPSS- transjugular intrahepatic porto-systemic stent shunt

ICP- intracranial pressure

LPS- lipopolysaccharide

SIRS- systemic inflammatory response syndrome

IL- interleukin

BCAA- branched-chain amino acid

LTx- liver transplantation

UNOS- United Network Organ Sharing

DIC- disseminated intravascular coagulation

FPSA- fractionated plasma separation and adsorption

SPAD- single pass albumin dialysis

DF- discriminant function

CO- cardiac output

WHVP- Wedged hepatic venous pressures

FHVP- Free hepatic venous pressures

HVPG- hepatic venous pressure gradient

HBE- hepatic blood flow

ICG- indocyanine green

IHVR- intrahepatic vascular resistance

TBA- thiobarbituric acid

MDA- malondialdehyde

BHT- butylated hydroxytoluene

SDS- sodium dodecyl sulfate

EPR- electron paramagnetic resonance

HPLC- high-pressure liquid chromatography

MRM- multiple reaction monitoring

AH- alcoholic hepatitis

OLT- orthotopic liver transplantation

SBP- spontaneous bacterial peritonitis

ALT- alanine aminotransferase

INR-international normalised ratio

SMT- standard medical therapy

NOx- nitrate and nitrite

NH_3- ammonia

TNF-R- TNF-receptor

PBN- α-phenyl N-tert-butyl nitrone

PRA- plasma rennin activity

CBF- cerebral blood flow

CVVHF- continuous veno-venous haemofiltration

PUBLICATIONS ARISING FROM THIS THESIS

1. **Jalan R, Sen S, Steiner C, Kapoor D, Alisa A, Williams R.** Extracorporeal liver support with molecular adsorbents recirculating system in patients with severe acute alcoholic hepatitis. ***J Hepatol*** 2003; 38: 24-31.
2. **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; 43: 142-148.
3. **Sen S, Davies NA, Mookerjee RP, Cheshire LM, Hodges SJ, Williams R, Jalan R.** Pathophysiological effects of albumin dialysis in acute-on-chronic liver failure: A randomised controlled study. ***Liver Transplantation*** 2004; 10: 1109-19.
4. **Sen S, Rose C, Ytrebo LM, Davies NA, Nedredal GI, Drevland SS, Kjonno M, Prinzen FW, Hodges SJ, Deutz NEP, Williams R, Butterworth RF, Revhaug A, Jalan R.** Effect of albumin dialysis on intracranial pressure increase in pigs with acute liver failure: A randomised study. ***Crit Care Med*** 2006; 34: 158-164.
5. **Sen S, Ratnaraj N, Davies NA, Mookerjee RP, Cooper CE, Patsalos PN, Williams R, Jalan R.** Treatment of phenytoin toxicity by the Molecular Adsorbents Recirculating System (MARS). ***Epilepsia*** 2003; 44: 265-67.
6. **Sen S, Ytrebø LM, Rose C, Fuskevaag O-M, Davies NA, Nedredal GI, Williams R,Revhaug A, Jalan R.** Albumin dialysis: A new therapeutic strategy for intoxication from protein-bound drugs. ***Intensive Care Med*** 2004; 30: 496-501.