

The role of human albumin solution in preventing infection in patients with acute decompensation of liver cirrhosis

A Thesis submitted for the degree of Doctor of Philosophy,
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'I, Louise China confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

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Abstract

Liver disease is the only major cause of mortality currently increasing in the UK and is the fifth most common cause of death. Patients with symptoms of liver failure secondary to cirrhosis are described as acute decompensation (AD) patients. They are highly prone to bacterial infection secondary to immune dysfunction. Elevated circulating Prostaglandin E₂ (PGE₂) levels contribute to immune suppression in AD patients. The plasma protein albumin can bind and catalyse inactivation of PGE₂. Albumin is synthesised in the liver and levels fall as the synthetic function of the liver declines in AD and binding capacity becomes defective, making PGE₂ more bioavailable. Previous work has shown a serum albumin of < 30g/L predicted immune dysfunction in a small cohort of AD patients and low serum albumin is associated with increased risk of nosocomial infection. Finally, a pilot study suggested that albumin infusions to raise levels above 30g/L may improve immune function in AD. There is a widespread belief in Hepatology that albumin holds additional therapeutic benefit, other than volume resuscitation. However, there are no randomised clinical studies to support this.

My thesis examined whether prophylactic intravenous human albumin infusions to increase serum albumin >30g/L would prevent AD patients from developing infection.

A new IV 20% Human Albumin Solution (HAS) treatment regimen was tested in a 79 patient single-arm feasibility study in busy healthcare settings. Clinical data collected during this study allowed for the modification and improvement of the protocol and outcomes to move to a multicenter randomised control trial of >800 patients. I developed a plasma bioassay to explore the impact of IV albumin on plasma mediated macrophage dysfunction that was feasible in a large trial setting to investigate possible underlying mechanisms of any effect. Samples from this feasibility study supported a beneficial effect of albumin infusions on immune function by binding plasma PGE₂.

Subsequent analyses from patients randomised to IV 20% HAS treatment versus standard care showed IV HAS decreased plasma PGE₂ and improved the functional ability of plasma albumin to bind PGE₂. However, there was no improvement in macrophage TNF α production nor any markers of systemic inflammation. This was despite patients in the treatment arm receiving 1000 mLs (700-1500) (median (interquartile range); Med(IQR) compared to 100 mLs (0-600) in standard care (P<0.0001, adjusted mean difference 710.4 (95% CI 631.9 to 788.8)).

This was consistent with no clinical impact of IV albumin on infection with no differences in incidence of new infection, nor outcome in patients admitted with infection or receiving antibiotics at enrolment. In addition, no improvement in renal dysfunction nor mortality was observed.

In summary albumin infusions to raise and maintain serum albumin >30g/L have no effect on immune function nor markers of systemic inflammation and do not decrease incidence of infection in AD patients. It should not be used for this purpose and perhaps the widespread use of albumin over other fluids in cirrhosis might be reconsidered.

Impact Statement

Benefits inside academia

Basic science

The work in my thesis developed two assays, which can be used in the field of decompensated cirrhosis to assess immune function and change in the functional quality of albumin. The 'lipopolysaccharide stimulated monocyte derived macrophage (MDM) assay' is a consistent functional bioassay of patient plasma induced MDM effects, which can be used in future multicenter studies focusing on immune function. It has already been used, and published, in work extending beyond the scope of this thesis.

Dysfunctional and low concentrations of plasma albumin have long marked the progression of end stage liver disease, hence the focus on human albumin solution as a therapy. The ^3H -PGE₂-plasma albumin bioassay demonstrates for the first time an assay which uses a physiological albumin binding site and a ligand which is relevant in vivo to assess changes in albumin. The assay could feasibly be used to assess other interventions ex vivo, which may improve outcomes in cirrhosis patients.

Clinical research

Defining clinically meaningful but transparent and objective outcomes for clinical trials is a huge challenge in liver cirrhosis studies, particularly in unwell inpatients. The work in this thesis demonstrates when it is possible to accurately record outcomes in relation to infection and its complications in a ward based setting. In particular, a transparent approach to critiquing and improving infection diagnosis in a clinical trial setting has been described and explored. These methods could be used in other clinical trials in decompensated liver cirrhosis patients.

Benefits outside academia

Albumin infusions were first used in cirrhosis more than 70 years ago and have long been considered the best fluid to prescribe to prevent or treat renal dysfunction in cirrhosis. Many pre-clinical papers describe potentially beneficial immune modulatory effects. However, although a large-scale trial showed benefit of albumin infusions in outpatients with cirrhosis, the vast majority of prescriptions are given to hospitalised patients and a firm evidence-base for much of our clinical practice is lacking.

Contrary to the overwhelming preference for albumin over other fluids in cirrhosis by liver specialists worldwide, this work unequivocally demonstrates no effect of targeted albumin therapy over current UK standard care. Given an overall three-fold difference in volumes infused between patient groups, an absence of effects across all subgroups and the large numbers involved, this work advocates a fundamental re-evaluation of perhaps the most commonly prescribed treatment for hospitalised patients with cirrhosis. These findings will stimulate substantial debate and support a change in clinical practice.

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Publications and Conferences arising from this Thesis

Publications:

ATTIRE: Albumin To prevent Infection in chronic liver failure: study protocol for a single-arm feasibility trial. China L et al. *BMJ Open*. 2016 Jan 25;6(1):e010132

Administration of Albumin Solution Increases Serum Levels of Albumin in Patients With Chronic Liver Failure in a Single-Arm Feasibility Trial. China L et al. *Clin Gastroenterol Hepatol*. 2018 May;16(5):748-755.e6.

Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease. China L et al *Clin Gastroenterol Hepatol*. 2018 May;16(5):738-747.

ATTIRE: Albumin To prevent Infection in chronic liver failure: study protocol for an interventional randomised controlled trial. China L et al. *BMJ Open*. 2018 Oct 21;8(10):e023754.

International Conference Presentations & Published Abstracts:

ATTIRE: Albumin To prevent Infection in chronic liver failure. China L et al. Poster presentation of trial protocol at EASL ILC 2015 (Vienna)

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution. China L et al. Poster presentation at AASLD annual conference 2016 (Boston)

Defining meaningful clinical trial endpoints in patients with alcohol induced chronic liver disease: results from a multicentre feasibility trial. China L et al. Poster presentation at EASL Special Conference 2017 (London). Full bursary awarded.

Accurately defining infection as a clinical trial endpoint in patients with alcohol induced chronic liver disease: results from a multicentre feasibility study. China L et al. Poster presentation at EASL Special Conference 2017 (London). Full bursary awarded.

Plasma Lipid Mediator (LM) Profiling Identifies Hyper- and Hypo-activated Groups of Patients with ACLF and Targeted 20% Human Albumin Solution Infusion Recalibrates Abnormalities. China L et al. Oral presentation at EASL ILC 2017 (Amsterdam).

Albumin Binding Capacity is Impaired in Decompensated Liver Cirrhosis and Dysfunction is Reversed by Targeted in vivo 20% Human Albumin Solution Infusions. China L et al. Poster presentation at EASL ILC 2017 (Amsterdam). Registration bursary awarded.

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution. China L et al. Poster presentation AASLD & EASL Masterclass 2018 (Florida).

Exploring treatment failures in a multicentre feasibility trial using human albumin solution to prevent infection in acute decompensation of liver cirrhosis. China L et al. Oral presentation (basic science) EASL ILC 2019 (Vienna). Full bursary awarded.

ATTIRE: Albumin To prevent Infection in chronic liver failure: an interventional randomised controlled trial. China L et al. Oral presentation (Late Breakers) EASL ILC 2020 (London). Full bursary awarded. Abstract selected for 'Best of ILC Summary'.

List of abbreviations

ACLF	Acute on chronic liver failure
AD	Acute decompensation
AE	adverse event
AKI	Acute kidney injury
ALBIOS	Albumin for Volume Replacement in Severe Sepsis trial
AML	acute monocytic leukemia
ANP	atrial natriuretic peptide
ANSWER	Albumin for the treatment of ascites in patients with hepatic cirrhosis
ARDS	Acute Respiratory Distress Syndrome
ATTIRE	Albumin To prevent Infection in chronic liver failure
BCP	bromocresol purple
BCG	bromocresol green
BDL	bile duct ligated
Bmax	maximum binding
CI	confidence interval
CLIF-SOFA	Chronic Liver Failure Sequential Organ Failure Assessment Score
COX	Cyclooxygenase
COX	cyclo oxygenase
CRF	Case Report Form
CRP	C reactive protein
CTU	clinical trials unit
Cys34	Cysteine residue at position 34
CVS	cardiovascular system
DAMPs	danger-associated molecular patterns
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EHOD	extrahepatic organ dysfunction
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EP receptor	E-prostanoid receptor
FA	fatty acids
FCS	foetal calf serum
FiO ₂	Inspired oxygen
GI	gastro intestinal
GFR	glomerular filtration rate
HAS	Human albumin solution
HBSS	Hank's balanced salt solution

HBV	hepatitis B virus
HCV	hepatitis C virus
HE	hepatic encephalopathy
HLA-DR	Human leucocyte antigen - antigen d related
HMA	human mercaptalbumin
HNA	human nonmercaptalbumin
HPLC	high performance liquid chromatography
HRS	Hepatorenal syndrome
HSA	Human serum albumin
HV	Healthy volunteer
ICU	Intensive care unit
ID	identification
IDMC	independent data monitoring committee
IL	interleukin
IMP	investigational medicinal product
IQR	interquartile range
IV	intra venous
Kd	equilibrium dissociation constant
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LVP	Large volume paracentesis
LRTI	lower respiratory tract infection
MACHT	Midodrine and Albumin in the Prevention of Complications in Cirrhotic Patients Awaiting Liver Transplantation
M-CSF	Macrophage Colony-Stimulating Factor
MCP-1	monocyte chemo attractant protein-1
MDM	Monocyte derived macrophage
MELD	model for end stage liver disease
MERTK	MER receptor tyrosine kinase
MHC	major histocompatibility complex
MHRA	Medicines and Healthcare products Regulatory Agency
MRSA	methicillin resistant staphylococcus aureus
MM6	Mono-Mac 6
MMP	metalloproteinases
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non alcoholic fatty liver disease
NEQAS	National External Quality Assessment Service
NIHR	National Institute for Health Research
NHS	National Health Service
NSAIDS	non steroidal anti inflammatory drugs
OPS	orthogonal polarization spectroscopy

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCT	procalcitonin
PGE ₂	Prostaglandin E ₂
PTG	peptidoglycan
RBC	Red blood cell
RCT	Randomised control trial
ROC	Receiver operating analysis
SAEs	Serious Adverse Events
SBP	Spontaneous bacterial peritonitis
SD	standard deviation
SIRS	Systemic Inflammatory Response Syndrome
SOFA	sequential organ failure assessment
S1P	sphingosine-1-phosphate
SpO ₂	Oxygen saturations
Th	T helper
TLR	Toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF α	Tumour necrosis factor alpha
TSC	trial steering committee
UCL	University College London
UCLH	University College London Hospital
UTI	Urinary tract infection
VD3	vitamin D3 (1 α , 25 dihydroxycholecalciferol)
VRE	vancomycin resistant enterococcus
WCC	white cell count

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

1.1.1 Background

Liver disease is the only major cause of mortality currently increasing in the UK and is the fifth most common cause of death after heart disease, cancer, stroke and respiratory disease¹. Liver disease deaths increased by 12% from 2005-2008 and, at the current rate, are predicted to double over the next 20 years¹. Liver disease kills more people than diabetes² and road accidents combined.

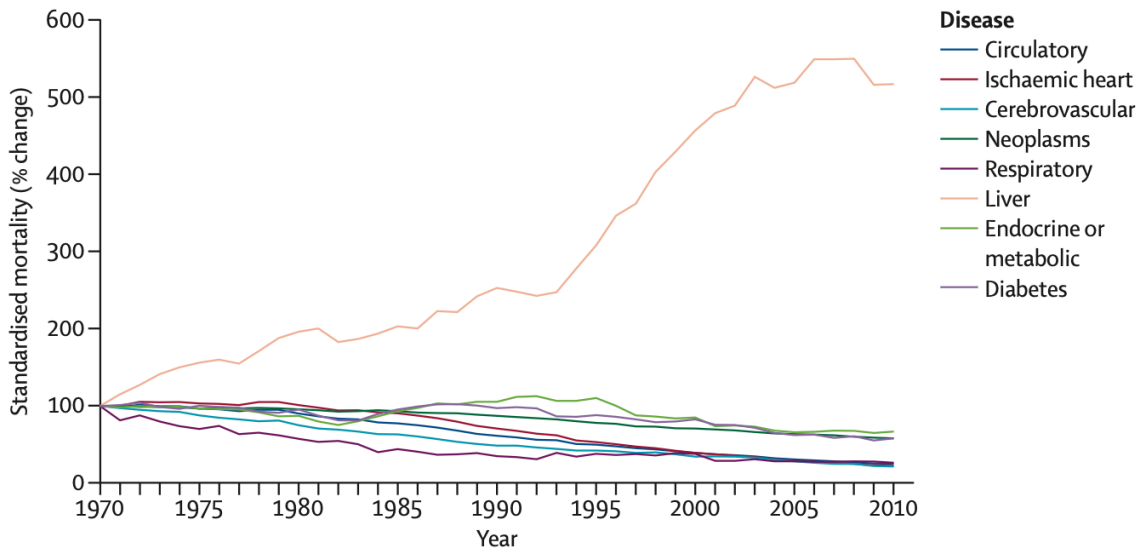


Figure 1.1. Standardised UK mortality rate data

Data were normalised to 100% in 1970, and subsequent trends plotted using the software Statistical Package for the Social Sciences. Taken from Williams, et al. ²

Furthermore, people can survive with 70% liver damage and so there is a substantial burden of morbidity, a high cost to the NHS and a huge economic and human cost from liver-related ill health. Although a considerable problem, most research efforts focus on underlying causes (e.g. alcohol and obesity) or industry trials for viral hepatitis.

Liver Cirrhosis

Liver cirrhosis is a result of advanced liver disease. It is characterized by replacement of liver tissue by fibrosis (scar tissue) and regenerative nodules (lumps that occur due to attempted repair of damaged tissue). These changes lead to loss of liver function.

Liver cirrhosis is a pathological definition based on liver biopsy. However, this is an invasive procedure and uncommonly performed in patients admitted with complications

of cirrhosis. Patients are considered to have cirrhosis based on clinical judgment (including radiological imaging) in standard UK practice.

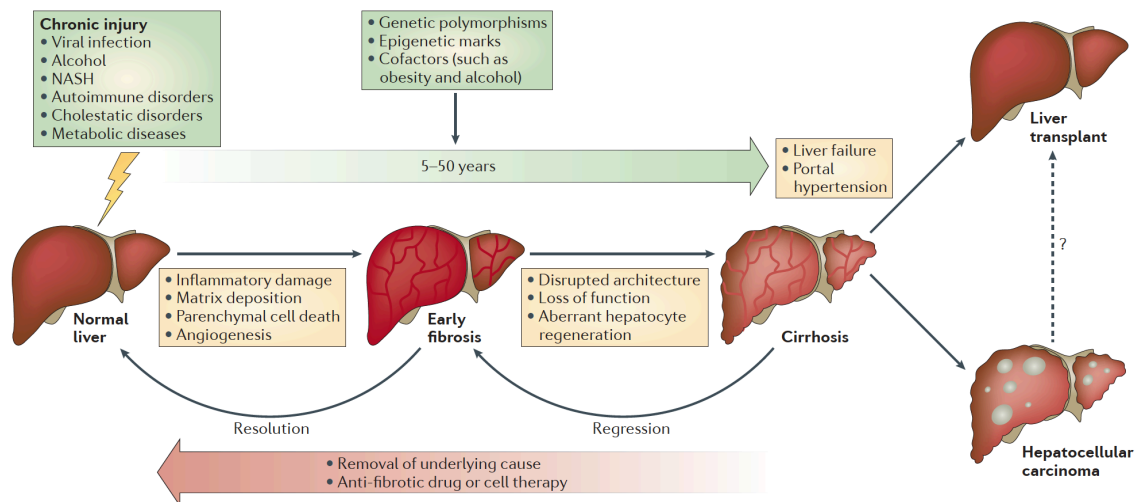


Figure 1.2. Natural Progression of chronic liver disease

Taken from Pellicoro, et al. ³

Cirrhosis is most commonly caused by alcohol, chronic viral hepatitis (B and C) and fatty liver disease, but has many other causes including idiopathic (of unknown cause).

Complications of Liver Cirrhosis

The common complications of advanced liver disease are:

1. **Ascites:** refers to fluid retention within the abdominal cavity. It is the most common complication of cirrhosis. It is associated with a poor quality of life, increased risk of infection, and a poor long-term outcome. Paracentesis refers to the drainage of ascites.
2. **Jaundice:** yellow discolouration of the skin and sclera of the eyes due to high bilirubin levels in the blood.
3. **Varices:** these are enlarged blood vessels within the oesophagus and stomach which can burst causing significant bleeding which commonly manifests as vomiting of blood.
4. **Hepatic encephalopathy:** confusion and coma as a result of liver failure.

Development of any of these complications is termed decompensation. Acute decompensation refers to the acute development/worsening of these complications and is the main cause of hospitalisation in these patients. When these complications occur it marks the onset of a deterioration which often leads to death⁴ (figure 1.2).

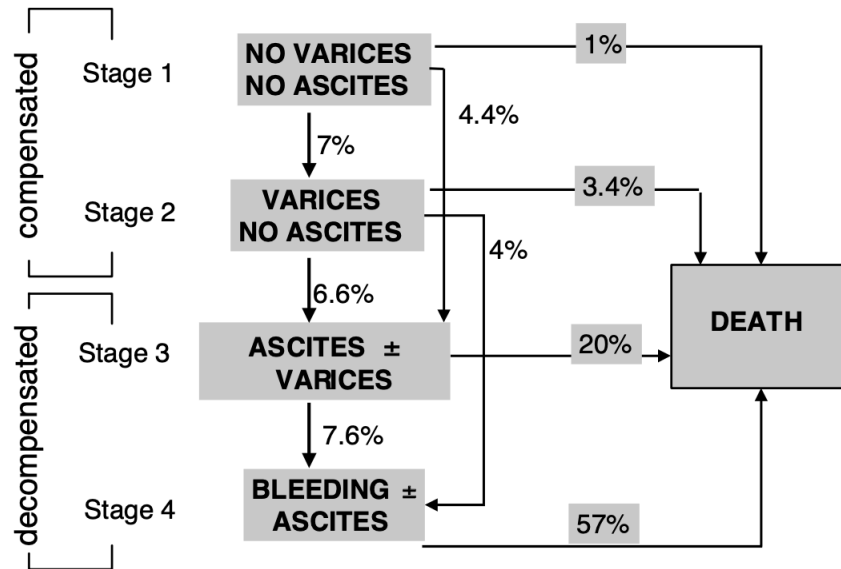


Figure 1.3. Clinical course of cirrhosis: 1-year outcome probabilities according to clinical stages
Taken from D'Amico, et al.⁴

There were nearly 60,000 patients admitted to English hospitals during 2011-12 with liver disease, e.g. encephalopathy, jaundice, gastro-intestinal bleeding, ascites and alcoholic hepatitis (source Public Health England). Of those cirrhosis patients who develop sepsis and organ dysfunction, 60-95% die, often following prolonged intensive care (ICU) admission. In the most recent figures available (2006-2008) cirrhosis patients accounted for over 5% of all UK ICU admissions⁵.

These patients are highly prone to bacterial infection⁶ secondary to immune dysfunction⁷, with nosocomial (hospital-acquired) infection rates of 35 per cent compared to five per cent in non-cirrhotic patients^{8,9}. Of those that develop infection with organ dysfunction, 60-95% die, often following prolonged intensive care unit admission¹⁰. There is, however, no medical strategy to restore immune competence. The only current curative treatment option for these patients is liver transplant which is limited to <900 patients per year. Therefore current strategies are aimed at preventing clinical deterioration and patient optimisation prior to liver transplant, if this is an option.

My supervisor demonstrated that elevated circulating Prostaglandin E₂ (PGE₂) levels contribute to immune suppression in AD patients¹¹. The plasma protein albumin has been demonstrated to bind and catalyse inactivation of PGE₂¹². Albumin is synthesised in the liver and levels fall as the synthetic function of the liver declines in advanced cirrhosis, making PGE₂ more bioavailable. In addition the binding capacity of endogenous albumin is known to be defective in cirrhosis^{13,14}. Work within our group found a serum albumin of < 30g/L predicted plasma induced macrophage dysfunction in a small cohort of AD patients¹¹ and this was reversed when albumin levels were increased to >30g/L.

Acute on Chronic Liver Failure (ACLF)

The most recent consensus working definition states that “ACLF is a syndrome in patients with chronic liver disease with or without previously diagnosed cirrhosis which is characterized by acute hepatic decompensation resulting in liver failure (jaundice and prolongation of the INR) and one or more extrahepatic organ failures that is associated with increased mortality within a period of 28 days and up to 3 months from onset.”

Such a definition identifies patients with decompensated cirrhosis (of any aetiology)¹⁵ WITH extra hepatic organ failure.

Prognostic Scores in Chronic Liver Disease & assessing organ dysfunction

The Model for End-Stage Liver Disease (MELD)¹⁶ is a scoring system for assessing the severity of chronic liver disease. It was initially developed to predict death within three months of surgery in patients who had undergone a transjugular intrahepatic portosystemic shunt (TIPS) procedure, and was subsequently found to be useful in determining prognosis and prioritizing for receipt of a liver transplant. This score is now used by the United Network for Organ Sharing (UNOS) and Eurotransplant for prioritizing allocation of liver transplants instead of the older Child-Pugh score. MELD uses the patient's values for serum bilirubin, serum creatinine, and the international normalized ratio for prothrombin time (INR) to predict survival. The UK Model of End-Stage Liver Disease (UKELD)¹⁷ uses MELD but also incorporates serum sodium, it is used in the UK to prioritise for liver transplantation¹⁸.

Child-Pugh score¹⁶ is used to assess the prognosis of chronic liver disease, mainly cirrhosis. Although it was originally used to predict mortality during surgery, it is now used to determine the prognosis, as well as the required strength of treatment and

the necessity of liver transplantation. The score employs five clinical measures of liver disease: total bilirubin, serum albumin, INR, ascites, hepatic encephalopathy

The sequential organ failure assessment (SOFA) score¹⁹, is widely used to diagnose organ failure in general intensive care units. The SOFA score has been used in a number a large randomized control trials to assess organ dysfunction as a primary outcome¹⁹⁻²¹. However, some components of this score do not take into account specific features of cirrhosis. Therefore the Chronic Liver Failure Consortium has developed a modified SOFA score, called the CLIF-SOFA score¹⁵.

Renal injury/dysfunction is particularly predictive of a poor outcome with mortality increased 10 fold following kidney injury²². Acute kidney injury/dysfunction has recently been defined by the North American Consortium for Study of End-Stage Liver Disease as a >50% increase in serum creatinine level from the stable baseline value in <6 months or an increase of ≥ 0.3 mg/dL (26.5 μ mol/L) in <48 hours.

1.1.2. Impaired innate immune function in liver cirrhosis

Patients with cirrhosis have an increased predisposition to infection due to multimodal defects in the innate immune system. Impaired monocyte and neutrophil function was first identified more than 30 years ago^{7,23} however, the exact causative mechanism has not been established.

Patients with advanced cirrhosis have enteric dysbiosis with increased translocation of bacteria and their products across a leaky gut epithelial barrier^{24,25}. Once bacteria have passed into the circulation the first organ they should encounter is the liver, via the portal circulation. However in liver cirrhosis patients this pathway is often bypassed due to increased portal pressure and the development of a collateral circulation. The liver contains more than 80% of the reticuloendothelial system (kupffer and sinusoidal endothelial cells) which are in part responsible for removing circulating bacteria. Katz et al²⁶ administered ³⁵S-radiolabelled E.Coli to rats with or without portocaval shunting. 77% of e.coli was found in the liver within 10 minutes as opposed to 45% in the rats with portocaval shunting, this highlights the importance of circulation of blood through the liver in pathogen removal. Therefore a greater burden is placed on circulating immune cells.

During inflammation, monocytes move quickly to sites of tissue infection and differentiate into macrophages to elicit an immune response. Numerous studies have demonstrated the role of monocyte deactivation in cirrhosis associated immune suppression²⁷⁻²⁹. AD patients have reduced monocyte expression of HLA-DR. Wasmuth et al²⁷ isolated monocytes from stable cirrhotics, AD patients and patients without liver disease who were septic. Monocytes from septic and AD patients expressed significantly lower HLA-DR compared to stable patients and produced lower levels of TNF α production following stimulation with LPS. Monocyte dysfunction was independent of cause of liver cirrhosis.

Neutrophil migration and phagocytic activity is also decreased in AD patients. Fiuza et al³⁰ used a skin blister to analyse migration of neutrophils. Patients with liver cirrhosis had lower numbers of neutrophils after a chemoattractant was administered as opposed to healthy individuals. In addition neutrophils that were isolated were less effective at phagocytosing *E.coli*.

Plasma from cirrhotic patients can decrease healthy neutrophil phagocytic function suggesting a responsible circulating mediator. A study performed in 63 patients with alcoholic hepatitis demonstrated a reduced phagocytic capacity was transmissible by treating normal neutrophils with patients' plasma and this could be restored by addition of normal plasma³¹. The ex vivo removal of endotoxin from patients' plasma decreased the resting burst and increased the phagocytic function. However subsequent work from the same group found no correlation with neutrophil function and endotoxin levels³² but did find that there was increased expression of Toll-like receptors (TLRs) 2 and 4 in poorly functioning neutrophils. TLRs are specific for the recognition of bacterial components and are key drivers of the early inflammatory response to pathogens.

Another potential circulating mediator of neutrophil dysfunction is ammonia³³. Patients with liver cirrhosis often have increased ammonia levels due to decreased hepatic clearance and increased production in the dysbiotic bowel. Shawcross et al fed rats a high ammonia diet to induce high circulating ammonia levels and found neutrophils from these rats to have decreased phagocytosis and increased spontaneous oxidative burst. They then went onto feed 8 stable human cirrhotics an amino acid solution inducing hyperammonaemia and ex vivo analysis of their neutrophils found decreased phagocytosis of *E.coli* compared to stable cirrhotic control neutrophils. Similar effects were replicated in healthy volunteer neutrophils isolated in a high ammonia solution.

More recently MER receptor tyrosine kinase (MERTK), a transmembrane protein receptor, has been introduced as a potential cell mediated factor in the down-regulation of the immune response in patients with AD and organ failure³⁴. Patients (n=41) had increased numbers of immunoregulatory monocytes and macrophages that expressed MERTK and the number of these cells correlated with disease severity and a poor inflammatory response. MERTK inhibitors restored patient monocyte production of inflammatory cytokines. The authors did not investigate why this receptor might be upregulated in unwell cirrhotic patients and it was unclear how many of the patients had active infection.

1.1.3. The role of PGE₂ in suppression of the immune response

1.1.3.2. Role in suppression of the innate immune response

PGE₂ plays a key role in regulation of the innate immune cells. Macrophage function is inhibited by PGE₂, which acts to inhibit FcγR phagocytosis and NADPH oxidase activity in alveolar macrophages via EP2 receptors³⁵⁻³⁷. PGE₂ also induces the degranulation-independent production of monocyte chemo attractant protein-1 (MCP-1) by mast cells³⁸. MCP-1 is a chemokine that regulates migration and infiltration of monocytes/macrophages. PGE₂ also plays a role in the induction of mast cells, as well as the local chemotaxis and degranulation via EP1 and EP3 receptors³⁹⁻⁴².

1.1.3.3. Effects on the adaptive immune response

PGE₂ plays an inhibitory role in the initiation of the adaptive immune response. It inhibits the production of IL-2 and decreases expression of IL-2 receptors, causing a decrease in T cell expansion^{43,44}. In addition, PGE₂ shifts the balance from Th1 responses to Th2 responses⁴⁵. Cytotoxic T lymphocyte activity is also severely impeded by the presence of PGE₂ which decreases cell motility and adherence to target cells⁴⁶. PGE₂ can also suppress the expansion and differentiation of human B cells⁴⁷

1.2.3.4. The role of PGE₂ in the dysfunctional immune response in liver cirrhosis

Work conducted within our group was the first to establish a link between PGE₂ and immune dysfunction in decompensated liver cirrhosis¹¹ which I will summarise in this section.

Plasma from AD patients (n=7) showed significantly elevated levels of PGE₂ as compared to healthy volunteers plasma (HV), approximately 0.1ng/mL versus

0.025ng/mL. This concentration of PGE₂ was seen to dampen the release of TNF α from LPS stimulated healthy volunteer monocyte derived macrophages (MDMs). Addition of patient plasma to these MDMs (n=35) caused the same effect which was reversed after PGE₂ receptor blockade (EP1-3, see below). In a different assay macrophages were isolated with E.coli in the presence of AD plasma, there was decreased bacterial killing compared to HV plasma (and stable cirrhotic plasma) which again was reversed with PGE₂ receptor blockade. The only clinical marker which correlated with extent of ex vivo plasma mediated MDM suppression was serum albumin concentration. In these 35 patients a cut off of <30g/L predicted an 'immunosuppressive' response in ex vivo plasma analysis.

There was increased expression of COX-2 (part responsible for the production of PGE₂ from arachidonic acid) in peripheral blood mononuclear cells (PBMC) of AD patients compared to HV PBMCs. In addition it was found that inhibiting production of PGE₂, with the COX inhibitor indomethacin, increased bacterial killing and restored survival in two different mouse models of liver cirrhosis. COX-2 up regulation in these models was seen in organ tissue from kupffer cells and alveolar macrophages, suggesting these cells from these organs as a source of increase PGE₂ synthesis in these models.

It was postulated that the identified low albumin levels could be contributing to immunosuppression, as albumin is known to bind and catabolise PGE₂. Therefore bile duct ligated (BDL) mice were treated with IV 20% HAS to restore near normal albumin levels and as a consequence these mice were found to have lower levels of blood bacteria, after a bacterial challenge, compared to BDL mice treated with saline.

Finally 20% HAS (median 200mL) was given to 6 AD patients with a serum albumin <30g/L. Serum albumin levels rose to a 30.1g/l (+/- 3.1g/L). Ex vivo plasma analysis from these patients showed a significant improvement in LPS stimulated MDM TNF α production post treatment with albumin. This study was conducted at one site over a short time period and no clinical patient outcomes were recorded.

1.1.3.4. PGE₂ cell membrane receptors

PGE₂ effects changes in target cells via signaling through four distinct cell membrane-associated G protein-coupled E-prostanoid (EP) receptors, termed EP1, EP2, EP3, and EP4⁴⁸. It is unclear by exactly which pathway PGE₂ mediates its effects in the immune cells of cirrhosis patients. Alveolar macrophage activity is thought to occur via an EP2

dependent mechanism⁴⁹. Our group has previously found an EP1-3 dependent effect¹¹ however the receptor antagonist used only worked at very high concentrations (300µM) and therefore could have been causing an additional element of EP4 blockade. Subsequent work within our group (J.Fullerton, PhD thesis 2015, unpublished) using a differentiated monocyte cell line is more supportive of an EP4 related mechanism of action. It is likely that available receptor blockers are insufficiently selective and mechanisms should be fully investigated using methods other than simple receptor blockade.

1.1.4. Potential therapeutic interventions to remove PGE₂'s potential immunosuppressive effect

When considering PGE₂ as a mediator of immune dysfunction in AD patients several potential therapeutic options are available. In the mouse models described in 1.1.3.3 indomethacin reversed immune suppression and improved following bacterial infection. However, non-steroidal anti-inflammatory drugs (NSAIDs) are contraindicated in cirrhosis due to risk of renal impairment and gastrointestinal bleeding⁵⁰. PGE₂ receptor (EP) antagonists are an alternative but are not yet available for clinical use⁵¹.

Non-biological artificial liver support devices aim to remove albumin-bound and water-soluble toxins arising as a result of liver failure. However, recent multicentre controlled trials failed to show a benefit on transplant-free survival. Their use at present seems only justified as a bridge to liver transplantation⁵².

1.1.4.1. IV 20% HAS as an immunorestorative treatment in AD

Exploring the use of albumin as a modulator PGE₂ mediated immunosuppression in AD patients offers substantial advantages over other options. It is safe, cheap and simple to administer. There would be low regulatory hurdles to clinical use and, as albumin is already used for liver patients (see 2.2.1) a 20% HAS treatment regimen could be administered in any hospital and would not be limited to specialist centres.

20% HAS is already recommended for use in cirrhotic patients with spontaneous bacterial peritonitis (SBP)⁵³ due to positive effects seen in preventing renal dysfunction⁵⁴. There have been two randomised control trials evaluating the potential benefits of HAS in treating non-SBP infection in liver cirrhosis^{55,56}. In the first 110 patients admitted to hospital with non-SBP infection were randomly assigned to HAS (1.5g/kg day 1, 1g/kg day) plus antibiotics or antibiotics alone⁵⁵. Their pre-defined primary outcome of

reduction in 3-month mortality was not met. After multivariate analysis independent predictors of outcome (this appeared to be bilirubin, renal function and 'nosocomial infection') were corrected for and a 3-month survival benefit was then shown ($p=0.04$, log rank). There was also a suggestion of a benefit in preventing renal dysfunction. There was lack of clarity in the paper reporting outcomes and much over evaluation of results in this study. In a second study, of very similar design, 193 patients with liver cirrhosis (Child's Pugh score >8) and non-SBP infection were recruited and randomised⁵⁶. HAS infusion delayed onset of renal failure but did not improve renal function or survival at 3 months however the study was stopped prior to full recruitment due to an increased rate (8.3%) of pulmonary oedema in the HAS treatment arm. This will be further discussed in section 2.1.3.

There has never been an interventional study using HAS in cirrhotic patients with the aim of preventing infection. In addition HAS dose is usually given according to body weight therefore we do not know the effectiveness of serum targeted HAS infusion protocols, and whether these are safe. Finally previous PGE₂ work from our group was in a very small number of patients therefore outcomes need to be validated in a larger, more heterogeneous patient group prior to more widespread application.

1.2. Research question and hypothesis

Hypothesis

Prophylactic intravenous human albumin infusions increase serum albumin and subsequently prevent patients with acute decompensation of liver cirrhosis from developing infection

Proposed mechanism:

1. Circulating Prostaglandin E₂ levels are elevated in acutely decompensated (AD) liver cirrhosis and have been shown to contribute to immune suppression
2. Albumin binds and inactivates PGE₂
3. AD patients have low serum albumin which is also functionally deficient
4. Human Albumin Solution (HAS) could thus be used as an immune restorative drug in these patients – by improving quantity and functional quality of circulating albumin

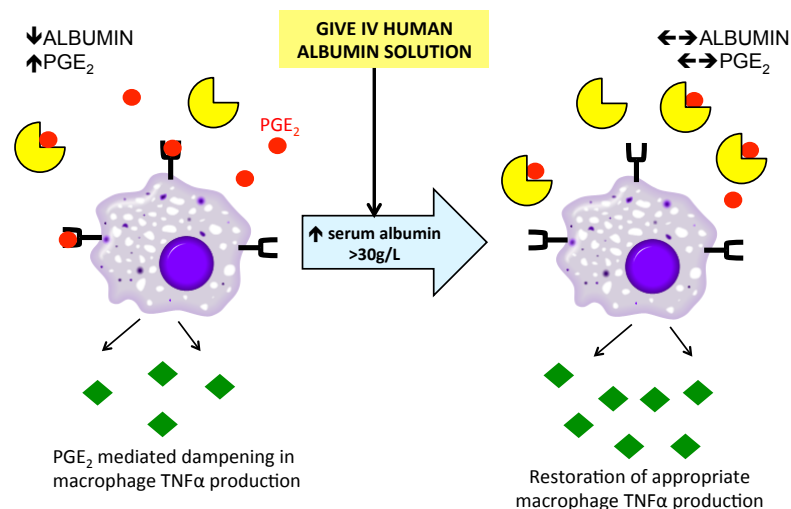


Figure 1.4. Proposed mechanism behind hypothesis that albumin can improve immune response in patients with decompensated liver cirrhosis

A macrophage is shown with increased and more bioavailable PGE₂ which binds to EP receptors. PGE₂ inhibits Fc receptor mediated phagocytosis and NADPH oxidase mediated bacterial killing and leads to a downregulated Th1 response leading to decreased pro-inflammatory cytokine production. TNFα is one of these pro-inflammatory cytokines and is a validated marker of monocyte function in critical illness. Albumin binds and catalyses PGE₂ however albumin levels are low in AD. This could contribute to raised free levels of PGE₂. If albumin is given to patients in order to increase circulating levels to above the previously identified cut off (30g/L) there will be more albumin to bind and possibly catalyse PGE₂, as a consequence there may be restoration of an appropriate immune response

Research questions

1. Can patient serum albumin levels be increased to near normal (>30g/L) using intravenous 20% HAS and is this safe?
2. Is there an assay that could be used to assess the PGE₂ dependent **mechanism** via which HAS is functioning that was suitable for large-scale clinical trials?
 - a. To assess immune function after IV HAS
 - b. To assess improvement in the function (as well as concentration) of circulating plasma albumin after IV HAS
3. What is the most accurate and feasible way of diagnosing infection and in patients with acute decompensation of liver cirrhosis in a large-scale interventional study?
 - a. What are the most commonly reported methods being used to diagnose infection in acutely unwell liver cirrhosis patients in clinical trials?
 - b. Is there a method of more accurately diagnosing infection for use in a large-scale clinical trial?
4. Does HAS infusion versus standard medical care reduce diagnoses of infection in patients with acute decompensation of cirrhosis?
 - a. Do these infection diagnoses correlate with lab assays connected to an underlying mechanistic role for albumin?
 - b. Can we identify those patients who are at a higher risk of developing infection?

CHAPTER 2: THE FEASIBILITY AND SAFETY OF ADMINISTERING SERUM ALBUMIN TARGETTED DAILY HUMAN ALBUMIN SOLUTIONS TO PATIENTS WITH ACUTE DECOMPENSATION OF LIVER CIRRHOSIS

Publications relating to this chapter:

ATTIRE: Albumin To prevent Infection in chronic liver failure: study protocol for a single-arm feasibility trial. [China L*](#) et al. *BMJ Open*. 2016 Jan 25;6(1):e010132

Administration of Albumin Solution Increases Serum Levels of Albumin in Patients With Chronic Liver Failure in a Single-Arm Feasibility Trial. [China L*](#) et al. *Clin Gastroenterol Hepatol*. 2018 May;16(5):748-755.e6.

Presentations relating to this chapter:

ATTIRE: ALBUMIN TO PREVENT INFECTION IN CHRONIC LIVER FAILURE [China L*](#) et al. EASL 2015 (Vienna) CT-1481 – *Trial protocol presented*

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution [China L*](#) et al. AASLD 2016, Boston. – *Clinical results presented*

Defining meaningful clinical trial endpoints in patients with alcohol induced chronic liver disease: results from a multicentre feasibility trial. [China L*](#) et al. Poster presentation at EASL Special Conference 2017 (London).

Accurately defining infection as a clinical trial endpoint in patients with alcohol induced chronic liver disease: results from a multicentre feasibility study. [China L*](#) et al. Poster presentation at EASL Special Conference 2017 (London).

Contributions by others to this chapter:

- Ethics approval and site set up: Led by Zainib Shabir (Trial Manager)
- Trial protocol: Written by Alastair O'Brien and myself (clinical), Simon Skene (statistics), Zainib Shabir (trial administration)
- Statistical analysis: Led by Simon Skene, sub analysis conducted by myself alone.
- Data collection and entry: James Blackstone & Zainib Shabir
- Patient screening and recruitment: Individual hospital research teams (10)

2.1. Introduction

To date there has not been an albumin dosing trial aimed at increasing serum albumin levels in the context of acutely decompensated (AD) liver cirrhosis. Therefore it was essential to complete a feasibility study before proceeding to a large, interventional randomised control trial (RCT) investigating whether targeted albumin treatment is beneficial compared to standard of care.

Therefore the aim of the first part of this work is to verify that daily intravenous human albumin infusions will restore serum albumin levels to near normal in AD patients, that this is safe and that there is physician equipoise in terms of prescribing albumin prior to proceeding to a large RCT. Despite multiple studies and systematic reviews^{57,58} evaluating albumin in septic intensive care patients, there is a lack of interventional RCTs in patients with liver cirrhosis in which the mechanism of the action of albumin may be different⁵⁹⁻⁶¹.

2.1.1. Different potential intravenous albumin treatment protocols

Albumin is currently used as standard care in three particular clinical scenarios in liver cirrhosis (table 2.1).

Indication	Amount of 20% HAS advised	Evidence
Large volume paracentesis	8 g albumin/L of ascites removed (that is 100 mL of 20% albumin/3L ascites) ⁵³	Bernardi, et al. ⁶² GINE`S A, et al. ⁶³ Arora, et al. ⁶⁴
Spontaneous bacterial peritonitis (with developing signs of renal impairment)	1.5 g albumin/kg in the first six hours followed by 1 g/kg on day 3 ⁵³	Sort P ⁵⁴ Chen, et al. ⁶⁵ Fernandez, et al. ⁶⁶ XUE, et al. ⁶⁷
Hepatorenal Syndrome (type 1)	<i>Optimal dose not defined.</i> Expert consensus advises: 1 g/kg of body weight on the first day, up to a maximum of 100 g, followed by 20–40 g/day ⁶⁸	Afinogenova and Tapper ⁶⁹ Martin-Llahi, et al. ⁷⁰ Guevara, et al. ⁵⁵ Fernandez and Arroyo ⁷¹

Table 2.1. Current indications as per national/international guidance for albumin use in liver cirrhosis

Interventional studies using albumin in AD in different clinical scenarios have generally used weight-based regimens. However this is problematic in liver cirrhosis as between 24-80% of inpatients have significant ascites⁷² making dry weight assessment difficult. This may lead to excessive albumin being prescribed and complications such as fluid overload⁶⁹.

In work from our group a treatment target of serum albumin >30g/L was identified to improve immune dysfunction. Receiver operating analysis (ROC) of patients (n=35) found that a cut off of <30g/L predicted a suppressed immune response in their ex vivo analysis with a sensitivity of 70% (CI 47-87) and a specificity of 67% (CI 35-90)¹¹. Therefore treatment with albumin infusions could be targeted to increase the AD patient's serum albumin to >30g/L rather than a non-specific weight based regimen.

Caironi, et al.²⁰ used an albumin treatment protocol in 1818 patients with severe sepsis on ICU. It took 9 days for patients to reach a median serum albumin of 30g/L and patients were treated with IV albumin (or saline in the control group) for a maximum of 28 days. There were no differences in survival at 90 days although subgroup analysis suggested a benefit of albumin treatment in severe sepsis and renal failure. In patients with sepsis there is a high consumption of albumin and clinicians were targeting an endpoint of 30g/L serum albumin but did not reach this level. A slightly higher treatment target (e.g. 35g/L) may have ensured that the patients incremented to this defined 30g/L endpoint and perhaps would have led to a positive outcome in this study. Importantly the infusion protocol that Caironi, et al.²⁰ used was shown to be safe with no increased incidence of Serious Adverse Events (SAEs) in the treatment group compared to the control group.

Recently conflicting results have been published from studies evaluating long-term HAS administration to outpatients with chronically decompensated cirrhosis. The ANSWER study⁷³ administered a higher dose of HAS to patients and saw a sustained increment in their serum albumin levels with subsequent reduction in mortality incidence rate ratio and other complications such as infection over an 18 month period. In contrast the MACHT study⁷⁴ found no mortality benefit with a lower dose of albumin, although over a much shorter time period in a group of patients whom many went onto receive a donor liver shortly after study inclusion. In another small outpatient study (n=18) the patients

who received a higher dose of HAS with an increase in their serum albumin to normal ranges over a 12 week period were found to have a sustained improvement in markers of systemic inflammation as opposed to those who received a smaller HAS dose without a sustained increase in their albumin levels⁷⁵. This provides some insight into the differing results seen in the ANSWER and MACHT studies and highlights the potential importance of targeted improvement in serum albumin in these patients. Neither study had a primary aim of increasing serum albumin and the infusion regimen was not adapted for individual patients (set amount administered at set time points).

2.1.2. Measuring patient serum albumin in UK NHS Hospitals

Albumin levels are measured in hospital laboratories from serum samples taken with a gold top vacutainer tube known as a serum separating tube. It contains two agents; silica particles (activate clotting) and a serum separating gel. Dye binding methods are used to measure serum albumin. The UK National External Quality Assessment Service (NEQAS) assesses accuracy of UK hospital laboratory reporting using blinded control samples to be tested by hospitals on a bi-monthly basis⁷⁶. The standard deviation of all UK laboratories in the scheme (private and NHS) when measuring albumin is 1g/L. UK-NEQAS indicates that 60% of laboratories use bromcresol green (BCG) and 40% use bromcresol purple (BCP) methods to measure albumin. The albumin assay is inaccurate with patients who have IgM gammopathy (Waldenstrom's macroglobulinaemia) and in significant haemolysis. Only very high bilirubin levels (>1026umol/l) will cause interference with the assay.

The regulation of UK laboratories via NEQAS and the lack of interference with physiological high bilirubin levels means that reported serum albumin levels from different hospital sites are comparable in a clinical trial setting.

2.1.3. Safety concerns with intravenous albumin treatment.

Human albumin for infusion is produced via ethanol fractionation from pooled donated healthy donor blood⁷⁷. There are at least 5 current UK manufacturers of 20% HAS and production processes vary slightly between manufacturers. All are required to check donated plasma for known testable transmissible viruses. Manufacturers are also required to heat the albumin fraction to >60 degrees for 10 hours and check samples for endotoxin/bacterial/fungal contamination.

A recent meta-analysis of 16 albumin-interventional RCTs (4190 patients) in patients with sepsis concluded that albumin infusion was safe and a signal towards harm was not detected⁵⁷.

Virus transmission

There have been no reports of viral transmission via 20% HAS infusion in the UK in the last 20 years (email communication from the MHRA). There remains a theoretical risk of prion transmission however there have been no cases of Creutzfeldt-Jakob disease linked to albumin transfusion.

Sensitivity reaction

There are rare reports of hypersensitivity reactions to albumin infusions. This may be a reaction to the albumin itself or the stabilisers used in the solution as prepared for infusion.

Fluid overload

Administering excessive amounts of any intravenous fluid carries a risk of fluid overload and this risk is much higher in patients with cardiac dysfunction. Alcoholic cardiomyopathy is frequently seen in patients with liver cirrhosis with rates of up to 50% reported in some case series⁷⁸. Therefore causing harm with excessive fluid administration is a concern in the AD patient group. However it is often the case that AD patients are intravascularly deplete and require intravenous fluid administration in order to prevent organ failure when they are admitted to hospital, therefore a careful balance is required with regular clinical assessment. There is also a theoretical concern in patients who present with variceal bleeding that giving excessive intravenous (IV) fluid could further increase portal pressure and worsen bleeding. Ongoing studies are assessing a potential beneficial effect of restricted transfusion in the GI bleed setting⁷⁹ (with and without portal hypertension) and the mechanism of benefit of a restricted strategy demonstrated thus far could involve the component of controlling increases in portal pressure.

Interventional albumin studies in liver cirrhosis for SBP and HRS have shown no increased rates of pulmonary oedema or variceal bleeding^{54,70}. However a study using albumin infusions (day 1=1.5mg/kg and day 3=1mg/kg) to assess a potential benefit in treating non SBP infection in AD patients was stopped early due to an excessive high

rate of pulmonary oedema in the albumin treatment group⁵⁶ (8.3% in 96 patients with 1 death). However the mean albumin dose in this study was 106g+/-22g which equates to 5 x 100mLs 20% HAS. This equates to an average weight of patients of 70kg+/- 14kg, much higher than reports of dry weights in nutritional studies in liver cirrhosis⁸⁰. In addition ascites was more frequently observed in the albumin treatment arm. Therefore one could theorise that patients were not given albumin according to their dry weight and the already high 1.5mg/kg dose of albumin was given in excess.

A retrospective analysis of 169 patients in one US liver centre calculated an optimal albumin dose for survival in AD patients with renal failure +/- SBP to be 87.5g (no patient weights analysed) for improved survival in multivariate analysis⁶⁹. However they concluded that higher doses (>100g) were associated with increased ICU admissions related to fluid overload. The authors did not take into account that these patients may have just been more unwell and hence were the ones that received more albumin and no attempt at correction for prognostic score was made in their analysis of ICU admissions. There are also huge challenges in the inpatient AD group in accurately diagnosing pulmonary oedema secondary to fluid overload as opposed to alternative feasible diagnosis such as Acute Respiratory Distress Syndrome (ARDS) or bilateral pneumonia.

2.1.4. Defining endpoints in clinical trials involving AD patients

2.1.4.1. Formulating composite endpoints for open label, pragmatic clinical trials

An endpoint in a clinical trial is an event such as occurrence of a clinical problem (e.g. death) or a particular laboratory result (e.g. creatinine rise marking renal failure). Once someone reaches the primary endpoint, they are generally excluded from being able to contribute further to that endpoint and may be withdrawn from participating in the trial completely. Endpoints are often described as 'soft' and 'hard'. A soft endpoint is a subjective measure, for example the impact a particular intervention has on a patient's quality of life which, when measured un-blinded, could be effected by the person receiving the measurement or the patient themselves. In contrast, a hard endpoint is an endpoint that is well defined and can be measured objectively. For example a blood test result taken at a specific pre-defined time to measure an organ failure with an established definition. In open-label studies it is important that primary endpoint

measurements are as objective as possible. Modifying endpoint definitions in this scenario, to remove subjectivity, can reduce bias considerably⁸¹.

Composite endpoints in clinical trials are composed of primary endpoints that contain two or more distinct component endpoints⁸². Composite endpoints should include components that are similar in importance, that occur with similar frequency and that are affected to a similar degree by the intervention⁸³. The benefits can include increased statistical efficiency, decrease in the required sample size, a shorter trial and subsequent decreased cost. However, the possible benefits must be weighed against the challenges in interpretation. The larger the gradient in importance, frequency, or results between the component endpoints, the less informative the composite endpoint becomes, thereby decreasing its utility for medical-decision making.

2.1.4.2. Infection as an endpoint in liver cirrhosis clinical trials

End points for trials should be clinically relevant to patients and clinicians. In clinical practice infection it is often difficult to diagnose, particularly in patients who may have a dysfunctional immune response such as AD patients⁸⁴. Previously positive bacterial cultures have been used as 'hard evidence' of a diagnosis of infection, however even in the presence of sepsis these cultures can be negative^{85,86}. In a recent large clinical trial in liver cirrhosis patients with alcoholic hepatitis who were treated with steroids infection rates were reported as 11-13%⁸⁷ which is much lower than the usual $\approx 30\%$ reported in multiple other observational cohort studies in liver cirrhosis patients^{4,72,88,89}. Infection was highly relevant in this study as patients with alcoholic hepatitis are thought to be at an even higher risk of infection than patients with other causes of acute decompensation⁹⁰ and steroids could increase this risk further therefore it was surprising that such low infection rates were reported. However on further reading it is apparent that infection in this study was recorded using the number of Serious Adverse Events (SAEs) reported with infection as a cause. An SAE is a patient event, in a clinical trial, which is defined as an event which is life threatening, prolongs hospital admission or death. Reporting depends on nurse training at site, although this should be consistent. Therefore in this alcoholic hepatitis main study report only severe infections are reported as 'infection' due to the definition. This may mislead the reader and highlights the importance of transparency with definitions in clinical trials.

A standardized criteria for the diagnosis of infection in liver cirrhosis trials exists⁸⁹. However in real life practice clinicians are unlikely to adhere to a rigid set of clinical criteria and will additionally use their own clinical judgment prior to the initiation of therapy (usually antimicrobials) for suspected infection. The only proposed gold standard of infection diagnosis is positive microbiological culture, however this poses a huge problem in liver cirrhosis studies as cultures are often negative in these patients when clinical infection is apparent⁹¹.

For the purposes of this project I will evaluate initiation of therapy for an infection as a clinical surrogate for the diagnosis of infection.

2.1.4.3. Organ failure in liver cirrhosis in clinical trials

There are multiple prognostic scores for use in patients with chronic liver disease which were developed with different intentions for use⁹²⁻⁹⁶. Many of these scores incorporate markers of liver function, such as coagulopathy or bilirubin, as these are important markers of long term prognosis. However in the acute setting, particularly with infection and acute decompensation, extra hepatic organ dysfunction is most closely correlated with outcome⁹⁷.

The sequential organ failure assessment (SOFA) score¹⁹, is widely used to diagnose organ failure in general intensive care units (ICUs). The SOFA score has been used in a number a large randomised control trials to assess organ dysfunction as a primary outcome¹⁹⁻²¹. The Albumin for Volume Replacement in Severe Sepsis (ALBIOS) trial²⁰ used a change in a component score from 0, 1 or 2 to a score of 3 or 4 to define new organ failures.

However, some components of this score do not take into account specific features of cirrhosis. Therefore the Chronic Liver Failure Consortium has developed a modified SOFA score, called the Chronic Liver Failure Sequential Organ Failure Assessment Score (CLIF-SOFA) score¹⁵ (see table 2.2).

Organ/system	0	1	2	3	4
Liver (bilirubin, $\mu\text{mol/L}$)	<20	≥ 20 to ≤ 34	>34 to <102	≥ 102 to <205	≥ 205
Kidney (creatinine, $\mu\text{mol/L}$)	<106	≥ 106 to <176	≥ 176 to <309	≥ 309 to <442	≥ 442
			or use of renal replacement therapy		
Cerebral (HE grade)	No HE	I	II	III	IV
Coagulation (INR)	<1.1	≥ 1.1 to <1.25	≥ 1.25 to <1.5	≥ 1.5 to <2.5	≥ 2.5 or plt count $\leq 20 \times 10^9/\text{L}$
Circulation (mean arterial pressure, <i>mm Hg</i>)	≥ 70	<70	Dopamine <5 or dobutamine or terlipressin	Dopamine >5 or E <0.1 or NE <0.1	Dopamine >15 or E >0.1 or NE >0.1
Lungs PaO ₂ /FiO ₂ or SpO ₂ /FiO ₂	>400	>300 to ≤ 400	>200 to ≤ 300	>100 to ≤ 200	≤ 100
	>512	>357 to ≤ 512	>214 to ≤ 357	>89 to ≤ 214	≤ 89

Table 2.2. CLIF-SOFA score.

HE, hepatic encephalopathy; E, epinephrine; NE, norepinephrine; PaO₂, partial pressure of arterial oxygen; FiO₂, fraction of inspired oxygen; SpO₂, pulse oximetry saturation. E: epinephrine. NE:Noradrenaline [infusion rates are in mcg/kg/min]

Renal injury/dysfunction is particularly predictive of a poor outcome with mortality increased 10 fold following kidney injury²². Acute kidney injury/dysfunction has recently been re defined by the North American Consortium for Study of End-Stage Liver Disease as a >50% increase in serum creatinine level from the stable baseline value in <6 months or an increase of ≥ 0.3 mg/dL (26.5 $\mu\text{mol/L}$) in <48 hours²². A revised statement from the international ascites club has defined AKI as an increase in sCr ≥ 0.3 mg/dl (≥ 26.5 $\mu\text{mol/L}$) within 48 hours or a percentage increase sCr $\geq 50\%$ from baseline which is known, or presumed, to have occurred within the prior 7 days⁹⁸.

The above described scores often use complicated clinical measures only available in ITU/transplant units. I hypothesised that detecting early, rather than advanced, extrahepatic organ dysfunction (EHOD) in ward settings would be more clinically relevant for interventional studies in AD patients as this is the tipping point for progression to multi organ failure and death. Therefore modified extra hepatic components of the CLIF scoring system will be used as the criteria, in this project, in an attempt to detect organ dysfunction at an earlier stage when it is more clinically relevant and an intervention may be effective. The patient clinical details required to assess outcomes against these criteria also need to be recorded accurately in a pragmatic way

by research nurses at multiple sites and therefore this also has to be taken into account when developing them.

Chapter aims:

- Using a daily 20% HAS IV treatment protocol targeted towards increasing serum albumin levels, to determine:
 - Efficacy of increasing AD patient serum albumin to >30g/L
 - How long it takes, on average, for a patient to reach a serum albumin of 30g/L after treatment
 - What volume of 20% HAS, on average, is required to raise and maintain serum albumin to >30g/L in a 2 week treatment period
 - If there are any safety concerns with the protocol
 - If it is feasible to continue in a multi-site NHS setting
- In a multi centre study of hospitalized AD patients in the UK, to determine:
 - Baseline characteristics using a selection criteria based on low serum albumin levels according to the previously identified cut-off (<30g/L)
 - Clinically important event rates using a new pragmatic criteria to detect earlier extra hepatic organ dysfunction
 - Event rates of infection using a surrogate marker for diagnosis, and whether this is an accurate approach
 - An event rate for a primary composite endpoint of inpatient infection, extrahepatic organ dysfunction and death which could be used to power an interventional RCT comparing daily 20% HAS infusions to standard of care
 - And how the components of this primary composite endpoint might be altered to improve reliability

2.2. METHODS

This was a multicenter (10), open label single-arm feasibility trial in which all patients were treated with daily IV 20% HAS to target near normal serum albumin levels (>35g/L) with an endpoint of >30g/L.

2.2.1. Patient selection

Patient population

This included all patients admitted to hospital with complications of liver cirrhosis and serum albumin < 30 g/L, aged over 18 years with anticipated hospital length of stay of 5 or more days at trial enrolment, which was no later than 72 hours from admission. The exclusion criteria are detailed in table 2.3. The diagnosis of cirrhosis was made by the clinical team as per standard UK practice and did not require liver biopsy or imaging.

Patient Inclusion Criteria	Patient Exclusion Criteria
All patients admitted to hospital with acute onset or worsening of complications of cirrhosis	Advanced hepatocellular carcinoma with life expectancy of less than 8 weeks
Over 18 years of age	Patients who will receive palliative treatment only during their hospital admission
Predicted hospital admission > 5 days at trial enrolment, which must be within 72 hours of admission	Patients who are pregnant
Serum albumin <30g/l at screening	Known or suspected severe cardiac dysfunction
Documented informed consent to participate (or consent given by a legal representative)	Any clinical condition which the investigator considers would make the patient unsuitable for the trial
	The patient has been involved in a clinical trial of Investigational Medicinal Products (IMPs) within the previous 30 days that would impact on their participation in this study
	Trial investigators unable to identify the patient (by NHS number)

Table 2.3. Patient inclusion and exclusion criteria

Consent

Patient information sheets were given to and discussed with potential patients before consent was sought. Informed consent was obtained from each participant or their legal representative. Patients who lacked mental capacity, for any reason, were not excluded from the trial. An important subgroup of patients will have hepatic encephalopathy and these patients may lack capacity to consent. However these patients may be amongst those that receive maximum benefit from the intervention^{97,99,100}. In this case consent

was sought from an appropriate legal representative independent of the research team as per current UK clinical trials regulations¹⁰¹. This process was approved during ethical board assessment of the protocol (NRES:15/LO/0104).

2.2.2. Intervention

It was intended that all patients would receive a daily infusion of 20% HAS intravenously (100mLs/hour) for a maximum of 14 days or until discharge (if less than 14 days). The volume of HAS prescribed each day was determined by the patient's serum albumin level on that day, or if this was not known (as no bloods were taken as part of standard of care) an estimate was made by the trial site clinician.

Table 2.4 shows the suggested dosing protocol for albumin administration. This is based on the reported regimen used in the ALBIOS study²⁰ and clinical experience as there are no prior studies in cirrhosis patients. In ALBIOS²⁰ patients with a very low albumin (<20g/L) incremented to a higher value within 4-5 days therefore I expected 20% HAS requirements, as according to this trial protocol, to decrease after a few days with a subsequent decrease in cost and time of administration.

Differing regimens may be used to cover large volume paracentesis (8g of albumin per litre of ascites drained) or treat Hepatorenal syndrome (1g of albumin per kilogram of body weight) as per international guidelines^{98,102} but HAS must be prescribed and given if serum albumin <35g/L. Trial clinicians were given flexibility in the prescription if they were concerned about the patient's safety (e.g. risk of fluid overload). It was requested that volume variations or complete lack of prescription were recorded in the patient's daily Case Report Form (CRF). If a patient's serum albumin reached normal levels (>35g/L) but subsequently fell back below this level during the 14 day treatment period HAS should again be prescribed according to the protocol. In a pragmatic approach, to account for absence of research staff at weekends at many hospital sites or lack of daily blood tests, site clinicians were able to use previous treatment days albumin levels to prescribe the 20% HAS.

Patient's Serum Albumin Level	Amount of 20% HAS to be administered
≥35 g/L	none
30-34 g/L	100mLs
26-29 g/L	200mLs
20-25 g/L	300mLs
<20 g/L	400mLs

Table 2.4. Dosing protocol for 20% HAS administration (amounts per day) as advised by measured serum albumin level on that day (or previous days if there were no standard of care blood tests on that day).

2.2.3. Evaluations during and after treatment

Clinical, biochemical and microbiological data was collected daily during the trial treatment period (figure 2.1) using information from hospital notes that is recorded as standard of care. There was no follow up beyond the treatment period other than recording mortality at 30 days. The blood samples collected for ex vivo laboratory analysis by myself will be analysed in a blinded fashion at UCL (see chapters 3 and 4).

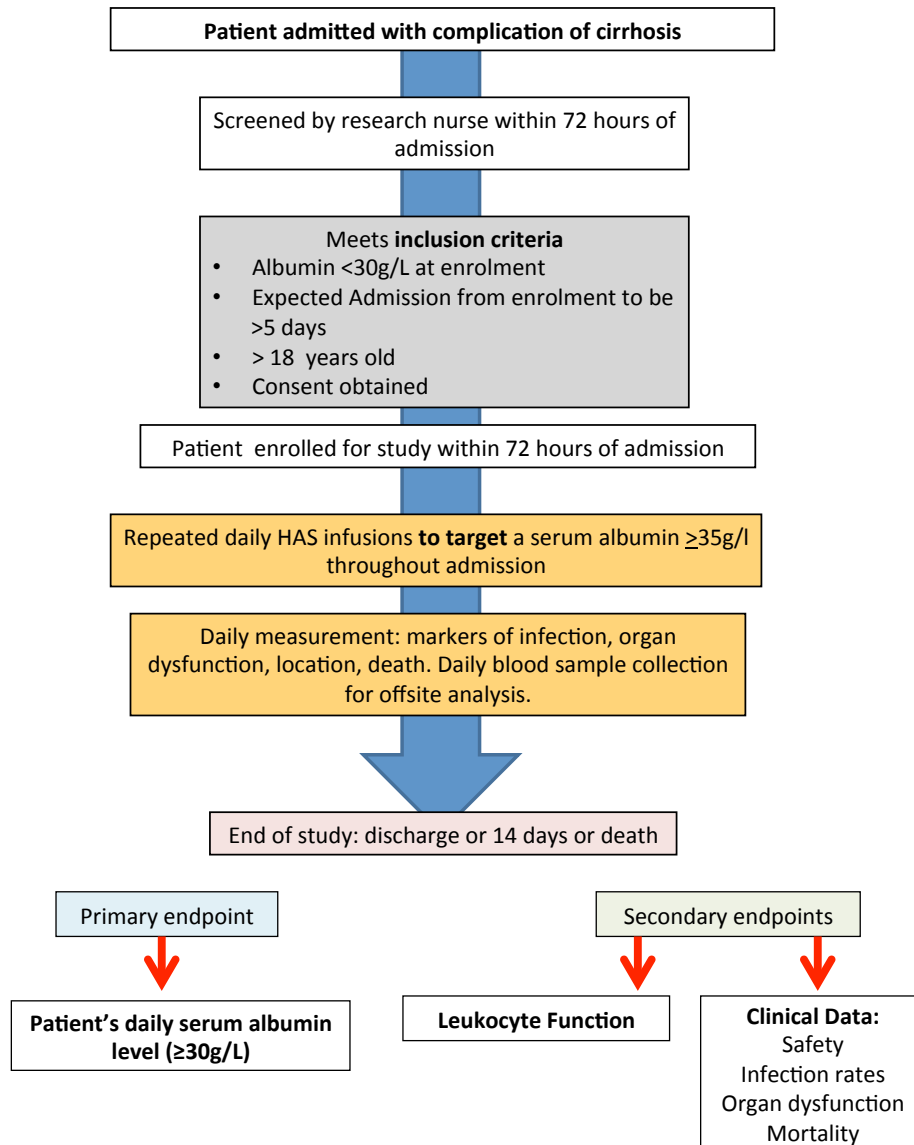


Figure 2.1. Flow chart of patient screening, intervention and relation to clinical outcomes.
Taken from China, et al. ¹⁰³

Criteria for labelling 'infection' in analysis

For the purposes of this study a surrogate was used to record infection as marked by a new or change in antibiotics > 2 days after treatment with albumin infusions have started (between days 3-15 of the trial treatment period). This was chosen as clinicians often disagree regarding a diagnosis of infection and initiation of antibiotics was thought to be indicative that a clinical decision had been made.

Infection can be defined according to the peer reviewed criteria⁸⁹ in table 2.5.

1. Spontaneous bacteraemia: positive blood cultures without a source of infection.
2. SBP: ascitic fluid polymorphonuclear cells >250 cells/mm³
3. Lower respiratory tract infections: new pulmonary infiltrates in the presence of: i) at least one respiratory symptom (cough, sputum production, dyspnoea, pleuritic pain) with ii) at least one finding on auscultation (rales or crepitation) or one sign of infection (core body temperature >38°C or less than 36°C, shivering, or leukocyte count >10,000/mm³ or <4,000/mm³) in the absence of antibiotics.
4. Clostridium difficile Infection: diarrhoea with a positive C. difficile assay.
5. Bacterial entero-colitis: diarrhoea or dysentery with a positive stool culture for Salmonella, Shigella, Yersinia, Campylobacter, or pathogenic E. coli.
6. Soft-tissue/skin Infection: fever with cellulitis.
7. Urinary tract infection (UTI): urine white blood cell >15/high-power field with either positive urine gram stain or culture.
8. Intra-abdominal infections: diverticulitis, appendicitis, cholangitis, etc.
9. Other infections not covered above.
10. Fungal infections as a separate category.

Table 2.5. Details regarding classification of infection

Therefore to investigate whether prescription of antibiotics as a surrogate marker for infection diagnosis was accurate research nurses were asked to complete an infection case report form (CRF) every time antibiotics were prescribed for a new infection. These CRFs record microbiological, clinical, radiological and biochemical data to support the infection diagnosis. Using the CRFs the diagnosis will then be assessed according to the criteria in table 2.5.

2.2.4. Statistical considerations

The primary purpose of this trial was to demonstrate that repeated 20% HAS infusions can raise and maintain serum albumin at ≥ 30 g/L in liver cirrhosis patients presenting with AD. As this was a single arm, feasibility study the emphasis was on producing data summaries rather than hypothesis testing. 80 patients were to be recruited. Success would be demonstrated if 60% of these were able to achieve and maintain serum albumin levels at or above 30 g/L on at least 1/3 of days in which the level was recorded.

The trial was performed at 10 sites with the assumption that 8-10 patients per site would allow identification of any variability in the delivery of the albumin-targeting dose protocol between centres. It was compulsory to record reason for protocol variation in the daily CRF.

Primary outcome

Serum albumin levels were summarised for each of days 1-15. Day 1 represents the baseline serum albumin level before the first administration of 20% HAS according to the protocol. The number of patients on each day whose serum albumin level exceeds 30g/L were reported as a percentage of those evaluated, together with the overall percentage of patients whose serum albumin level exceeds 30g/L on at least 1/3 of the days on which it was recorded. Success was defined as more than 60% of patients having a serum albumin level of >30g/L on at least 2/3 of the days that they were treated.

Secondary outcomes.

Information was summarised regarding the total volume of albumin infused and duration of hospital stay, together with the rates of nosocomial infections, new organ dysfunction (see table 2.6 for definitions) and in-hospital mortality. Safety was assessed by the number of SAEs reported during the trial. Infection and organ failure rates were reported from day 3 onwards, as patients should have had 2 days of HAS treatment by that point.

Data was further summarised within 'groups' defined by baseline serum albumin levels (<20g/L, 20-25 g/L and 26-29g/L) to investigate whether there were any apparent differences in primary outcome by group.

Organ dysfunction	Definition of new dysfunction			
Renal	Serum creatinine increases by $\geq 50\%$ as compared to serum Creatinine at randomisation OR the patient initiated on renal replacement support (either haemodialysis or haemofiltration). <i>Note: if the patient is receiving renal replacement support at baseline they cannot reach this endpoint</i>			
Cerebral	Grade III (drowsy) or grade IV encephalopathy (coma) using the Westhaven Criteria to grade hepatic encephalopathy. <i>Note: if the patient has grade III encephalopathy somnolent but rousable at baseline, they will need to progress to grade IV to reach this endpoint</i>			
Circulatory	i) Mean Arterial Pressure (MAP) falls to <60mmHg, OR ii) patient is started on inotropic/vasopressor support (not including terlipressin if given for renal dysfunction) <i>Note: if the patient has a MAP < 60mmHg at baseline, they will need to be started on inotropic/vasopressor support to reach this endpoint</i>			
Respiratory	Any single point increase in SpO ₂ /FiO ₂ as classified on the following scoring system as compared to SpO ₂ /FiO ₂ at randomisation:			
		0	1	2
	SpO ₂ /FiO ₂	>357	>214 to ≤ 357	≤ 214 or mechanical ventilation
	<i>Note: if the patient is receiving mechanical ventilation at baseline they cannot reach this endpoint</i>			

Table 2.6. Definitions of a new organ dysfunction (endpoint will be recorded after day 3 of recruitment)

Nurses recorded the highest and lowest values from existing patient observations in a 24 hour period on the daily CRF.

Clinical measurements used to define extrahepatic organ dysfunction were recorded on a daily basis by research nurses. As it was not possible for them to be present for a whole 24 hour period the 'worst' (most extreme value e.g. lowest blood pressure) measurement needed to calculate dysfunction for each individual organ component was measured in an attempt to reflect the previous 24 hour period.

Exploration of a proposed primary composite endpoint for a future RCT

This single arm study allowed exploration and confirmation of reliable and meaningful endpoints for a future open-label randomized controlled trial comparing daily targeted 20% HAS to standard of care. The purpose of the study was to use albumin infusions to prevent infection, however infection diagnosis, as discussed, can be a subjective measure. The development of extrahepatic organ dysfunction is closely related to infection and is a meaningful event to patients and clinicians as it often marks the tipping point 'of no return' in the patient pathway. Occasionally patients deteriorate very rapidly and die prior to infection or organ failure being identified. Therefore the proposed composite endpoint, to increase validity and statistical power, for a future RCT was the occurrence of infection, extrahepatic organ failure (table 2.6) or death during the trial treatment period after patients have had at least 48 hours of 20% HAS treatment.

2.2.5. Ethics and MHRA approval and trial registration

The recruited patients involve a potentially vulnerable patient group that have hepatic encephalopathy and therefore lack the capacity to consent. However patients with encephalopathy are at high risk of infection and could be those that potentially receive maximal benefit from the intervention and therefore should not be denied access to the trial treatment. The trial team undertook steps to ensure these patients were appropriately recruited to the trial (described in 'Consent' section 2.2.1) and provided individual site training.

Research Ethics positive opinion was given by the London-Brent Research Ethics Committee (ref: 15/LO/0104) which specialise in trials involving patients who lack the capacity to consent. The Clinical Trials Authorisation was issued by the Medicines and Healthcare products Regulatory Agency (MHRA, ref: 20363/0350/001-0001). The trial is registered with the European Medicines Agency (EudraCT 2014-002300-24) and has been adopted by the NIHR.

2.3. RESULTS

2.3.1. Patient characteristics

2.3.1.1. Numbers recruited and exclusions

517 patients were screened at 10 hospital sites over a 6-month period (figure 2.2). 124/517 were eligible for recruitment and 80/124 (65%) consented to take part in the trial. 1 patient was excluded from analysis as incorrect serum albumin levels were entered at randomisation and the patients correct serum albumin was >30g/L at recruitment therefore they did not fulfill inclusion criteria. The most common reasons for ineligibility during screening were: albumin level of 30 g/L or greater, admission more than 72 hours before screening and predicted hospital stay of fewer than 5 days.

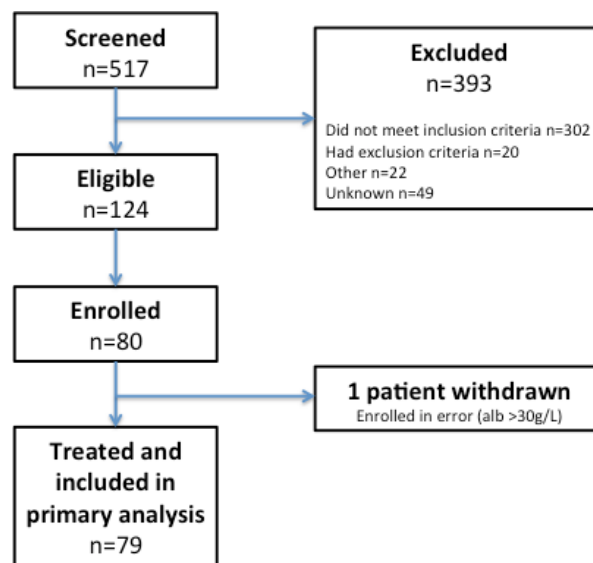


Figure 2.2. Albumin To Prevent Infection In Chronic Liver Failure feasibility study Consolidated Standards of Reporting Trials flowchart.

2.3.1.2 Baseline Characteristics and reasons for admission

Mean age was 53.4 years (standard deviation (SD) 11.63) and 66% of patients were male. Patients were recruited on average 1.8 days after admission to hospital and only 2/77 patients were recruited in the Intensive Care Unit (ICU).

Mean Model for End Stage Liver Disease (MELD) score was 20.9 (SD 6.6.2) (15 patients were excluded from this analysis due to missing data). A total of 21/79 patients had ACLF of any grade at recruitment. Using criteria based on those listed in table 2.6, 69/79 patients had none or one extra hepatic organ dysfunctions at baseline. Circulatory dysfunction at baseline occurred most commonly (table 2.7).

Characteristic	Mean (s.d.)
Age (years)	53.41 (11.63)
Serum albumin (g/L)	23.95 (3.51)
Days since admission	1.81 (0.88)
MELD	20.90 (6.62)
Creatinine	91.2 (78.2)
	n (%)
Male	52 (66)
Admitted to ICU	2 (3)
Prescribed antibiotics	41 (52)
Diagnosis of infection	27 (34)
Aetiology of cirrhosis*	n (%)
Alcohol	76 (96)
Hepatitis B	1 (1)
Hepatitis C	11 (14)
NAFLD	4 (5)
Other aetiologies	2 (3)
Organ dysfunction	n (%)
Renal (<i>Creatinine > 133μmol/L</i>)	8 (10)
Respiratory (<i>SpO₂/FiO₂ < 357</i>)	9 (11)
Circulatory (<i>MAP < 60</i>)	13 (16)
Cerebral (<i>HE Grade \geq3</i>)	3 (4)
ACLF Grade**	n (%)
Grade 0	58 (73)
Grade 1	11 (14)
Grade 2	6 (8)
Grade 3	4 (5)

Table 2.7. Baseline clinical characteristics and demographics of the analysis population.
*some patients have more than one liver cirrhosis aetiology. **according to EASL-CLIF criteria.

27/79 (34%) of patients already had a clinically suspected infection at recruitment to the study. More than this (41/79) were prescribed antibiotics at baseline, it is likely the additional prescriptions were for infection prophylaxis following variceal bleeding and SBP.

The most commonly listed aetiology for liver cirrhosis listed was alcohol (76/79 patients). With some patients having dual aetiology listed (most commonly alcohol with hepatitis C virus (HCV)). Other reported aetiologies were HCV (11/79), HBV (1/79), NAFLD (4/7) and unknown (1/79).

Research nurses reported 68/79 patients to have active alcohol misuse with a mean self-reported intake of 109.05 (SD 90.24) units of alcohol per week. 28/79 patients were being treated for alcohol withdrawal on recruitment to the trial. Most patients had more than one reason for admission, other common reasons listed were: Jaundice (48/79), GI Bleed (18/79), Hepatic Encephalopathy (21/79), Infection (17/79), alcoholic hepatitis (24/79) and renal failure (7/79).

Mean amounts of IV fluid given to all 79 patients prior to recruitment were: crystalloid 107mL (SD 281), 20% HAS 78mLs (SD 179), 4.5% HAS 11mLs (SD 62).

2.3.2. Change in serum albumin levels with treatment

Mean serum albumin level on day 1 of treatment (at recruitment) was 23.95g/L (SD 3.51). 13% of patients had a serum albumin <20g/L, 54% a serum albumin between 20 to 25g/L and 33% between 26 to 29g/L.

By day 3 of the trial period (2 days post intervention) the median serum albumin level was >30g/L (mean 30g/L, SD 4) and remained so from this point onwards (figure 2.3a). 68/79 patients (86%, 95% CI 76%-92%) achieved the primary endpoint of albumin ≥30g/L on at least 1/3 of days treated, more than half reached this by day 3 and more than 75% by day 7.

On average patients were treated for 10.3 days (SD 4.8, range 1-15 days) and were administered a total of 1042mL HAS (SD 677.8mL, range 0-3200mL). As expected mean levels required decreased as the trial proceeded (mean 155mLs on day 2 with 73 patients treated to 98mLs on day 6 with 57 patients treated). The amount of other IV fluids given, that were recorded, was low (maximum range in any one day recorded, to day 10, was 500mLs).

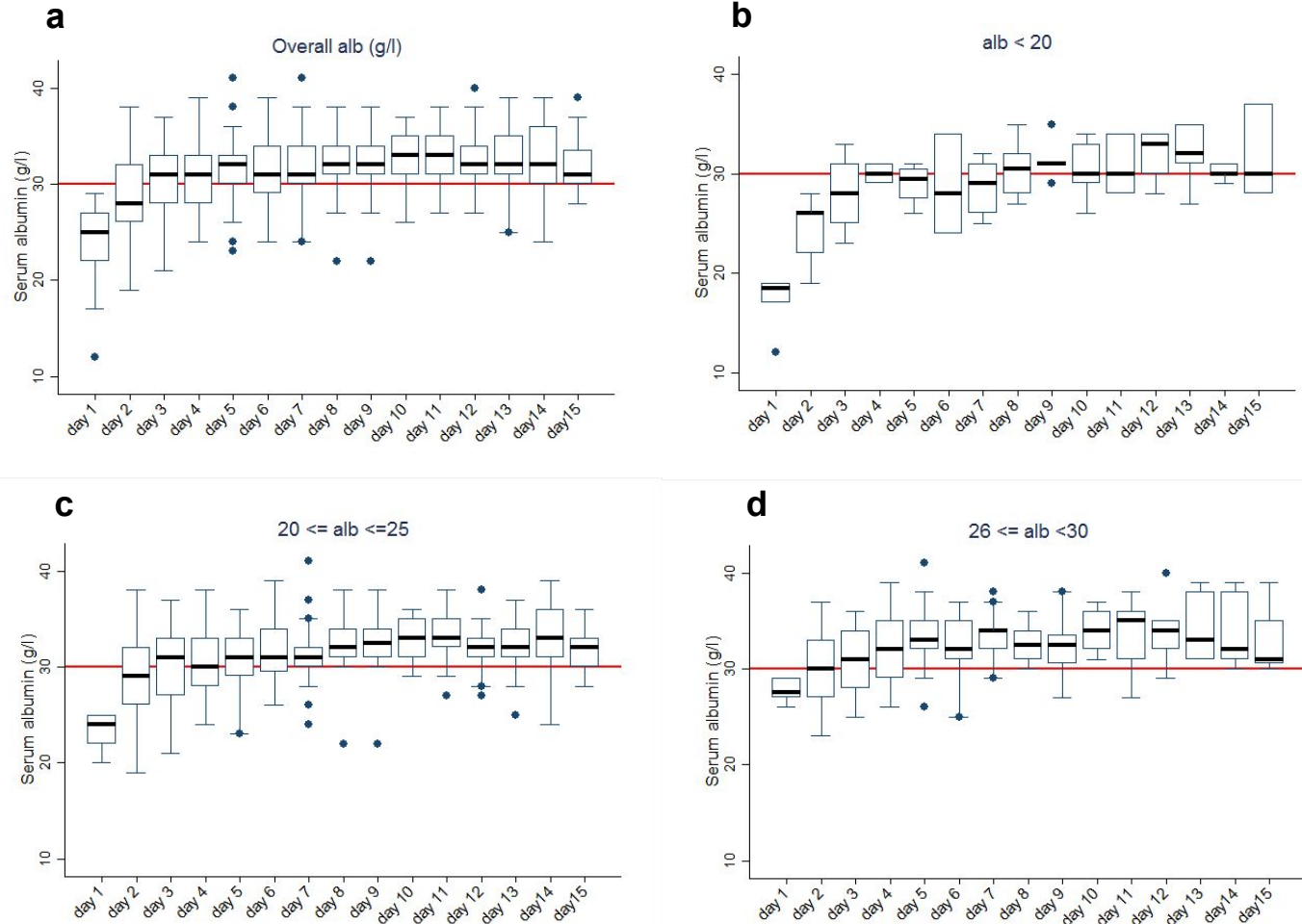


Figure 2.3. (a) Median serum albumin levels throughout the study period. (b–d) Data are expressed according to baseline serum albumin (alb) level. Day 1 was defined as the time of recruitment (pretreatment). The horizontal line in the boxes indicates the median, the top and bottom of the box indicate the interquartile range; dots represent individual outliers, defined as data points greater than 1.5 times the interquartile range from the median

The regimen was effective across all serum albumin subgroups, with the highest success in the 26 to 29 g/L group (Figure 2.3d, 96% success; 95% CI, 80%–100%) compared with less than 20 g/L (Figure 2.3b, 50% success; 95% CI, 19%–81%).

2.3.3. Protocol compliance

Clinicians were given the prescription protocol (table 2.4) with the option to amend as long as 20% HAS was prescribed if serum albumin was <35/L, unless there was a safety concern for example if the patient was felt to be at risk of fluid overload. Reasons for non-prescription in this situation were requested in free text on the CRF. 64% percent of administrations were in accordance with the suggested protocol, with 88% within +/-100 mL of the suggested dose.

On 161 of 657 occasions, 20% HAS was either not prescribed or prescribed but not administered despite a serum albumin level less than 35 g/L, suggesting an adherence rate of 75% (table 2.8).

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
not prescribed nor administered	1	5	10	10	11	7	9	4	9	10	13	11	7	7	114
Prescribed, not administered	10	6	8	2	1	2	2	3	2	4	2	1	3	1	47
Administered but not prescribed	2	2	1	1	3	0	0	0	0	2	0	2	1	0	14
Total	13	13	19	13	15	9	11	7	11	16	15	14	11	8	175
N (alb <35)	79	73	64	58	56	48	48	44	40	32	29	34	28	24	657

Table 2.8. Number of occasions albumin neither prescribed or administered when albumin <35 (g/l)

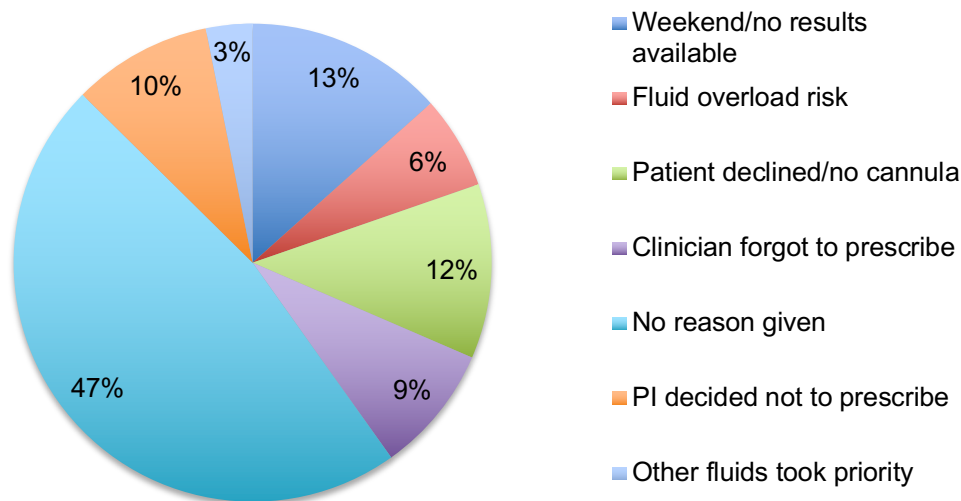


Figure 2.4. Free text reasons for non-prescription of 20% HAS when serum albumin was <35g/L

Reasons for non-administration in this circumstance are detailed in figure 2.4. The most common cause of non-administration given was that there were no results available to guide prescription, in this case the clinician should have prescribed according to the previous days results.

2.3.4. Incidence of Infection

Between days 3 and 15 of the trial treatment period 21/79 (27%) of patients developed a new infection, this was defined by a new or change in antibiotics. 12/21 of these patients had an 'infection CRF' completed in this time period with a further 23 infection CRFs submitted before day 3 (mixture of treatment days, most day 1 therefore reflective of the baseline infection). Using the pre-defined codes, pneumonia and spontaneous bacteraemia were the most common types of infection (table 2.9). 11 patients had culture sensitivities reported, 6 of these were resistant organisms. Patients who had been prescribed antibiotics on admission had increased subsequent nosocomial infection rates compared with those who were not prescribed antibiotics on admission (24% vs 8%, respectively).

Classified Infection	Number of times confirmed ^b	Antibiotic sensitivity		
		Resistant	Sensitive ^a	Unknown
Spontaneous bacterial peritonitis	4	0	1	3
Pneumonia	6	0	1	5
Cellulitis	4	0	1	1
Bacterial enterocolitis	1	1*	0	0
Fungal infection	1	0	0	1
Spontaneous bacteraemia	7	2**	2	3
Other infection	8	3***	0	5
Urinary tract infection	0	n/a		
Other intra-abdominal infection	0			
C.Difficile	0			
TOTAL	31	6	5	18

Table 2.9. Details from infection data matched to 35/62 antibiotic prescriptions.

*VRE ** klebsiella oxytoca ***MRSA

^aIncluded: Enterobacter cloacae, Stapholococcus Aureus and e.coli

^bNote some patients had multiple infections. (4/35 cases did not meet criteria for an infection diagnosis).

4 patients diagnosed with infection between day 3-15 had an infection CRF which did not contain adequate evidence to support an infection diagnosis. Reviewing longer term outcomes for these patients (figure 2.5) the patients who did not have evidence to support an infection diagnosis had better outcomes in terms of mortality and subsequent organ failure.

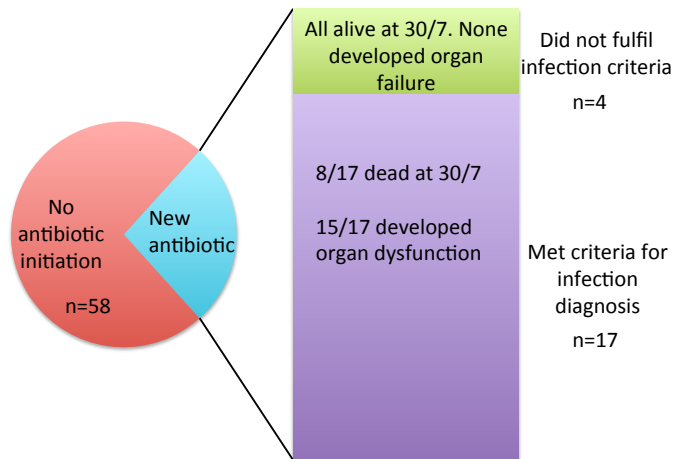


Figure 2.5. Patients who were diagnosed with a new infection from day 3 to day 15 of the trial treatment period as marked by a new or change in antibiotics.

4 patients did not fulfill required criteria for a new diagnosis of infection and subsequently had better clinical outcomes.

Patients were more likely to develop a new infection after 48hours of IV HAS treatment (day 3 onwards) if they had a baseline infection diagnosed or had ACLF or renal failure alone (table 2.10).

	New infection after day 3 (n=21) Mean (s.d)	No Infection (n=58) Mean (s.d)
Age	54 (13.46)	53 (10.9)
MELD	22 (6.0)	20 (6.83)
ACLF	32% (1:14%, 2: 9%, 3: 9%)	23% (1:14%, 2: 5%, 3: 4%)
Albumin (g/L)	22.8 (4.05)	24.39 (3.21)
Bilirubin (µmol/L)	137.2 (113.9)	165.6 (156.2)
Creatinine (µmol/L)	136 (122.42)	74.07 (43.2)
Sodium (mmol/L)	133.3 (5.6)	135.2 (5.4)
Infection at baseline	59.1%	24.6%
Antibiotics prescribed	68.2%	47.4%
CRP (mg/L)	76.8 (61.9)	28.24 (22.9)
WCC (x10⁹/L)	12.7 (8.7)	8.6 (5.0)
Temperature (°C)	37.0 (1.1)	36.7 (0.6)

Table 2.10. Baseline characteristics divided into patients who went onto develop a new infection after day 3 of recruitment versus those that did not.

2.3.5. Incidence of organ dysfunction and death during the trial treatment period

Respiratory dysfunction was the most commonly occurring organ failure (19/79 patients, 24%, on days 3-15) (table 2.11) closely followed by circulatory dysfunction. A total of 8 patients died within the study treatment period, 3 of these within the first 48 hours.

Endpoint	Numbers of patients (Days 3-15 of trial treatment)
Extra Hepatic organ dysfunction	
Renal	7/79 (9%)
Respiratory	19/79 (24%)
Circulatory	15/79 (19%)
Cerebral	1/79 (1%)
Death	5/79 (6%)

Table 2.11. Number of patients developing organ dysfunction or dying from day 3 to 15 during the trial treatment period. Some patients developed more than 1 organ failure.

Five patients (6%) died during day 3 to 15 of the treatment period, and 14 patients died within 30 days of study entry (18%). No patient underwent liver transplantation within 30 days of study entry. Of the 5 patients that died during days 3-15 of the trial, 2 had grade 2-3 ACLF at baseline with MELD scores between 30-37. 2 patients with ACLF died on

day 2 of the trial. 12 of the 21 patients with baseline ACLF (any grade) reached a new organ failure or infection endpoint from day 3-15 of the trial.

Rates of respiratory and circulatory dysfunction were higher than expected and hepatic encephalopathy (termed cerebral dysfunction) was lower than expected, therefore this was explored further.

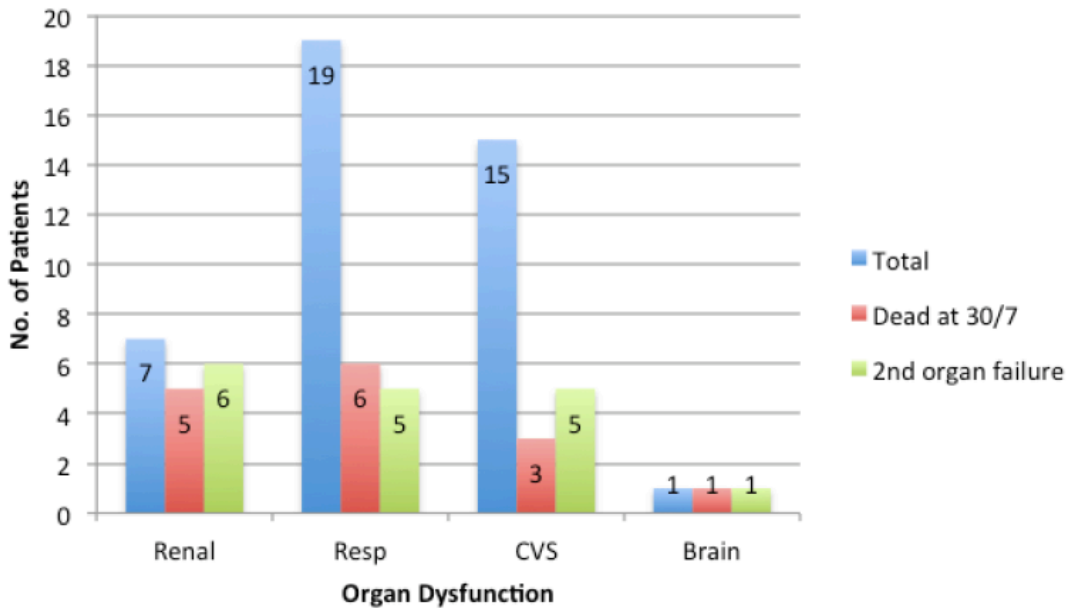


Figure 2.6. Number of patients (out of 79 recruited) developing organ failures as defined during the 15 day trial period, those patients that died 30 days post recruitment and those that developed a 2nd organ failure.

The majority of patients that only triggered a respiratory or cardiovascular endpoint had a good outcome with several discharged within a few days (figure 2.6). This is counterintuitive as organ dysfunction is a key predictor of poor prognosis. It is possible that assessment was subject to technical difficulties such as standard size blood pressure cuffs used in sarcopaenic patients or SaO₂ /FiO₂ recording of respiratory dysfunction greatly influenced by amount of oxygen administered. Only one patient developed hepatic encephalopathy (>grade 3) suggesting under reporting and therefore objective assessment being challenging. These factors suggest that the above endpoints may not be reliable in multi-centre trials. However renal dysfunction uses an objective measurement, creatinine and patients developing this had poor prognosis, as expected supporting that this measure can be reliably used as an endpoint.

2.3.6. Contribution of infection, organ failure and death to the planned primary composite endpoint for an RCT

Thirty-eight of 79 patients (48%) reached the planned composite end point during the treatment period. The breakdown of components that triggered the composite end point are summarised in table 2.12. First events that triggered the endpoint component were divided as follows:

- 13 patients triggered the infection component first
- 3 patients triggered the renal component first
- 12 patients triggered the respiratory component first
- 9 patients triggered the circulatory component first
- 0 patients triggered the brain dysfunction component first
- 1 patient died without triggering any other prior organ dysfunctions first

As previously discussed in section 2.3.6 many patients who developed circulatory and respiratory dysfunction had good outcomes in terms of low subsequent organ failure and mortality, where as most that developed infection and renal dysfunction had poor outcomes and a longer length of hospital stay.

First component recorded	Day	Subsequent or concurrent component	Day	Subsequent or concurrent component	Day	# days in hospital	# days from comp out to discharge	Alive at 30 days
<i>Respiratory</i>	13					13	0	Yes
<i>Respiratory</i>	7					23	16	Yes
<i>Respiratory</i>	15					16	1	Yes
<i>Respiratory</i>	8					23	15	No
<i>Respiratory</i>	3					12	9	Yes
<i>Respiratory</i>	9					17	8	Yes
<i>Respiratory</i>	4					6	2	Yes
<i>Respiratory</i>	3	Infection	9			15	12	Yes
<i>Respiratory</i>	3	Infection	5			28	25	Yes
<i>Respiratory</i>	3	Infection	6			17	14	Yes
<i>Respiratory</i>	3	Death	3			3	0	No
<i>Respiratory</i>	3	Infection	5	Circulation	9	23	20	Yes
<i>Circulatory</i>	6					15	9	Yes
<i>Circulatory</i>	9					21	12	Yes
<i>Circulatory</i>	3					3	0	Yes
<i>Circulatory</i>	3					6	3	Yes
<i>Circulatory</i>	3					15	12	Yes
<i>Circulatory</i>	9					14	5	Yes
<i>Circulatory</i>	4	Infection	8			32	28	Yes
<i>Circulatory</i>	11	Infection	15			27	16	Yes
<i>Circulatory</i>	10	Renal	12			14	8	Yes
<i>Renal</i>	3					11	8	Yes
<i>Renal</i>	3	Infection	4	Death	5	5	2	No
<i>Renal</i>	5	Inf+Resp+ Circ.	8	Cerebral	9	24	19	No
<i>Infection</i>	3					31	28	No
<i>Infection</i>	11					18	7	Yes
<i>Infection</i>	8					13	5	Yes
<i>Infection</i>	3					22	19	Yes
<i>Infection</i>	13					43	30	Yes
<i>Infection</i>	3					88	85	Yes
<i>Infection</i>	13	Respiratory	13			101	88	Yes
<i>Infection</i>	3	Respiratory	3			23	20	No
<i>Infection</i>	3	Circulatory	10			14	11	Yes
<i>Infection</i>	7	Renal	14	Death	15	15	8	No
<i>Infection</i>	3	Circulatory	5	Respiratory	6	34	31	Yes
<i>Infection</i>	3	Resp. + Circ.	3	Renal	4	13	10	No
<i>Infection</i>	3	Resp. + Circ.	3	Renal, Death	5, 10	10	7	No
<i>Death</i>	6					6	1	No

Table 2.12. Outcomes for Individual Patients Who Triggered the Planned Composite End Point for an RCT

2.3.7. Safety

As IV 20% HAS is commonly used in clinical practice adverse events (AEs) were not reported centrally in this trial and only recorded at site. Serious Adverse Events (SAEs) as defined by a clinical deterioration that occurs that: prolongs hospital stay, is life threatening or causes death were reported in the trial treatment period (up to day 15 post recruitment).

SAE Description	Number of events
New ascites	1
Renal impairment	1
Variceal Bleeding (death)	3
Variceal Bleeding	1
Pneumonia (death)	1
Death (decompensated cirrhosis)	4
Bronchogenic carcinoma & pleural effusion (death)	1
<i>Total deaths in trial treatment period</i>	<i>8 (10%)</i>

Table 2.13. Details of Reported Serious Adverse Events throughout trial treatment period (days 1 to 15)

Table 2.13 lists all reported SAEs. As there was not a control arm it is difficult to definitively conclude that IV 20% HAS as used in this study did not have increased serious adverse event rates. However reported rates were low in comparison to those reported in other studies involving patients with similar characteristics. **No SAEs were deemed to be related to the trial treatment (albumin infusion) by the site investigators or by an independent data monitoring committee.** SAEs were only required to be reported within the study treatment period. There was no relationship between SAEs and larger volumes of HAS being prescribed.

2.4 SUMMARY

- Daily infusions of 20% HAS given to patients with AD according to a suggested infusion protocol are effective at restoring serum albumin levels to near normal (>30g/L) within 3 days
 - This appears to be a safe intervention
 - It is a feasible intervention across multiple NHS hospital sites
 - On average patients were treated for 10.3 days and were administered a total of 1042mL HAS
- Deviation of HAS administration from the suggested protocol was common. However, a serum albumin of >30g/L was achieved in 86% of patients for at least 1/3 of the trial treatment period
 - The main reasons for deviations were non prescription or administration for logistical reasons and safety concerns.
- Alcohol abuse, as an aetiology of liver cirrhosis, has a higher incidence in this UK cohort of AD patients than in other studies in liver cirrhosis around the world.
- My data demonstrates that measures of new respiratory, circulatory and brain dysfunction used in the study are likely to be subject to recording bias and therefore unreliable for use in a larger, randomized study as part of a primary composite endpoint
- New infection, as marked by a new antibiotic prescription, was not a robust endpoint with overall high rates of antibiotic prescription.

2.5. CONCLUSIONS

2.5.1. Daily 20% HAS according to an infusion protocol targeting serum albumin levels is effective at increasing and maintaining AD patient levels above 30g/L.

At baseline there was a spread of patient albumin levels with a good proportion of AD patients in each subgroup of albumin level (<20, 20-25, 26-29g/L). Despite there being a higher number of patients than was expected recruited with serum albumin levels <20g/L the defined primary endpoint was still achieved. This success was demonstrated at 10 busy UK NHS hospitals and patients were recruited quickly in less than 6 months suggesting the protocol is easy to use in these settings.

The inclusion criteria were broad and straightforward and selected patients with almost exclusively alcohol-induced liver disease, a substantial spread of albumin values, and an ACLF score of 1 to 3 in 25% of cases. It is proposed that the HAS intervention would be more successful in preventing infection in ward-based patients rather than in patients with established multi-organ failure, and these criteria appeared to capture this population. Although ward-based, these patients were unwell, as expected with inpatient AD, with a mean MELD score of just over 20.

In the UK alcohol is the most common cause of liver cirrhosis and this was reflected in the patients recruited to this study. This is in contrast to other European and North American studies that report a higher prevalence of viral hepatitis as a cause of liver cirrhosis⁹⁴. Many recruited patients were reported to be actively drinking alcohol on admission to hospital. Alcohol is an independent mediator of a suppressed immune response¹⁰⁴ and therefore this should be taken into consideration when interpreting future findings.

There were a significant number of deviations from the suggested targeted infusion protocol, although the primary endpoint was achieved. Flexibility was given to treating site doctors for reasons previously discussed. On some occasions HAS was prescribed but not given by ward nurses or an advanced 'weekend prescription' was not arranged. These reasons reflect real life practice and therefore make any future clinical findings more applicable outside of a clinical trial setting. There were not a high number of non-prescriptions due to safety concerns which strongly supports that the protocol and targeted approach were acceptable at these 10 sites. Asking clinicians to target a normal serum albumin (>35g/L) which was higher than our desired endpoint (>30g/L, previously

identified cut off) was likely to have contributed to the success of the protocol. The ALBIOS trial²⁰ failed to reach the desired albumin endpoint and did not use this approach.

The average amount of 20% HAS infused during the trial treatment period was just over 1L for a 10 day average treatment period. Depending on local site agreements, 100mL 20% HAS costs between £23-40 in the UK. Therefore the cost of this intervention would be £230 - £400.

Finally there were no serious adverse events or deaths which were deemed to be related to the HAS infusions as judged by an independent committee. Adverse event rates appeared to be lower than in other studies with similar patient groups^{72,87,93}. Four variceal bleeds were reported, which potentially can be precipitated by increased portal pressure after albumin. Although not reported in previous albumin trials, this remains a concern. However, a 5% incidence during treatment is similar to expected rates. The study was single arm with a relatively small number of patients therefore no absolute conclusions can be made with regards to safety but there did not appear to be a signal that HAS infusions, used in this way, were unsafe. Ultimately the only way to judge this is in a larger, randomised control trial.

2.5.2. New infection, as marked by a new antibiotic prescription, was not a robust endpoint with overall high rates of antibiotic prescription

A robust diagnosis of infection in AD/ACLF is challenging due to high rates of culture-negative sepsis¹⁰⁵. The on-site clinician-reported infection rate at admission was 34%, which is in line with other studies; however, antibiotics were prescribed in substantially more patients (52%). This perhaps reflects a tendency to overprescribe, as reported elsewhere⁹¹ and therefore using a new/changed antibiotic prescription as a surrogate for infection diagnosis, as originally intended, appeared subject to potential bias and difficult to standardise across multiple sites.

21/79 (26.5%) of patients were diagnosed with a new infection, according to this definition, after they had had at least 48hours of HAS treatment (from day 3 of the trial onwards). However 4 (19%) of these patients did not have enough evidence to support a diagnosis of infection (as defined in table 2.5). These 4 patients went on to have no subsequent organ failures and were all alive at 30 days. This is in contrast to the other 17 patients who nearly all had subsequent organ failure and high rates of mortality, this

would be expected with true infected and has been observed in other large cohort studies⁹⁷. Collected data indicated that patients were more likely to develop a new infection after day 3 of trial inclusion if they had already been diagnosed with infection at baseline recruitment and subsequently had poor outcomes, again in line with existing larger studies⁸⁹. However due to missing data (infection CRFs) I was unable to pair all second infections with baseline to ensure this was a new infection and not a change of antibiotics for an existing infection due to lack of response or antimicrobial sensitivities. This is a second problem encountered with using 'change in antibiotics' as a surrogate for new infection.

2.5.3. Measures of organ dysfunction using clinical ward observations are likely to be unreliable for primary endpoint use in a larger study

Respiratory and circulatory dysfunction

Rates of respiratory and circulatory dysfunction, recorded after at least 48 hours of HAS treatment, were higher than expected in this single arm study (24% and 19% respectively). 75% of patients with hitting the respiratory dysfunction endpoint and 66% of patients hitting the circulatory dysfunction endpoint did not go onto develop a second organ failure. In fact many of them were discharged from hospital within days of reaching the definition required to mark the endpoint making the definitions clinically irrelevant for an interventional study.

Many large studies in unwell liver cirrhosis patients define respiratory failure as a requirement for mechanical ventilation⁹⁷ and circulatory failure as when a patient requires inotropes to support blood pressure. In an attempt to define a respiratory endpoint that would be more meaningful in a study that is aiming to prevent patients with a new infection deteriorating and requiring ICU level care, we used measures which could be defined in a ward based setting. Respiratory failure used a ratio of oxygen saturations (finger probe measurement, lowest in a 24 hour period) divided by maximal inspired oxygen (matched to the oxygen saturation reading) on a point increment score (table 2.6). Research nurses were asked to review vital signs charts for the previous 24hours to record this information. However it is not uncommon in clinical practice that a patient may have had a single inaccurate low oxygen saturation reading (e.g. with cool peripheries) or have been administered more inspired oxygen than was necessary – falsely giving the appearance of a raise SpO₂/FiO₂ ratio. Similarly circulatory dysfunction

was recorded as a MAP <60mmHg using lowest blood pressure measurements in a 24hour period to record this. Cirrhosis patients are often sarcopaenic and it is not uncommon for incorrect blood pressure cuffs to be used to record blood pressure giving a falsely low reading.

Therefore although these outcomes may be more meaningful in terms of detecting the tipping point for deterioration and provide a higher event rate, which is of use when powering a clinical study, they are subject to bias and not reliable enough to contribute towards a primary endpoint in a larger randomised study.

Brain dysfunction (Hepatic Encephalopathy)

Hepatic encephalopathy (HE) is difficult to diagnose in its subclinical form¹⁰⁶. For the purposes of this investigation development of overt encephalopathy (grade 2, confusion) on the ward would be highly relevant clinically and prognostically, especially as it has been suggested in other studies that albumin may prevent this¹⁰⁰. However our pragmatic study relied on the use of NIHR funded clinical research nurses who had one hour a day to record clinical changes in the previous 24-hour period. Multiple studies in HE have demonstrated that, in the absence of a clinical expert, only grade 3 HE (onset of drowsiness) can be diagnosed reliably^{107,108} therefore this was used for this study as we did not have a clinical expert at sites. However only 1 out of 79 patients were diagnosed with grade 3 HE from day 3-15 of the inpatient treatment period. This is a very low rate in comparison to other reports of unwell cirrhotic inpatients^{93,97,109} and therefore it is quite possible that HE was under-diagnosed in this study. It may be that nurses (and clinical teams) thought patients were drowsy secondary to other reasons e.g. sepsis or medications or just did not understand the clinical sign to be recorded despite education at the outset of the trial.

Renal dysfunction

Renal dysfunction was defined according to a set increase in serum creatinine (table 2.6) therefore a 'hard' marker which could be extracted from patients' daily blood tests by research nurses. Rates from day 3-15 of the treatment period were slightly lower than expected (9%) however this was a single arm study and all patients were treated with HAS which is known to benefit renal function in many situations in acute decompensated patients^{54,71,110}. Therefore this is the only extrahepatic organ function assessed which was deemed to be reliably recorded in this feasibility study.

2.5.4. A primary composite endpoint for an interventional study comparing HAS to standard of care should only include infection, renal dysfunction and death as the components

A future RCT primary composite end point was proposed as infection, extra hepatic organ dysfunction (CVS/brain/respiratory/renal) and death because infection commonly triggers organ dysfunction and the combination substantially increases mortality. However, the feasibility of recording such data in a ward-based trial of AD/ACLF at multiple sites is unproven. Other than renal failure, there is also no universally accepted definition for early (reversible) organ dysfunction/failures in patients with cirrhosis. As discussed in section 2.4.3, I believe the data cast significant doubt over whether these dysfunctions can be recorded accurately in largely ward-based patients across multiple sites and therefore precludes use as part of an RCT primary composite end point, although these can still be reported as secondary outcomes. Table 2.14 shows the incidence of a revised primary composite end point of infection, renal dysfunction, and mortality and the impact on total event rate. As this data come from a single arm study where all patients are treated with HAS the event rate may be higher in the control arm if HAS is an effective treatment.

N=79 patients	Existing composite endpoint	Excluding respiratory, circulatory and brain dysfunction from the composite endpoint
	<i>Days 3-15</i> <i>n (%)</i>	<i>Days 3-15</i> <i>n (%)</i>
Composite endpoint	38 (48%)	25 (32%)
Infection	13	19
Renal	3	4
Respiratory	12	
Circulatory	9	
Brain	0	
Death	1	2

Table 2.14. Incidence of proposed composite endpoint and contributing components; with and without respiratory/circulatory dysfunctions from days 3-15

CHAPTER 3: THE VALIDATION OF AN EX VIVO FUNCTIONAL ASSAY TO ASSESS THE IMPACT OF ALBUMIN TREATMENT ON PROSTAGLANDIN E2 MEDIATED IMMUNE DYSFUNCTION

Publications in relation to this chapter

Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease.

China L*, Maini A, Skene SS, Shabir Z, Sylvestre Y, Colas RA, Ly L, Becares Salles N, Belloti V, Dalli J, Gilroy DW, O'Brien A. *Clin Gastroenterol Hepatol.* 2018 May;16(5):738-747.

International presentations in relation to this chapter

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution.

China L*, Skene S, Maini A, Shabir Z, Forrest E, O'Beirne J, Portal J, Ryder SD, Wright G, Gilroy D, O'Brien. *AASLD 2016, Boston. Poster presentation*

Plasma Lipid Mediator (LM) Profiling Identifies Hyper- and Hypo-activated Groups of Patients with ACLF and Targeted 20% Human Albumin Solution Infusion Recalibrates Abnormalities

China L*, Maini A, Colas R, Ly L, Dalli J, Gilroy D, O'Brien A *EASL 2017 (Amsterdam) JOURNAL OF HEPATOLOGY. ELSEVIER SCIENCE BV. 66: S390. Oral ePoster presentation.*

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution.

China L*, Skene S, Maini A, Shabir Z, Forrest E, O'Beirne J, Portal J, Ryder SD, Wright G, Gilroy D, O'Brien. *AASLD & EASL Masterclass, Florida. Poster presentation.*

Contributions by other people to this chapter:

- Technical work:
 - Plasma cytokine levels and endotoxin assay: Alex Maini (PhD student)
 - Plasma lipid measurements: R. Colas (Lab technician, J. Dali Laboratory, QMUL)
 - MDM samples analysis at day 10 (fig 3.10A): N.Becares (Post doc)

3.1 INTRODUCTION

Immune function is an extremely complex process for which there is no simple test or assay. During inflammation, monocytes move quickly to sites of tissue infection and differentiate into macrophages to elicit an immune response. Numerous studies have demonstrated the role of monocyte deactivation in cirrhosis associated immune suppression²⁷⁻²⁹. However in large clinical trials it is impractical to perform blinded, standardised, biological assays using fresh monocytes from multiple hospital sites throughout the UK. As it has been suggested that circulating plasma mediators, including PGE₂, are responsible for monocyte and neutrophil dysfunction^{31,111}, I aimed to validate and refine an assay in which frozen stored plasma from acutely decompensated cirrhosis (AD) patients was added to monocyte derived macrophages (MDMs) from healthy donors¹¹. This was in order to permit testing of patient samples from multiple sites at the same time in a blinded, controlled fashion.

I selected MDM production of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF α) as the immune-readout as this has been validated as a biomarker of monocyte function in critical illness (see chapter 1). Reduced capacity to produce TNF α is associated with adverse outcomes following sepsis^{112,113}.

Previous work by O'Brien and colleagues demonstrated a potential role for PGE₂ as a humoral mediator of MDM dysfunction in acutely decompensated liver cirrhosis patients and that targeted albumin therapy may reverse this effect¹¹. In their study, healthy volunteer donated blood was used to isolate and culture MDMs. These cells were then stimulated, in the presence of patient plasma, with 1ng/mL LPS to simulate a bacterial infection and TNF α production from cells measured in their supernatant. TNF α production was reduced by AD patient plasma and this was reversed by PGE₂ receptor blockade. A similar reversal was seen with albumin treatment either added to cell culture or intravenously to 6 patients (when compared to sample taken pre albumin treatment if serum levels of albumin had risen to >30g/L) (see figure 3). The PGE₂ dose response, as shown in figure 3.1, has not previously been investigated. In addition the study did not link the assay outcomes to clinical patient outcomes, as this study was not designed to do so and there were very small patient numbers (n=6) at one hospital site. Analysis was not blinded.

PGE₂ and other lipid measurements in plasma are expensive and time consuming. The gold standard measurement is via liquid chromatography tandem mass spectrometry. The process involves stripping the lipid of anything it is bound to hence the produced measurements are a total of lipid that may have previously been bound or unbound to albumin (or other proteins). Although absolute concentrations are useful; a bioassay has the added benefit of evaluating the impact of 'bioavailable' PGE₂ plus any other circulating plasma mediators which may have an impact on the function of MDMs.

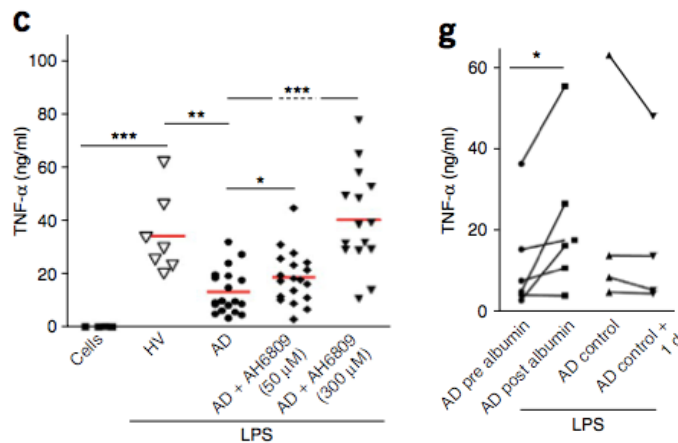


Figure 3.1. Figure 1c and 4g taken from O'Brien, et al. ¹¹
(c) LPS stimulated TNF α production from MDMs in the presence of Healthy (HV) and AD plasma with or without the addition of AH6809 (EP1-3r antagonist). **There was a significant decrease in TNF α with AD plasma which was reversed with AH6809 suggesting a PGE₂ dependant mechanism** (g) TNF α was increased when AD patients were treated with IV 20% HAS (n=6) and not in a control group of patients (n=4)

Work from our collaborators (R De Maeyer, Gilroy Group, UCL) has suggested that the additional step of negative selection using RosetteSep™ of monocytes prior to cell separation with a density medium increases the % of monocytes retrieved to 85%, suggesting not only a higher yield of the desired monocytes but less contamination with other cell types (e.g. lymphocytes). Therefore I used this additional step in the MDM isolation and culture protocol (see figure 3.2) which required validation for examination of LPS stimulated TNF α production in the presence of AD plasma.

A limitation of using MDMs cultured from healthy volunteers is that 100mLs of donated blood yields approximately 6×10^6 monocytes. Allowing for technical repeats this allows on average 25 plasma samples to be assessed per blood donation. Therefore if a larger number of samples are to be assessed either more than one healthy volunteer donor is

required or assessment needs to occur at different time points with the same donor's cells.

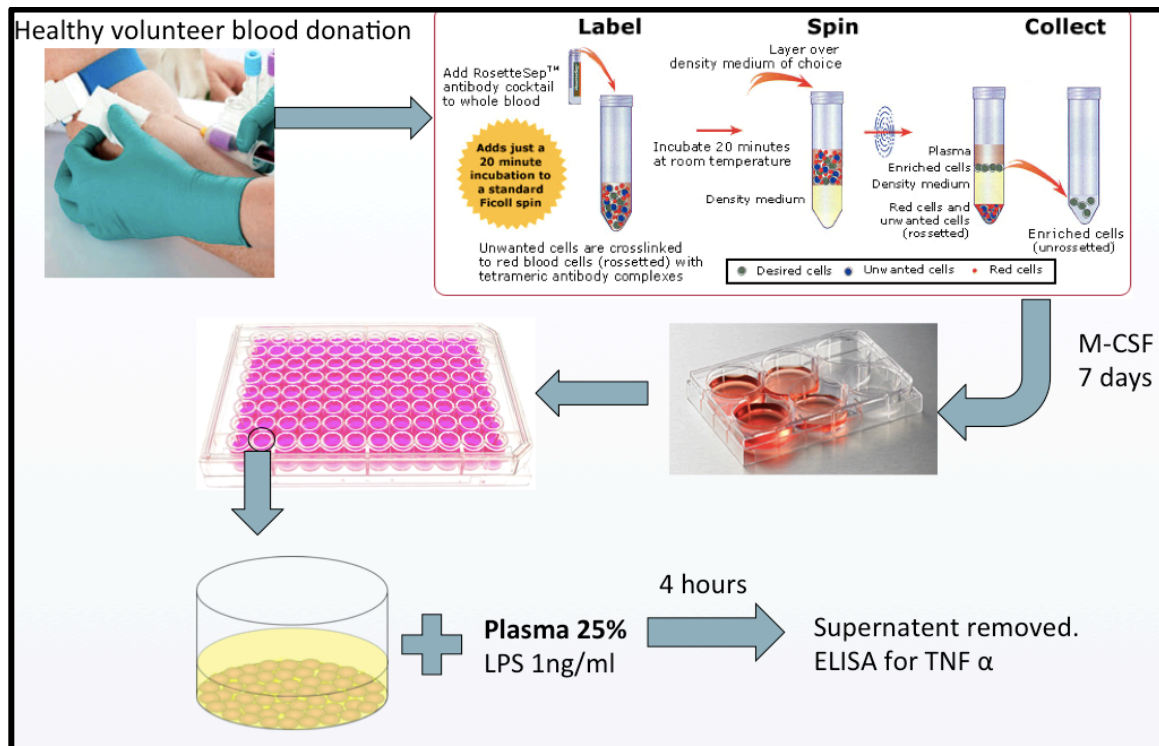


Figure 3.2. Pictorial overview of the method isolating monocytes and differentiating into macrophages from healthy volunteers.

Cells are then plated and +/- plasma stimulated with LPS for 4 hours prior to supernatants being removed.

An alternative is a monocyte cell line. MonoMac-6 (MM6) are a human cell line established from the peripheral blood of a 64-year-old man with relapsed acute monocytic leukemia (AML FAB M5) following myeloid metaplasia¹¹⁴. Morphologically these are single, round/multiformed cells or small clusters of cells in suspension that are occasionally loosely adherent. CD14 expression is highly dependent on cultivation conditions¹¹⁵. This monocyte cell line can be differentiated into MDMs using Vitamin D3. Using a cell line in the 'LPS-stimulated TNF α assay' would enable a large number of samples to be processed simultaneously, removing the potential for inter and intra donor variability. However this cell line will inevitably have differences to non-mitotic monocytes and results may not therefore correlate with in vivo mechanisms of MDM dysfunction, in particular in relation to PGE₂. A further complication is that previous work using this cell line has shown LPS-induced TNF α production falls by more than 50% in the presence of healthy volunteer plasma (unpublished work, thesis by J. Fullerton 2015). This is in contrast to the MDM assay and may affect interpretation of results.

Finally, when analysing plasma collected at multiple sites there may be differences in sample collection and processing that could affect the described assay. Engstad, et al.¹¹⁶ reported a suppression in LPS stimulated TNF α production in whole blood when blood had been taken using Ethylenediaminetetraacetic acid (EDTA) versus heparin as an anticoagulant. In addition if blood is left for some time after being collected and before plasma storage this could increase the breakdown of circulating plasma mediators of immune function or, if bacteraemia was present, could increase levels of endotoxin within the sample which could impact on the assay.

Chapter aims

This chapter aims to validate an approach to clinical trial plasma sample analysis, in relation to albumin treatment and PGE₂, using a new protocol to culture healthy volunteer monocyte derived macrophages in three stages:

1. Assessment of assay variability:
 - a. Examine variability in MDM TNF α production in response to LPS between:
 - i. Different healthy volunteer blood donors
 - ii. The same donor over time
 - b. Determine whether MDM/MM6 TNF α production is reduced by AD patient plasma compared to healthy volunteers
 - c. Explore how variations in sampling at peripheral clinical sites may affect MDM/MM6 production of TNF α in response to LPS
2. Characterise the PGE₂ impact on the assay
3. Trial the assays in a multi centre clinical study:
 - a. Comparing AD patient plasma pre treatment (serum albumin <30g/L) and post treatment (serum albumin >30g/L) with IV 20% HAS
 - b. Relate these findings to plasma cytokine and PGE₂ measures, endotoxin, patient clinical characteristics and outcomes
 - c. Explore the impact of infection development on the assay

3.2 METHODS

3.2.1 Peripheral Blood Collection

Whole blood was obtained from the median cubital vein of healthy volunteers using a 20g butterfly needle and aseptic non-touch technique. EDTA BD Vacutainer tubes (2 mM final, *Becton Dickinson*, UK) were used for blood collection for monocyte isolation (continued in 3.2.1).

For plasma collection, blood was collected in either EDTA (2 mM) or Lithium Heparin (17 IU/mL) vacutainers (*Becton Dickinson*, UK). Tubes were inverted repeatedly and immediately centrifuged at 1300x *g*, 10 min at room temperature. Plasma was aliquoted and stored at -80 °C.

3.2.1.2 Patients Samples and blinding

Patient samples initially used to validate the assay (section 3.3.1 and 3.3.2) were acquired through ongoing local research from UCLH NHS Trust and the Royal London Hospitals NHS Trust (Monocyte and Macrophage Phenotype and Function in Liver Failure; Harrow NHS Research Ethics Committee no. 12/LO/0167). Sequential patients admitted to hospital with acute decompensation of liver cirrhosis were recruited. After consent blood was taken once which was at varying time points from the patient's admission in EDTA or Lithium Heparin vacutainer tubes.

Samples analysed for section 3.3.4. were obtained via the ATTIRE feasibility study¹⁰³. Consent and timelines are explained in more detail in section 2.2. Patient's blood samples were taken using 9mL lithium heparin tubes prior to treatment with albumin and daily thereafter when usual standard of care blood was taken. These were then labeled with an individual 4 digit identifier (anonymous) for which a corresponding label was placed in the patient's clinical research file (CRF) for that day. Full lithium heparin tubes were transferred to site's hospital laboratories where samples were spun at 1300x *g* at 20°C. The plasma layer was removed and frozen at -80°C in 2mL cryovials with the corresponding 4 digit number. Samples were collected from 80 patients at 10 UK hospital sites. They were transferred to UCL (Rayne Building, O'Brien Lab) at the end of the recruitment period in December 2015. After CRF data entry of daily albumin levels at UCL Clinical Trials Unit (CTU) the trial statistician identified sample numbers corresponding to day 1 of treatment and the first day in which the patients' serum albumin level rose above 30g/L. A list of sample numbers was provided for analysis in

pairs (2 samples for each patient). It was not known by the analyser (myself) which sample from each pair was pre or post treatment. After analysis was complete all results were officially given to UCL CTU and I was unblinded enabling me to process the results by treatment group, albumin level and day of treatment.

3.2.2. *In vitro* differentiation of blood-borne monocytes into macrophages

Isolation of monocytes from donated whole blood

100mL of peripheral blood was collected as described in 3.2.1. In a laminar flow hood blood was separated into 4 x 50mL falcons (25mL per falcon) and 312.5µl of RosetteSep™ Human Monocyte Enrichment Cocktail (Stemcell, France) added to each falcon. RosetteSep™ is designed to isolate monocytes from whole blood by negative selection. Unwanted cells are targeted for removal with Tetrameric Antibody Complexes (TAC) recognizing CD2, CD3, CD8, CD19, CD56, CD66b, CD123 and glycoporphin A on red blood cells (RBCs). Blood was then left for 20 minutes at room temperature on an orbital shaker at slow speed.

1 volume blood was diluted with 2 volumes Hanks' Balanced Salt Solution (HBSS) (i.e. 25mLs HBSS was added to the 25mL of blood in each falcon). 35mLs mL of diluted blood was layered onto 15 mL Ficoll Paque in 50 mL Falcon tubes and spun at 1000x g, 30 min, 25 °C, brake off, low acceleration. RosetteSep™ causes the unwanted cells pellet along with the RBCs. The purified monocytes are present in a layer at the interface between the plasma and the Ficoll Paque. This layer was then removed from each of the 4 falcons and pooled into 2 x 50mL falcons. Falcons were then topped up to 50mL with PBS and spun at 300x g for 10mins at 10°C. The supernatant was discarded and pellets were re suspended in 1mL of ACK lysis buffer (lyses residual RBCs and removes residual antibody), pooled and left for 2-3 minutes. 30mLs of PBS was then added to the falcon which was centrifuged at 120x g at 20°C for 10 minutes to remove platelets. The supernatant was again removed and pellet was washed once more in 30mLs PBS and centrifuged at 120x g at 20°C for 10 minutes.

Finally, the pellet was re suspended in 1mL of media (ex vivo-15 (Lonza UK), 10% human serum (type AB male, Sigma), L-Glutamine 2mM and 1% Penicillin/Streptomycin (Gibco)) and passed through a 35micron strainer.

Culture of monocyte derived macrophages

After isolation monocytes (either from a cone or direct blood donor) were counted and then re-suspended at 4×10^6 cells/3mL media in a 6 well polystyrene plate (Corning®Costar®) and placed in an incubator at 37°C, 5% CO₂. After one hour media with any non-adherent cells was removed and replaced with fresh media which was then supplemented with 20ng/mL of macrophage colony-stimulating factor (M-CSF).

After 3 days media was changed and re supplemented with 20ng/mL M-CSF.

On day 6 media was aspirated and 1mL of lifting buffer (PBS plus 10mM EDTA and 4mg/mL lidocaine) at 10°C was added to each well and left for 20 minutes. Wells were then scrapped and suspended cells removed within the lifting buffer and placed in a 50mL falcon which was topped up to 50mLs with PBS and spun at 300x g at 20°C for 5 minutes. The supernatant was again removed and pellet washed in 30mLs PBS and centrifuged at 300x g at 20°C for 10 minutes. The pellet was then resuspended in 1mL of media and cells were counted and then plated in a 96 well tissue culture treated plate (Corning®Costar®) at 50,000 cells/well in 100µL of media containing 20ng/mL M-CSF. Plates incubated for 24 hours prior to experiments to allow cells to re-adhere.

This protocol has been shown to give a monocyte purity of >85% (day 1 of isolation) and monocytes cultured with M-CSF for 6 days expressed high levels of CD14 (expressed by macrophages).

3.2.3. MonoMac-6 (MM6) Cell Line

Mono Mac 6 (MM6) were obtained as a frozen culture from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany).

3.2.3.1 Culture Conditions

Mono Mac 6 cells were cultured under LPS free conditions in RPMI 1640 (Gibco) containing 10% FCS (invitrogen™), 200U/mL penicillin (Gibco), 200µg/mL streptomycin (Gibco), 2mM L-glutamine, 1mM sodium pyruvate (Gibco), 1mM oxaloacetic acid (Sigma), 1x MEM non-essential amino acids (Gibco) and 9 µg/mL human insulin (Sigma) in accordance with standard practice¹¹⁷. After addition of the supplements, the medium was ultra-filtered and stored at 4°C.

Following removal of MM6 from cryostorage, cells were cultured for 1 week in culture medium alone in 24 well plates (Orange Scientific, Belgium) at a density of 2×10^5 cells/mL

(2mL/well) and passaged every 48hours. Doubling time was initially 40-50hours, decreasing to 30-40hours, with cell viability increasing from 86-88% to $\geq 95\%$. MM6 were subsequently maintained in T75 flasks (25mL media), passaged every 48hrs with seeding at 2×10^5 cells/mL (5×10^6 /flask). Morphology was regularly monitored microscopically to evaluate for any apparent shift in phenotype (increased giant cells, increased multinucleated cells), clumping (reflecting potential LPS contamination) and/or clouding of the media (indicative of bacterial/fungal contamination). All tissue culture was carried out in sterile conditions. Experiments were carried out between passage 6 and 25. Significant deviation in cytokine production in control conditions from the established, expected range (1000-4000pg/mL TNF α in response to LPS 100ng/mL) led to discarding of the cells and re-instatement of the line from a frozen aliquot.

3.2.3.2. Differentiation of Mono Mac 6 Cells

MM6 may be further differentiated via incubation with various ligands to induce distinct cellular phenotypes and responses to stimuli^{115,118}. These reagents aim to transform the relatively immature MM6¹¹⁹ into cells with characteristics that resemble mature monocytes or macrophages¹²⁰.

In line with previous experience within our laboratory 1 α , 25 dihydroxycholecalciferol (VD3, dihydroxyvitamin D₃, calcitriol, Sigma, 10ng/mL)¹²⁰ was used to differentiate MM6 cells. MM6 were cultured with VD3 in T75 flasks (25mL) seeded at 2×10^5 for either 48 or 72 hours, scraped to ensure collection of newly adherent cells, washed, re-suspended in media alone to a density of 2×10^6 and plated in 96-well plates at 1×10^5 cells/well (50 μ L media) prior to stimulation.

3.2.4. LPS Stimulation

3.2.4.1. MDM Stimulation

Healthy volunteer MDMs were isolated and plated in 96-well plates as per 3.2.2 and incubated overnight (37°C/5% CO₂). The following day cells were treated sequentially with (dependent on experiment):

- i) PGE₂ receptor antagonist: AH6809 50 μ M (EP1-3 antagonist), MF498 1 μ M (EP4)
- ii) PGE₂ OR 25% v/v healthy volunteer or patient plasma OR plasma spiked with PGE₂

- iii) Lipopolysaccharide (LPS; *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science, 1ng/mL)
- iv) *Staphylococcus Aureus* peptidoglycan (PTG, Sigma Aldrich, 10µg/mL unless otherwise stated)

PGE₂ and MF498 were obtained from Cayman Chemicals (MI, USA), reconstituted in DMSO (<0.01%) to form stock solutions, and working concentrations made in appropriate culture media. AH6890 and PF-04418948 (Sigma Aldrich, USA) was reconstituted in DMF (<0.01%). 15 minutes was allowed between each addition step to allow receptor binding/activation. After addition of LPS, cells were incubated for 4 hours (37°C/5% CO₂) and supernatants removed and stored at -80°C prior to analysis.

These experiments were conducted to characterise:

- MDM response to gram negative stimuli (LPS; *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science)
- MDM response to gram positive stimuli (*Staphylococcus Aureus* peptidoglycan (PTG), Sigma Aldrich)
- Impact of healthy volunteer or patient plasma (anti-coagulated with Lithium Heparin) on cytokine release.
- Exploration of the inhibitory effect of PGE₂ in the form of a dose-response curve.
- A reversal of PGE₂ effect by selective EP-receptor:
 - Antagonists:
 - EP1-3/DP1: AH 6809
 - EP4: MF498

3.2.4.2. MM6 Stimulation

MM6 were differentiated as per 3.2.3.2, washed, plated in 96-well plates at 1x10⁵ cells/well in 50µL media and incubated for 1hr (37°C/5% CO₂) prior to reagent addition or stimulation. Reagents were added in a standardised order as 3.2.4.1.

After LPS addition cells were incubated (37°C/5% CO₂) for 4hrs (unless stated) prior to supernatant aspiration and storage at -80°C.

3.2.5. Calcein Cell Viability Assay

Calcein AM (Biotim UK) cell viability assay was used to detect effect of plasma/stimulation on cell viability at the end of some experiments. After supernatants

had been removed and frozen medium was aspirated from each well of the plate and wells were washed with PBS twice. 50 μ L 1 μ M Calcein AM in PBS was added to each well and left at 37°C for 30 minutes. The fluorescence on fluorescence plate reader with the excitation wavelength at 485 nm and the emission wavelength of 530 nm was read.

3.2.6. Single-Analyte Enzyme Linked Immunosorbent Assay

The concentration of TNF- α , IL-6, IL-8, LPS binding protein and sCD14 in cell culture supernatants and/or patient plasma was measured via enzyme-linked immunosorbent assay (ELISA). Pre-validated kits employing the 'sandwich' principle of analyte-specific capture and biotinylated detection antibodies were obtained from R&D systems (USA, DuoSet system) for the evaluation of analytes and conducted in half-volume (50 μ L) 96 well Corning CoStar high-binding, clear flat bottom polystyrene plates. Light absorbance of the streptavidin-horse radish peroxidase (HRP) catalysed breakdown of 3,3',5,5'-tetramethylbenzidine (TMB) was measured at 450nm against a reference wavelength of 595nm on a Tecan® GENios™ microplate spectrofluorometer and sample values interpolated from a standard curve of known antigen concentration on a plate by plate basis. Supernatants and plasma samples were thoroughly thawed and diluted in reagent diluent (PBS containing 5% bovine serum albumin) prior to addition to ensure working concentrations in the centre of the standard curve (1:4 MM6, 1:40 MDM) and the HRP-TMB reaction stopped via the addition of 1M sulphuric acid.

3.2.7. Cytokine bead array (conducted by AM Maini)

Beads with the appropriate cytokines (IL1b, IL6, IL8, IL10, TNF- α) were mixed with standards as provided to produce a standard curve. Samples were diluted in sample diluent. Assay was then performed as per the instructions. Beads were read on a BD FACSVerser flow cytometer (3 lasers: 405 nm, 488 nm, and 640 nm; 10-parameter analysis; BD Biosciences). Data were acquired using BD FACSuite (BD Biosciences). Data were analyzed using FCAP Array software v3.0 (Soft Flow Inc, Hungary).

3.2.8. Measurement of endotoxin (conducted by AM Maini)

HEK293 cells are transfected to stably express TLR4 and a nuclear factor-kB-inducible secreted embryonic alkaline phosphatase reporter gene. QUANTI-Blue detection medium changes colour in the presence of secreted embryonic alkaline phosphatase in the spectrum of 620–655 nm. Because the absorbance is in direct proportion to the amount of endotoxin present, the concentration of endotoxin can be calculated from a

3.3. RESULTS

3.3.1. Assay variability with healthy volunteer monocyte derived macrophages

3.3.1.1. *Inter-donor variability*

Seven healthy volunteers donated blood for the culture of MDMs. When these cells, in media with no plasma, were stimulated with 1ng/mL of LPS the inter donor variability of TNF α ranged from 15-28ng/mL with a mean of production of 20.34ng/mL for each donor (figure 3.3Ai). Some of this variability was due to difficulties with live cell counting; donors from some days had more cells plated per well (post plating manual counting). The yield of MDMs per donor ranged from 4.8×10^6 to 13.7×10^6 total live cells (viability ranged from 79 – 92% using trypan blue and Countess[®] cell counter, Invitrogen).

3.3.1.2. *The effect of plasma on the assay*

MDMs from different donors were stimulated with LPS in the presence and absence of 25% non-autologous plasma (4-8 different plasma donors). In one MDM donor (C) plasma decreased TNF α production by an average of 30% (range 10% - 41%) however there was no overall difference with the other 3 MDM donors (figure 3.3Aii). This was in contrast to MM6 (see 3.3.2.1) in which TNF α production was decreased by at least 50% in the presence of 25% plasma.

Looking at these results in more detail figure (3.3.Aiii) shows MDMs from 2 different healthy donors (C & D) in the presence of 25% plasma from 8 different healthy donors. Despite MDMs from donor C producing more TNF α without plasma, when the cells were in the presence of non-autologous plasma they always produced less TNF α than donor D.

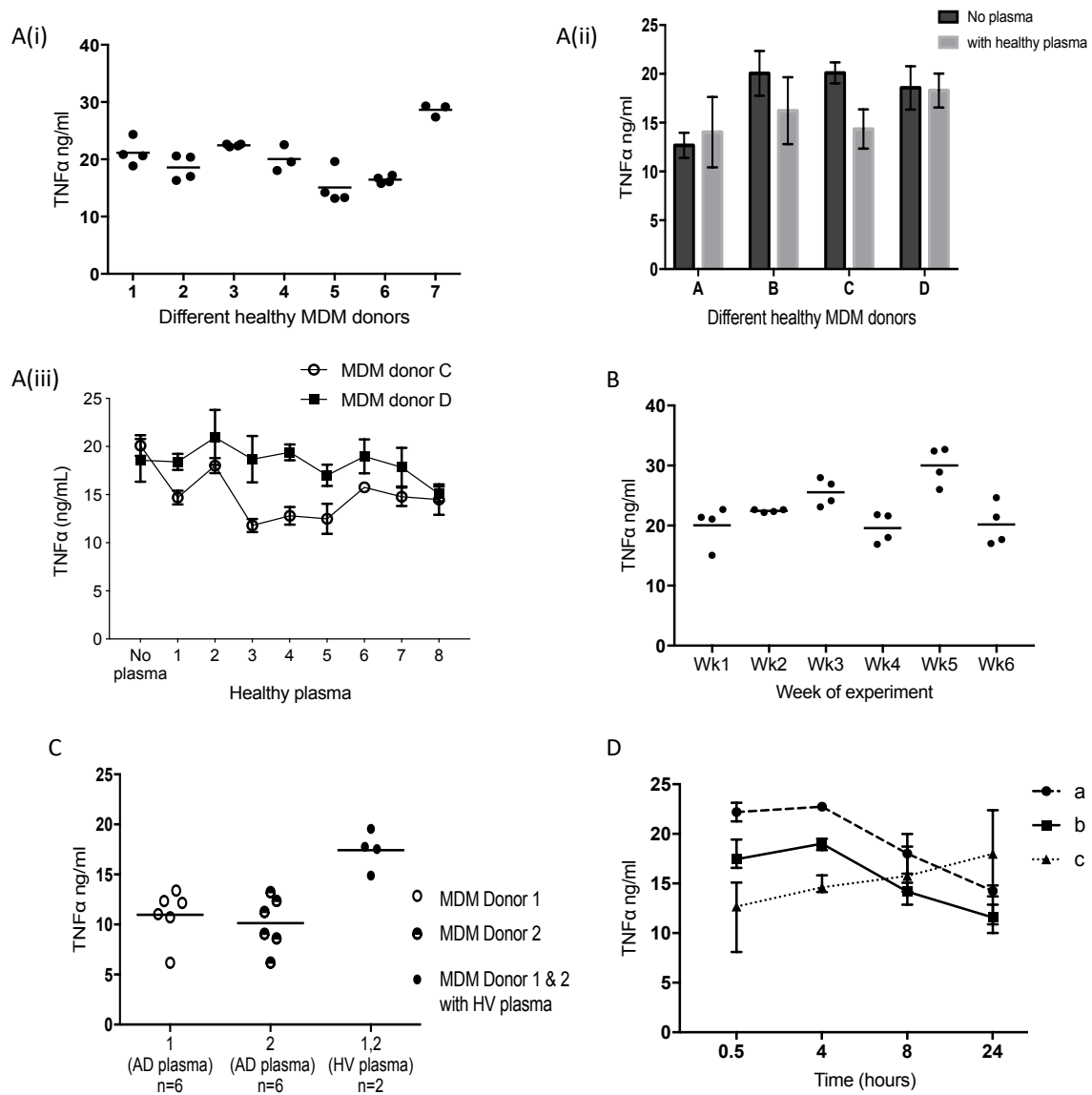


Figure 3.3. Factors effecting variability in healthy volunteer monocyte derived macrophage TNF α production after LPS stimulation

[A] LPS stimulated TNF α production varies between MDM donors: (i) in the absence of plasma: MDMs from n=7 healthy blood donors. (ii) in the presence of the same non autologous plasma: 4 different MDM healthy donors (A-D) with or without the same non-autologous plasma. (iii) In the presence of different (n=8) HV plasma, but the difference is consistent. TNF production from LPS stimulated MDMs from 2 different donors (C&D) in the presence of 8 different HV donated plasma. *Error bars are standard deviation. Dots represent technical repeats. Horizontal line represents mean*

[B]. Time variability in a single donor's MDMs (TNF α production in response to LPS stimulation) over the course of 6 weeks. Each dot represents a healthy volunteer (HV) plasma (n=4).

[C] AD plasma (n=6) consistently causes lower TNF α production in response to LPS versus non autologous HV plasma (n=2) even with different MDM donors.

[D] Effect of time to processing and freezing plasma on MDM TNF production.

AD patients (n=3) blood was left benchside for 1/4/8/24 hours prior to plasma separation. *Error bars are absolute range in technical repeats.*

When comparing the effect of AD patient plasma (n=6) on LPS stimulated TNF α production in two separate healthy volunteer MDM donors there was a consistent suppression of TNF α production between donor cells (figure 3.3C).

3.3.1.3. Intra-donor variability

There was variability in the amount of TNF α produced by MDMs from the same healthy blood donor from week to week. Mean TNF α production in the absence of any plasma was 25.92ng/mL (total range 19.88-36.99ng/mL). This variability was also reflected in the presence of 4 different healthy volunteer's plasma (figure 3.3B) although the effect of the same non autologous plasma is proportionally similar. This is likely to be secondary to variation in live cells counting and physiological donor factors over time.

Using calcien at the end of each experiment did not show any differences in live cell count between wells (data not shown).

3.3.1.4. Impact of time to plasma processing on the LPS stimulated MDM assay

The effect of leaving blood samples on the bench for different periods of time prior to spinning and removing plasma was assessed. 3 patient blood samples were left for 1, 4, 8 and 24 hours prior to spinning at 1300x g and removing plasma for storage at -80°C. When MDMs (same donor) were stimulated in the presence of this plasma (25% well volume) TNF α production decreased over the 24-hour period for 2 of the patient samples and slightly increased in the third sample. However there was no difference between 0.5 and 4 hours (figure 3.3D).

3.3.2. Variability with MM6

3.3.2.1. The effect of plasma on the assay

As expected the VD3 differentiated MM6 cells had a marked decrease in TNF α production with increasing well volumes of healthy plasma (figure 3.4A). This is similar to what has been observed previously within the laboratory (J.Fullerton, PhD thesis 2015). Cells in figure 3.4A were at passage 25 and starting to produce slightly less TNF α in response to LPS. These cells may have had an even higher sensitivity than usual to plasma (>50% decrease in TNF α production).

In the presence of plasma, the MM6 cell line had a further reduction in TNF α production if blood was taken from patients using EDTA as an anticoagulant rather than lithium heparin (figure 3.4B).

If samples were left for longer than 4 hours prior to centrifuging and removing plasma, TNF α production appeared to change however there was not a consistent increase or decrease in results (figure 3.4C).

Patient plasma appeared to slightly suppress TNF α production compared to healthy plasma (figure 3.4D) however this difference was small compared to the difference observed with the healthy volunteer MDMs (figure 3.3C).

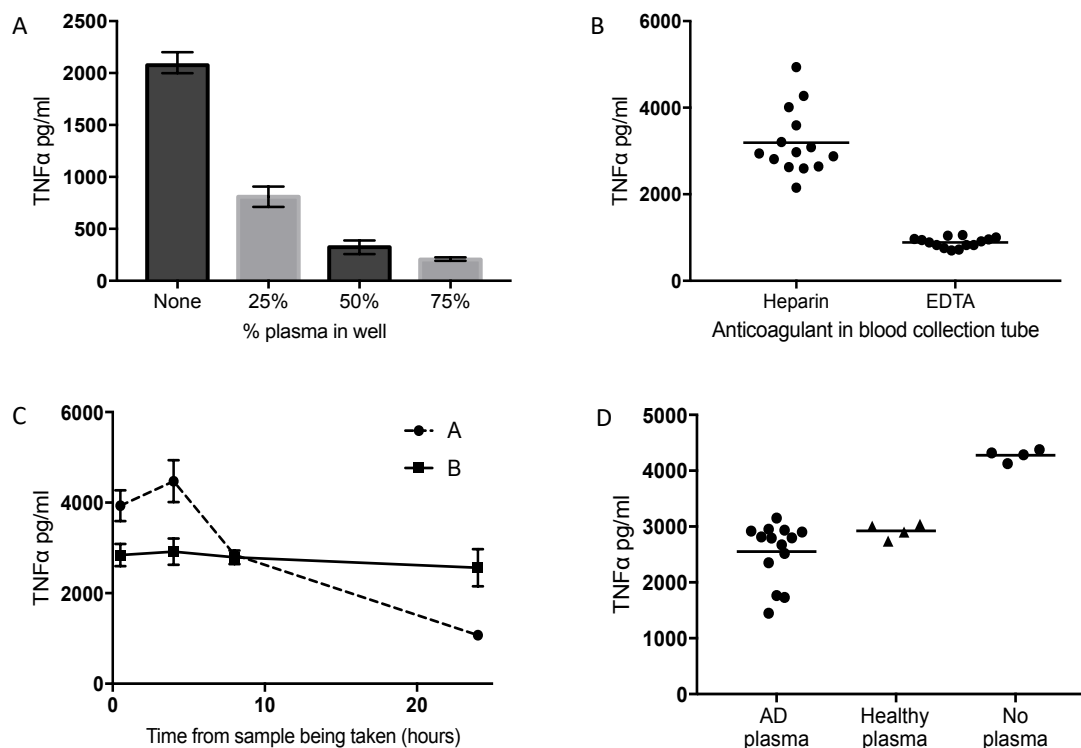


Figure 3.4. Factors effecting variability in MonoMac6 (MM6) TNF α production after LPS stimulation

[A] Healthy plasma suppresses LPS stimulated TNF α production from differentiated MM6 cells. *MM6 at passage 25. Error bars are absolute range of technical repeats.*

[B] Plasma collected with EDTA tubes suppresses LPS stimulated TNF α production from differentiated MM6 cells LPS. Patient blood samples (n=14) were taken with blood collection tubes container either lithium heparin or EDTA as an anticoagulant.

[C] Effect of time to processing and freezing plasma on MM6 TNF α production. 2 patients (A/B) with liver cirrhosis had blood taken in lithium heparin tubes which was left on the bench for 1/4/8/24 hours prior to plasma separation. *Error bars are absolute range in technical repeats.*

[D] LPS stimulated TNF α production from MM6 cells in the presence of patient (n=14), healthy (n=4) or no plasma (n=4). *MM6 at passage 9.*

3.3.3. MDM and MM6 response to PGE₂

3.3.3.1. PGE₂ dose response

LPS stimulated TNF α production decreases in a PGE₂ dose dependent manner in both MDMs and MM6 cells (figure 3.5Ai, 3.5Aii) with and without 25% healthy volunteer plasma. Levels of PGE₂ averaging 0.1ng/mL were previously measured in AD patients (opposed to 0.01ng/mL in healthy volunteers) using electrospray ionization liquid chromatography-tandem mass spectrometry¹¹.

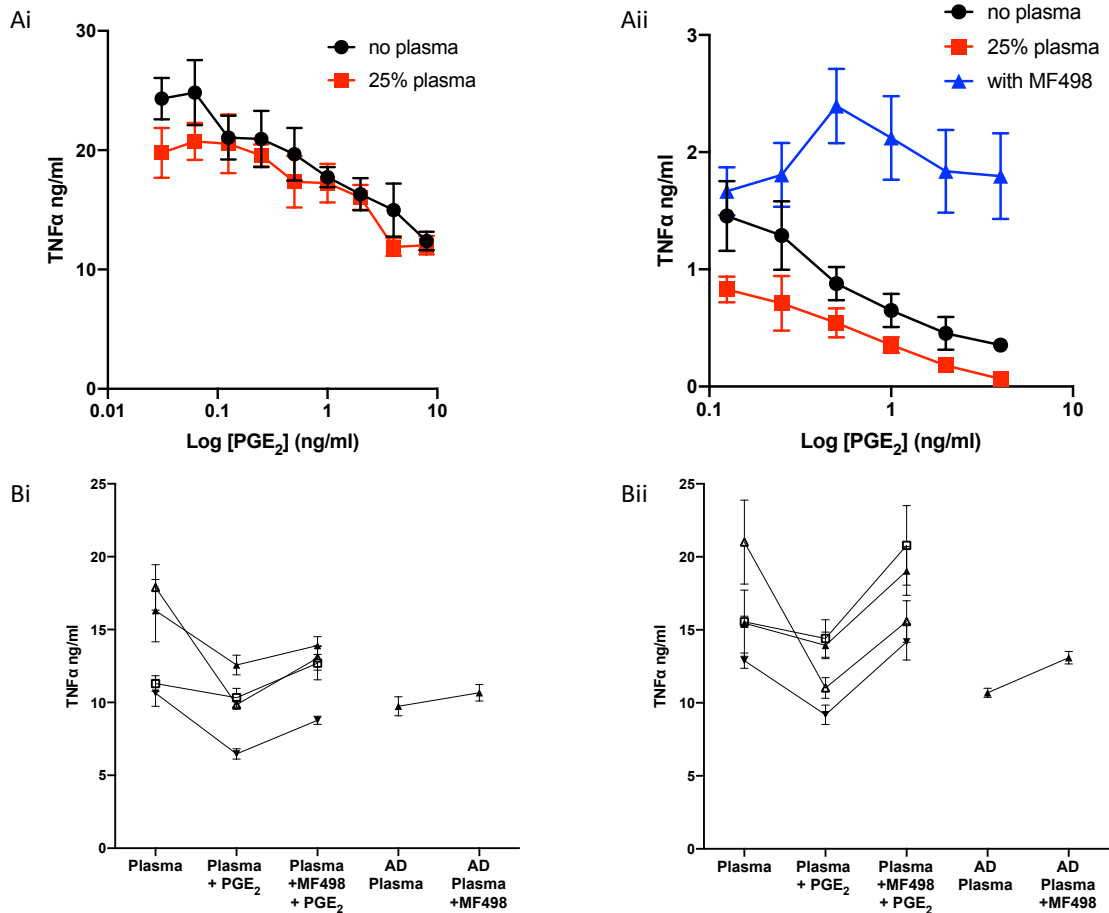


Figure 3.5. LPS stimulated TNF α production is decreased by PGE₂ and the effect is reversed by the EP4 receptor antagonist MF498

[A] LPS stimulated TNF α production from (i) Healthy MDMs and (ii) differentiated MM6 cells in the presence or absence of non-autologous plasma and increasing amounts of PGE₂. A set of MM6 cells were also pre treated with MF498 (1 μ M) prior to the addition of PGE₂ and LPS which reversed the suppressive effect of PGE₂.

[B] LPS stimulated TNF α production from healthy donor 1 (i) and healthy donor 2 (ii) in the presence of non-autologous healthy plasma (n=4). This was then spiked with 1ng/mL of PGE₂ and finally this spiked plasma was placed on cells which had been pre treated with the EP4 receptor antagonist MF498 (1 μ M). Plasma from 1 AD patient showed a lower TNF readout and this was improved with MF498 pre treatment.

Error bars s.d. (technical repeats). Points are mean. MM6 cells from passage 25.

Examining the 0.1ng/mL PGE₂ level using the MDM cells in figure 3.5Ai; this corresponds to around a 30% (no plasma) or 20% (plasma) decrease in TNF α production in response to LPS in this particular donor.

LPS stimulated total TNF α production is about a tenth of the amount in differentiated MM6 cells. However the MM6 PGE₂ dose response curve is smoother and has a higher sensitivity to increasing amounts of PGE₂ (figure 3.5Aii).

3.3.3.2. PGE₂ and EP4 receptor antagonist MF498

Previous unpublished work within our laboratory (J.Fullerton, 2016) identified the EP4 receptor antagonist MF498 as the most effective at reversing the suppressive effect of PGE₂ on differentiated MM6 cell function. Using two healthy donor MDMs, 2 different non-autologous healthy donors plasma was spiked with 1ng/mL of PGE₂ which caused a decrease in TNF α production from both MDM donor cells with all of the plasma donors. This was reversed when the cells were pre treated with 1 μ M MF498 (figure 3.5B). MDMs treated with MF498 alone did not produce more TNF α . Plasma from one patient with acute decompensation of liver cirrhosis decreased LPS stimulated TNF α production as compared to healthy non-autologous plasma in both MDM donors. However TNF α levels improved to that of the healthy volunteer plasma when cells were pre treated with MF498.

3.3.3.3. MDM stimulation with a gram positive bacterial source (Staph.Aureus Peptidoglycan) produces similar results as MDM stimulation with a gram negative bacterial source (LPS)

MDM cells reacted in a similar fashion to that described in previous sections when cells were stimulated with a component of Stapholococcus aureus cell wall as opposed to LPS (figure 3.6). Pre treating cells with PGE₂ in the presence or absence of plasma caused a down regulation in TNF α production as expected. There was a larger response with higher concentrations of PTG, however much higher concentrations in general were required to produce a similar TNF α output as compared to when the cells were stimulated with LPS (10 μ g/mL as opposed to 1ng/mL).

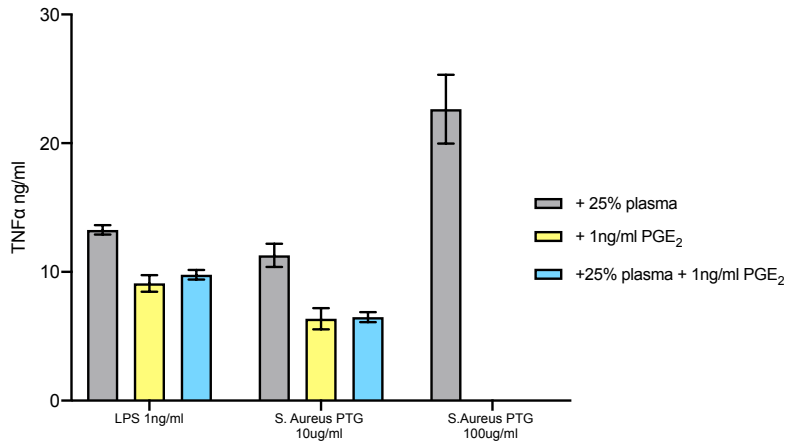


Figure 3.6. A comparison of LPS stimulation of MDMs versus S.Aureus PTG.

LPS 1ng/mL caused similar TNF α production from MDMs as 10 μ g/mL of S.Aureus PTG. There was a decrease when cells were pre treated with PGE₂ +/- plasma. Higher doses of S.Aureus PTG resulted in a higher production of TNF α .

3.3.4. The impact of patient administration of serum targeted 20% HAS on plasma mediated monocyte derived macrophage function ex vivo, in a single arm study

As a result of work evaluating the variability between MDM and MM6 cells lines and effects of processing the plasma samples to be evaluated, both types of cells were used in this blinded analysis. This was to add validity to any difference in the results between plasma pre and post treatment. In addition plasma was collected in lithium heparin tubes and processed within 4 hours at each hospital site. In order to reduce variability, all MDMs were obtained from the same donor.

In our single arm study, there were 52/80 patients that had a pre treatment plasma sample frozen on day one and a subsequent post treatment sample available for analysis. 45/52 of those patients had reached the primary endpoint of serum albumin >30g/L. The post treatment sample selected for analysis was the sample which corresponded to the first day on which the patient had reached a serum albumin of >30g/L this corresponded to a mean treatment day 3.29 (SD 1.27). These 45 patients had a mean pre treatment serum albumin of 23.98g/L (range 12-29g/L). 7/52 patients did not reach the target serum albumin of >30g/L therefore their post treatment plasma sample was selected as the sample corresponding to the day in which the patient's serum albumin was at its highest (mean day 2.57). These patients had a mean pre treatment serum albumin of 24g/L (range 19-28g/L).

3.3.4.1. Targeted 20% HAS infusions, to increase serum albumin >30g/L, improve plasma mediated MDM dysfunction in a PGE₂ dependent manner

LPS stimulated TNF α production from healthy volunteer MDMs significantly improved when in the presence of post albumin treatment plasma (serum albumin >30g/L) versus pre treatment plasma (serum albumin <30g/L). Mean increase in TNF α production was 1.75ng/mL (CI 0.72–2.77; P 0.0013) (figure 3.7Ai). This corresponded to a mean post treatment increase of 14.5% (CI, 5.1%–23.5%). TNF α production in presence of healthy volunteer plasma was 6.88ng/mL higher than in presence of pre HAS treatment AD plasma (p<0.0001).

In the 7 patients who had not incremented their serum albumin to >30g/L after HAS treatment there was still a trend toward improvement of TNF α production (figure 3.7Aii) despite these patient's post treatment samples not being correlated with achieving the target serum albumin >30g/L. This is a small number of patients but could reflect that treatment and an increase in serum albumin is having a positive impact itself rather than all patients having to achieve a set serum albumin level. There was no correlation between the magnitude of serum albumin increase and the size of MDM TNF α increase post treatment (figure 3.7D) in all samples analysed.

LPS-induced IL-6 (figure 3.7Aiii) and IL-8 (figure 3.7Aiv) production also increased significantly in the presence of post HAS treatment plasma (n=52) as compared to pre-treatment plasma.

The 52 patient analysis was split into subgroups based on:

- ACLF (any grade, n=9) at baseline versus no ACLF (n=43) at baseline
- High bilirubin (mean bilirubin >80 μ mol/L during trial period, n=29) versus lower bilirubin (mean bilirubin <80 μ mol/L during trial period, n=23)
- Known survival at 3 months (n=30) versus non survival (n=16)

There was no difference in the magnitude of effect between patients with a very high versus a lower bilirubin (high = mean improvement 1.9ng/mL TNF, low = mean improvement 1.7ng/mL TNF, p=0.8). There was no difference in the magnitude of effect between patients with ACLF versus no ACLF (ACLF = mean improvement 2.2ng/mL TNF, no ACLF = mean improvement 1.7ng/mL TNF, p=0.7) although the total number of ACLF patients was small. Survival outcomes were known for 46/52 patients in the

analysis. Again there was no difference in the magnitude of effect in this subgroup analysis (alive at 3 months = mean improvement 1.6ng/mL TNF, dead at 3 months = mean improvement 2.4ng/mL TNF, $p=0.4$).

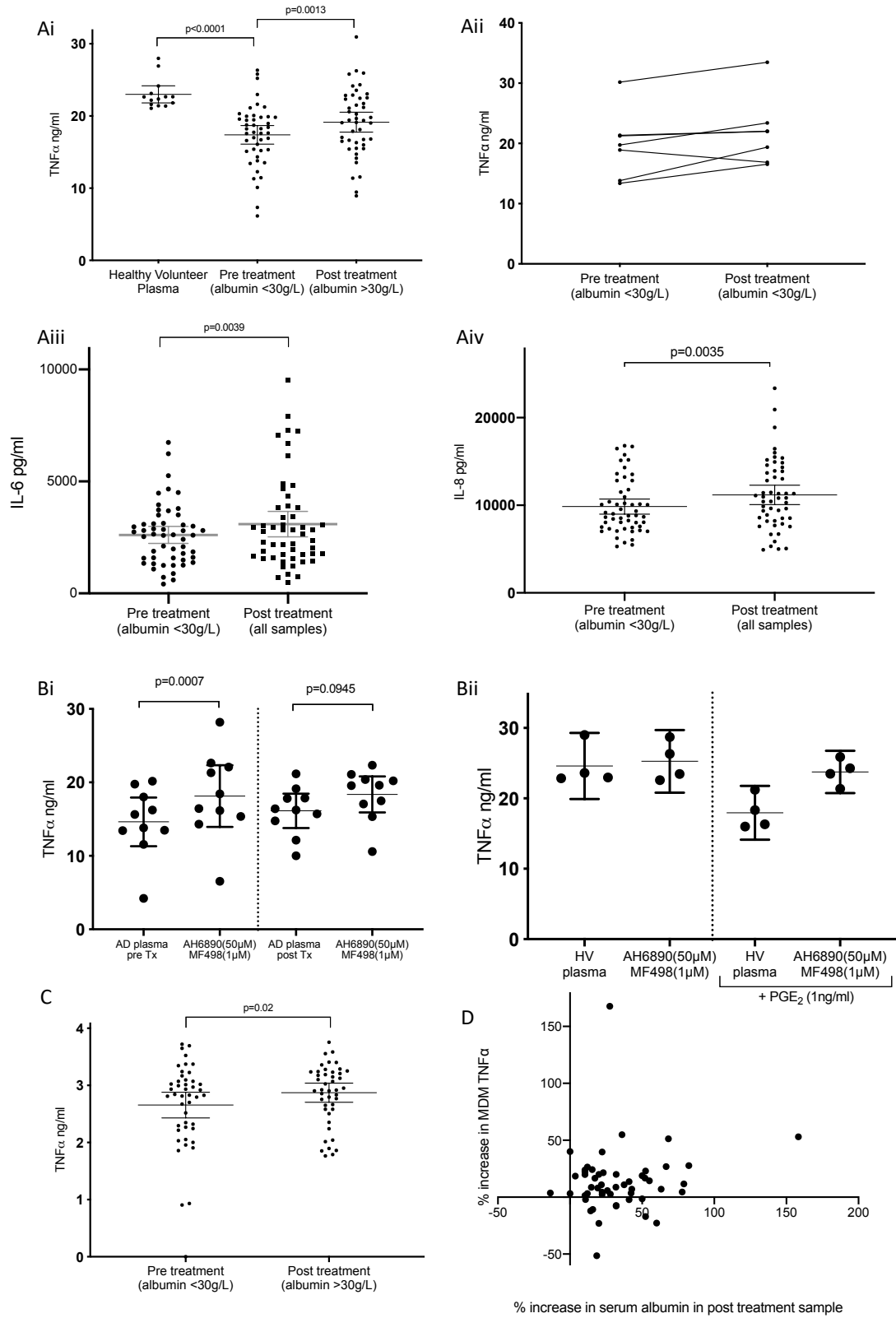


Figure 3.7 Targeted 20% HAS infusions, to increase serum albumin >30g/L, improve plasma mediated MDM dysfunction in a PGE $_2$ dependent manner

[A] (i) Endotoxin (LPS) stimulated MDM TNF α production in presence of patient (n=45) or non-autologous healthy volunteer plasma (n=12). TNF α production in presence of healthy volunteer plasma was 6.88ng/mL more than presence of pre HAS treatment AD plasma (CI, 4.85–8.91ng/mL; P < 0.0001). LPS MDM TNF α production in presence of plasma pre- and post-HAS treatment (n=45 patients incremented serum albumin >30 g/L). Mean post-treatment TNF increase 1.75ng/mL (0.72–2.77; P=0.0013), 14.5% (5.1%–23.5%). (ii) LPS stimulated HV-MDM TNF α production in the presence of patient plasma pre and post patient treatment with 20% HAS (n=7). Only patients whose serum albumin did not increment to >30g/L after treatment. LPS stimulated MDM IL-6 (iii) and IL-8 (iv) also increased significantly post HAS treatment (n=52) Mean post treatment increase in IL-6 480.5pg/mL (161.1 to 799.9, p=0.0039). Mean post treatment increase in IL-8 1337pg/mL (459.3 to 2215, p=0.0035).

[B]. (i) Addition of the EP2 (AH6890) and EP4 (MF498) receptor antagonists prior to LPS stimulation caused significant improvement in TNF α production in pre treatment plasma but not post treatment plasma (n=10). Mean increase of 2.9ng/mL (1.4-4.4ng/mL, p=0.0007). (ii) This is not simply an effect of the antagonist/solute on the MDMs. TNF α production from healthy volunteer MDMs stimulated with LPS in presence of healthy plasma (n=4) and presence/absence of AH6890 (50mM) and MF498 (1 μ M) and 1ng/mL PGE $_2$.

[C]. Endotoxin (LPS) stimulated MM6 TNF α production in presence of patient or non-autologous healthy volunteer plasma. (n=45). Mean post treatment increase in TNF α 0.2158ng/mL (0.0355-0.3961, p=0.02).

[D]. Percentage increase in serum albumin post treatment compared to percentage increase in LPS stimulated MDM TNF production. No significant correlation between values $r^2=0.022$. (n=52)

All: paired student's t-test, 95% CI, normal distribution. Error bars are CI.

Samples were selected from 10 patients whose initial sample analysis had shown at least a 15% difference between the pre and post treatment sample to explore whether MDM pre treatment with EP1-3 (AH6890) and EP4 (MF698) receptor antagonists (i.e. pan PGE $_2$ receptor blockade) prior resulted in an increase in TNF α production (i.e. by reversing the immune suppressive effect of PGE $_2$).

Pre-albumin treatment plasma showed a significant increase in TNF α production with pan PGE $_2$ receptor blockade (Figure 3.7Bi, P=0.0007) as opposed to post treatment plasma which showed no significant improvement with receptor blockade (p=0.0945). In the pre treatment plasma sample pan PGE $_2$ receptor blockade results an increase in TNF α production to that of the post treatment sample.

To ensure this was not simply an effect of the receptor antagonists themselves (or the solvent they were dissolved in) cells were pre treated with EP receptor antagonists in the presence of healthy plasma and there was no difference in LPS stimulated TNF α production. However when the HV plasma was spiked with 1ng/mL of PGE $_2$ (causing TNF suppression) pan PGE $_2$ receptor blockade normalised the suppression (figure 3.7Bii).

3.3.4.2. Targeted 20% HAS infusions, to increase serum albumin >30g/L, show a corresponding improvement plasma mediated MM6 cell line dysfunction

LPS stimulated TNF α production from MM6s significantly improved when in the presence of post albumin treatment plasma (serum albumin >30g/L) versus pre

treatment plasma (serum albumin <30g/L) (figure 3.7C). Mean post treatment increase in TNF α was 0.2158ng/mL (0.0355-0.3961, p=0.02) (figure 3.7C). This corresponded to a 10.2% increase in TNF α production (SD 25.7%).

3.3.4.4. Plasma Prostaglandin E₂

This analysis was undertaken in a subgroup of 10 patient samples due to expense of the analysis and the requirements of sample collection requiring absolute precision, Therefore all samples were collected from one trained site.

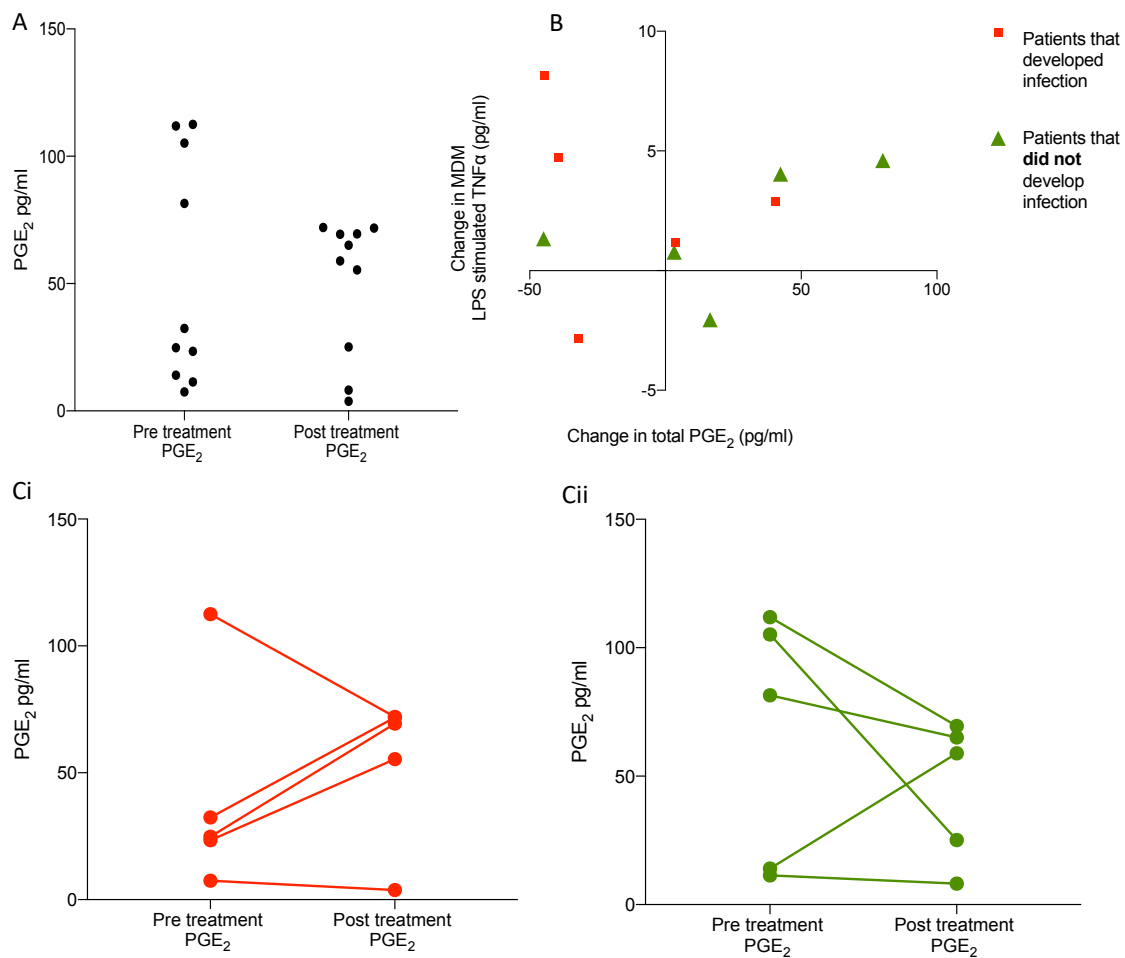


Figure 3.8. Changes in patient plasma PGE₂ post treatment (n=10).

[A] There are no differences in post treatment plasma PGE₂ (n=10 patients)

[B] Total change in plasma PGE₂ post treatment versus total change in LPS stimulated MDM TNF α production in the presence of patient plasma post treatment. Samples are divided into patients who developed a new infection (n=5, red) and those that did not develop a new infection (n=5, green).

[C] (i) Plasma PGE₂ in patients that developed infection (n=5) and (ii) those that did not develop infection

There were no overall changes in the 10/79 patients who had plasma PGE₂ measured pre and post treatment (figure 3.8A). There was no relationship between change in

plasma PGE₂ post treatment and change in TNF α produced from LPS stimulated MDMs in the presence of patient plasma in these samples with a broad spread of results (figure 3.8B). Although very small numbers, patients who developed infection tended toward an increase in measured PGE₂ post treatment (figure 3.8Ci) where as those that didn't tended towards a decrease in PGE₂ post treatment (figure 3.8Cii).

I D	Post Tx sample day	Infection day	Serum albumin (g/L) <i>pre Tx</i>	Serum albumin (g/L) <i>post Tx</i>	PGE ₂ (pg/mL) <i>pre Tx</i>	PGE ₂ (pg/mL) <i>post Tx</i>	MDM TNF α (pg/mL) <i>pre Tx</i>	MDM TNF α (pg/mL) <i>post Tx</i>
2	4	9	25	30	23.40	55.40	12.28	9.45
57	4	13	21	30	7.50	3.80	16.92	18.11
11	8	8	22	31	112.50	72.00	21.26	24.16
63	8	5	26	30	32.40	71.80	19.58	24.32
29	5	5	12	31	24.80	69.40	15.40	23.56
48	2	n/a	25	34	111.90	69.50	7.35	11.39
4	3	n/a	27	31	14.00	58.90	15.33	16.65
39	4	n/a	17	31	105.20	25.10	16.60	21.20
41	2	n/a	19	22	81.50	65.10	18.91	16.85
77	2	n/a	22	19	11.40	8.20	21.24	22.01

Table 3.1. Individual patient data for patients who had PGE₂ measured pre and post treatment.
Patients 48/4/39/41/77 did not develop an infection in the trial treatment period.

Mean post treatment sample day tended to be later in patients who developed infection (table 3.1) and often after or just as infection was diagnosed. All high starting PGE₂ concentrations (>50pg/mL) all decreased post HAS treatment. Interestingly these high concentrations were more commonly observed in the patients who did not go onto develop infection in the trial treatment period.

3.3.4.5. Plasma cytokine and endotoxin levels

	Healthy plasma <i>Mean (s.d)</i> (n=4)	Pre treatment patient plasma <i>Mean (s.d)</i> (n=45)	Post treatment patient plasma <i>Mean (s.d)</i> (n=45)	Mean change post treatment	<i>Confidence interval</i>
TNFα (pg/mL)	1.00 (1.62)	1.32 (2.40)	1.30 (2.27)	↓0.010	-0.416 to 0.396
IL-6 (pg/mL)	4.67 (1.24)	100.88 (141.22)	85.10 (133.67)	↓17.46	-49.05 to 14.13
IL-8 (pg/mL)	19.69 (6.17)	708.76 (1156.49)	458.61 (706.59)	↓252.8	-555.7 to 50.21
IL-10 (pg/mL)	2.14 (2.43)	2.78 (4.92)	3.24 (6.39)	↑0.442	-0.649 to 1.532
IL-1β (pg/mL)	0.00 (0.00)	1.28 (2.74)	1.14 (1.70)	↓0.156	-0.989 to 0.676
Endotoxin (pg/mL)	-	15.69 (18.69)	17.71 (17.28)	↓2.02	-4.792 to 0.748

Table 3.2. Plasma cytokine measurements show no significant differences post treatment after serum albumin has increased to >30g/L.

A panel of pro and anti-inflammatory cytokines were measured in patient plasma at baseline pre HAS treatment (serum albumin <30g/L) and post HAS treatment (serum albumin >30g/L). There was no significant change in in any of these cytokines post treatment with 20% HAS (table 3.2). Of note TNF α was in the low pg/mL range and therefore would not have had any impact on the LPS stimulated MDM and MM6 assays. Subgroup analysis in patients with ACLF (any grade according to EASL-CLIF criteria) versus those without and those with high bilirubin >80 μ mol/L versus those with bilirubin <80 μ mol/L did not show any significant differences in post treatment plasma cytokines as compared to pre treatment.

Patient plasma endotoxin pre HAS treatment (serum albumin <30g/L) and post HAS treatment (serum albumin >30g/L) was measured using HEK-blue TLR4 cell line. There was a non-significant mean 2.022pg/mL decrease in endotoxin level 17.71 v 15.69pg/mL (p=0.1484, -4.792 to 0.7477) (table 3.3). ACLF patients (n=21 total with 9/21 included in sample analysis) had a larger decrease in plasma endotoxin (18.31pg/mL to 12.35pg/mL, p=0.02, CI -10.60,-1.002).

3.3.5. In patients that develop infection there is a reversal in the initial improvement in plasma mediated MDM dysfunction

Samples were re-evaluated according to whether patients had developed a new infection during the trial treatment period or not. Clinical characteristics are described in chapter 2 (section 2.3.5). Baseline cytokines and endotoxin in these two groups are displayed in table 3.3.

	New Infection after D3 (n=21) Mean (s.d)	No New infection (n=57) Mean (s.d)
TNF α (pg/mL)	3.7 (6.5)	0.9 (1.72)
IL-6 (pg/mL)	213.6 (192)	178.8 (697.12)
IL-10(pg/mL)	5.1 (6.1)	2.8 (6.3)
IL-1 β (pg/mL)	3.4 (5.1)	0.8 (1.33)
IL-8 (pg/mL)	697.5 (662.3)	632.5 (1203.3)
Endotoxin	17.8 (17.7)	15.7 (19.2)

Table 3.3. There were no significant baseline differences in plasma endotoxin or cytokines in patients who went onto develop an infection after day 3 versus those who did not.

Patients diagnosed with infection had higher levels of plasma LPS binding protein and sCD14 than time matched samples from patients without infection (figure 3.9)

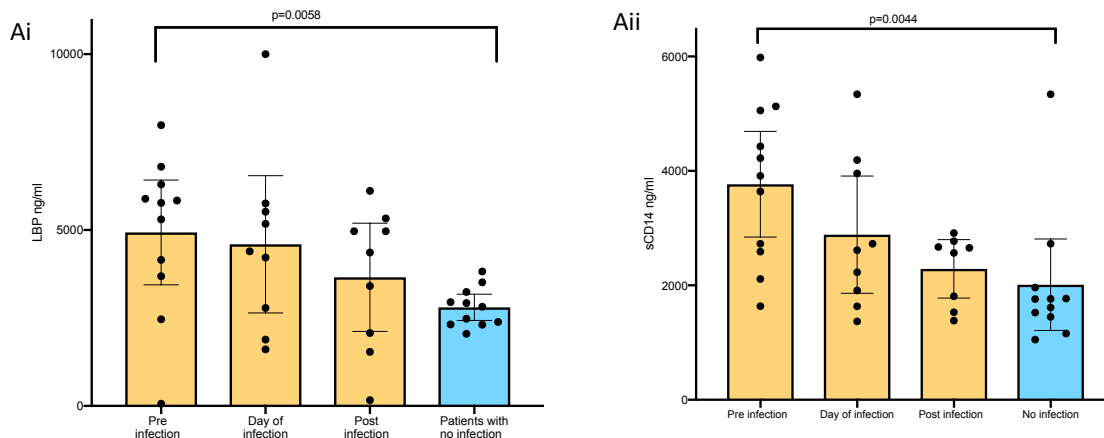


Figure 3.9. Plasma LPS binding protein (i) and sCD14 (ii) is increased in patients who develop infection at the time of infection as compared to time matched plasma samples from patients who did not develop an infection.

Increase was at its largest the day prior to diagnosis of infection (n=9) with a mean LBP increase of 2130ng/mL (3568 to 692.0, p=0.0058) and sCD14 increase of 1756ng/mL (2899 to 613.7, p=0.0044) compared to no infection patients (n=11).

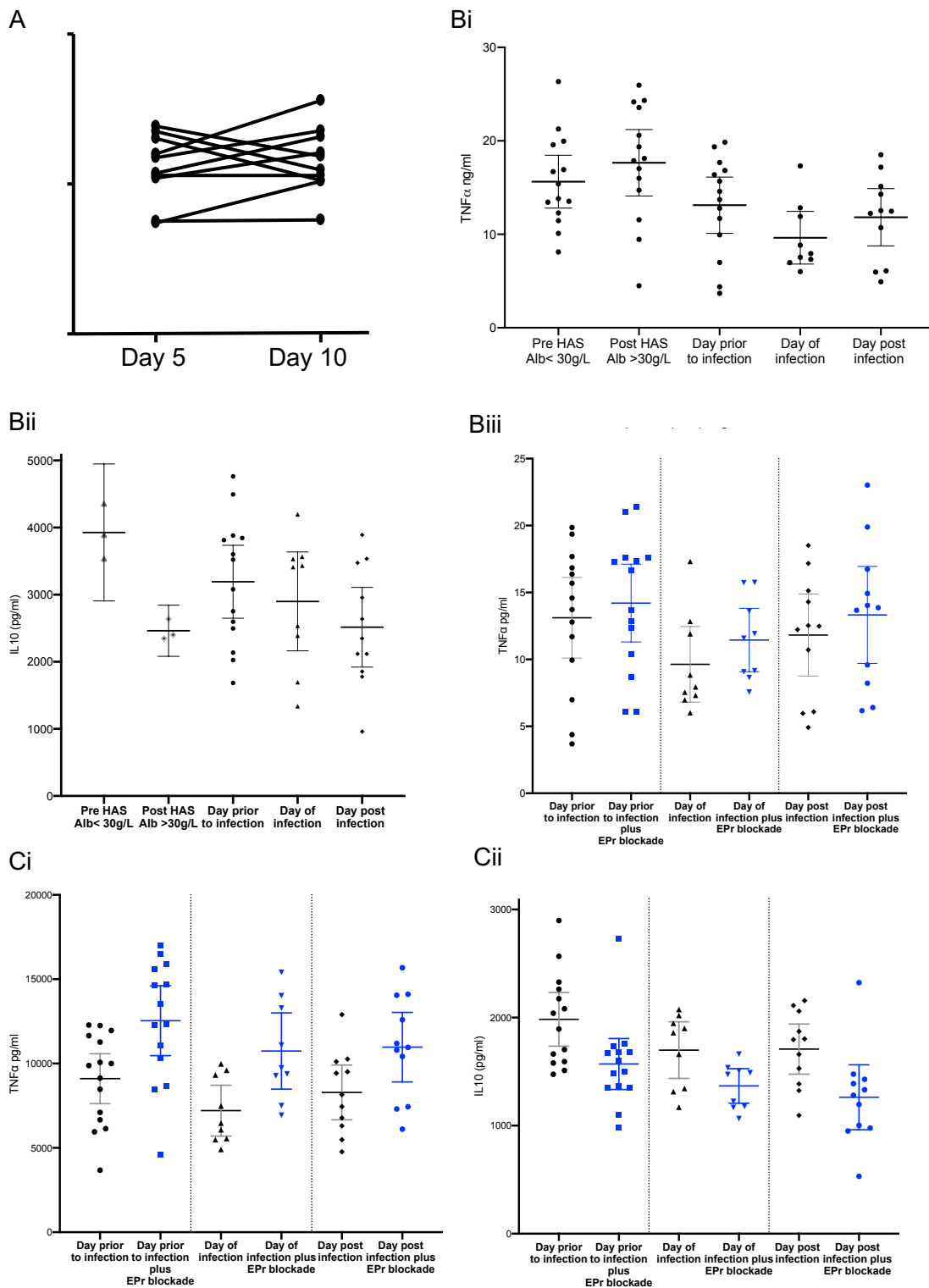


Figure 3.10. In patients that develop infection there is a reversal in the initial improvement in plasma mediated MDM dysfunction

[A] LPS mediated MDM TNF α production in presence of AD plasma Day 5 and 10 post-treatment with 20% HAS. No overall change over time is shown in this sample. (n=10)

[B] (i) LPS stimulated MDM TNF α in the presence of plasma samples over time from patients that went onto develop infection. Despite an initial mean 15.2% improvement (4.6-25.9%, $p=0.0008$) there was a 26% decrease in LPS stimulated TNF α production the day prior to infection ($p= 0.0448$, mean day 4.1) and a further decrease on the day of infection (mean day 6.65). There was a mirrored response in MDM IL-10 production (ii). (iii) EP receptor antagonists EP1-3 (AH6890 50 μ M) and EP4 (MF498 1 μ M) cause a partial reversal in decreased TNF α production indicating that PGE $_2$ present in the plasma was a probable factor in this down regulation.

[C] MDMs stimulated with *S.aureus* peptidoglycan in the presence of patient plasma from patients who developed an infection. There was a more pronounced impact of PGE $_2$ receptor blockade in this assay with a marked increase in production of TNF α at 4 hours (i) and a mirrored decrease in IL-10 at 24hours (ii).

Splitting the analysis shown in figure 3.10A into patients who developed new infection versus those that didn't; LPS stimulated MDM TNF α production increased by 15.2% (4.6,25.9, $p=0.008$) in the infection patients versus 8.7% (2.6,14.8, $p=0.006$) in the patients that did not develop infection. In a random sample of patients ($n=10$) there were no significant overall changes in LPS stimulated TNF production between day 5 and 10 (figure 3.10A). However in the infection patients, there was a 26% decrease in LPS stimulated TNF α production the day prior to infection ($p= 0.0448$, mean day 4.1) and a further decrease on the day of infection (mean day 6.65) (figure 3.10Bi). This was mirrored by the increase in MDM IL-10 production (figure 3.10 Bii). EP receptor antagonists EP1-3 (AH6890 50 μ M) and EP4 (MF498 1 μ M) cause a partial reversal in decreased TNF α production indicating that circulating PGE $_2$ was a probable contributory factor in this down regulation (figure 3.10Biii). There was a more pronounced impact of PGE $_2$ receptor antagonists when cells were stimulated with *S.Aureus* PTG (gram positive) rather than LPS (gram negative) (figure 3.10C).

3.4. SUMMARY

- LPS stimulated TNF α production from healthy volunteer MDMs is a reliable assay of cirrhosis plasma mediated MDM dysfunction:
 - There is a small amount of inter-donor variability. However, this can be eliminated by using the same donor over a number of weeks (if analyzing large batches of patient plasma samples) as there is less time variability with the same donor
 - TNF α production from the MM6 cell line is more consistent but significantly impaired in the presence of any plasma
- Both MDM and MM6 assays show a dose response to increasing concentrations of PGE₂
- LPS stimulated TNF α production from HV-MDMs and MM6 cells significantly improves in the presence of post HAS treated patient plasma (serum albumin >30g/L) versus pre treatment plasma (serum albumin <30g/L).
 - This validates and strengthens previous preliminary findings that ex vivo plasma mediators of immune suppression are reduced with 20% HAS treatment and verifies the immune restorative potential of daily HAS infusions targeted to a serum albumin of >30g/L
 - Results support a PGE₂ dependent mechanism for the suppressive effect of patient plasma on these cells
 - This finding needs to be evaluated with a control arm of patients
 - Initial improvement in plasma mediated dampening of MDM TNF α production, post 20% HAS treatment, is subsequently reversed in patients who go onto develop infection.
 - Targeted 20% HAS infusions had no overall effect on plasma PGE₂, cytokine or endotoxin levels in this group of AD patients

3.5. CONCLUSIONS

3.5.1. LPS stimulated TNF α production from healthy volunteer monocyte derived macrophages is a reliable assay of decompensated cirrhosis patient plasma mediated MDM dysfunction

3.4.1.1. Inter and Intra donor variability

There was variability between MDM donors response to LPS, however this was modest and most importantly the direction of response to different plasma samples was consistent between donors. Therefore when comparing plasma samples from the same patient (for example pre and post treatment) any differences observed would be expected to be consistent between donors.

In comparison to previous work by my supervisor O'Brien, et al. ¹¹ (see figure 3.11) the variability I saw was markedly less which may reflect improved consistency with cell selection with the addition of negative selection of monocytes with RosetteSep™ prior to culture.

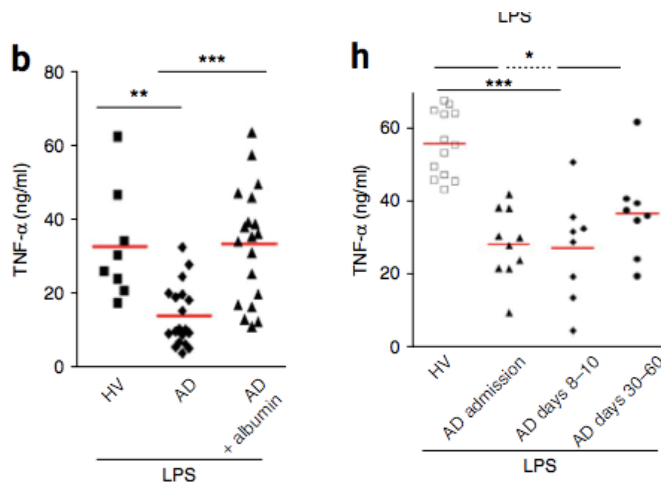


Figure 3.11. Taken from figure 4 from O'Brien, et al. ¹¹.

(b) TNF α with different healthy volunteer (HV) plasma ranges from 18ng/mL to 62ng/mL (h) TNF α with different HV plasma ranges from 42-65ng/mL

The variability over time when using MDMs from the same donor was less than the inter donor variability. Therefore for the purposes of assessment of a large number of patient plasma samples from the same clinical study, using the same donor's cells over a number of weeks would be more accurate than using a number of monocyte donors on

the same day. In addition the samples to be analysed were 'paired' meaning that each pre treatment sample acts as a control which should also account for variability.

3.4.1.2. PGE₂ mediates a reduction in LPS stimulated MDM TNF α production

Increasing concentrations of PGE₂ decreased LPS stimulated TNF α production in a concentration-response fashion, which was maintained even in the presence of plasma. Furthermore, the PGE₂ receptor antagonist, MF498, reversed the suppressive effect of PGE₂ on the bioassay as expected. The HV-MDMs were less sensitive to PGE₂ than MM6 cells. However I was concerned that the extremely low level of TNF α produced by the MM6 cells in the presence of PGE₂ **with** plasma might reduce the sensitivity of the assay in detecting a difference between pre and post treatment samples. Therefore I used both cell types in clinical trial ex vivo analyses as consistency in outcomes would increase, or reduce, confidence in results.

Gram positive infections are a growing problem in hospitalized decompensated cirrhosis patients therefore in an attempt to simulate a gram positive infection, ex vivo S.Aureus PTG was used to stimulate cells in the assay with good effect. However a much larger dose was required compared to LPS (simulating gram negative infection), which may be due to the solubility of the cell wall component.

One might argue that a more simplified approach would be to simply directly measure PGE₂ concentration in all pre and post HAS treatment samples. However this is technically difficult, very expensive and requires meticulous sample preparation which was not feasible with a large number of samples from different clinical sites. In addition, the processing for lipidomic analysis strips albumin from sample and therefore measures total PGE₂ levels which means that results may not truly reflect in vivo bioavailability. Finally, this functional bioassay has much more relevance to potential in vivo mechanisms, as an ex vivo simulation of an infection, and also accounts for any other unknown circulating mediators that may dampen monocyte derived macrophage response.

3.5.2. LPS stimulated TNF α production from HV-MDMs and MM6 cells significantly improved in the presence of post HAS treated patient plasma (serum albumin >30g/L) versus pre treatment plasma (serum albumin <30g/L).

This bioassay suggested a significant reversal in the immunosuppressive effects of patient plasma ex vivo after patients had been treated with targeted 20% HAS infusions.

This strengthens and validates previous preliminary findings¹¹ (which were in a small number of selected patients) that plasma mediators of immune suppression are reduced with 20% HAS treatment and verifies the immune restorative potential of daily HAS infusions targeted to a serum albumin of >30g/L.

The effect size is difficult to interpret. Comparing the difference in MDM TNF α seen in figure 3.7Ai and the MDM PGE₂ dose-response (figure 3.5A) a 14.5% lower TNF α production seen in the presence of pre treatment samples could correspond to PGE₂ increasing to concentrations previously observed in AD patients¹¹. Again with the MM6 bioassay results, a 10.2% lower TNF α production (pre treatment) corresponded to PGE₂ increasing to pathophysiological concentrations previously observed in AD patients.

Exploring the effect of PGE₂ receptor antagonists in the assay supported a PGE₂ dependent mechanism for the suppressive effect of patient plasma. LPS induced TNF α production from MDMs pretreated with pan-PGE₂ receptor antagonists before addition of pre-HAS treatment plasma was increased to a similar level as when post-HAS plasma was added (without PGE₂ antagonists). However pan-PGE₂ receptor blockade had no significant effect on MDMs treated with post 20% HAS plasma.

Albumin is thought to bind and catalyse the breakdown of PGE₂ therefore a simplistic expectation would be that there would be a correlation between the amount a patient's serum albumin had increased and the amount of improvement in MDM TNF α production post HAS treatment, if the impact of plasma on MDMs was entirely due to PGE₂. In this study there was no consistent relationship seen between these two measures. This could be due to the impact of other plasma mediators on the assay, other ligands binding albumin and preventing it from functioning.

These study samples were from a single arm study and all patients were treated with 20% HAS. Thus, the effect seen in this ex vivo assay could simply have been a time effect with patient's plasma becoming 'less immunosuppressive' to the monocyte derived macrophages over time as their overall clinical condition improved after hospitalization and treatment. Median time between pre/post treatment samples was 4 days, with an overall 25% improvement in bilirubin observed during that time, therefore this cannot be excluded as a confounder. However when patients are split into those with a high bilirubin throughout the trial (>80 μ mol/L) versus those with a lower bilirubin, there was no difference in magnitude of improvement in this bioassay. The same was seen for

patients with ACLF versus those without and those who had died at 3 months versus those that had not. The only way of accurately assessing the impact of treatment time on the assay is to have a control group of patients that are not treated with 20% HAS.

3.5.3. Targeted 20% HAS infusions had no overall effect in PGE₂ concentration in a small subgroup of patients

I was able to directly measure PGE₂ in 10 patient samples pre and post treatment which represented just under a fifth of samples analysed. There was no overall difference in PGE₂ levels post HAS treatment as compared to pre treatment. In the O'Brien, et al.¹¹ study plasma PGE₂ in AD patients was around 100pg/mL. In this study 4/10 patients had pre treatment PGE₂ in this range, 3 of this patients had a large decrease in measured PGE₂ post treatment and did not go onto develop infection. There was a trend toward increase in PGE₂ post treatment in those patients (n=5) who did go onto develop infection suggesting a lack of response. However, larger numbers may be needed to strengthen any conclusions. There was no consistent relationship between change in PGE₂ concentration in plasma and change in plasma mediated dampening of TNF α production in the MDM bioassay. This is possibly because measured levels are representative of total PGE₂ (bound plus unbound to albumin) and therefore may not be representative of true bioavailable PGE₂.

3.5.4. Targeted 20% HAS infusions had no effect on plasma pro/anti-inflammatory cytokines or endotoxin levels in this group of AD patients

There was no change after HAS treatment in circulating pro inflammatory cytokines (TNF α , IL1 β , IL6, IL8), anti-inflammatory cytokine (IL10) or endotoxin levels in 52 patient samples analysed. As shown previously, baseline levels of proinflammatory cytokines were overall higher than in healthy volunteers but varied.

There is conflict within the literature with regards to levels of circulating pro-inflammatory cytokines in decompensated liver cirrhosis^{29,32,34,122,123}. Historically acutely decompensated cirrhosis has been labeled a purely 'pro inflammatory state' with reported very high levels of inflammatory mediators^{15,72,94,124}. With so many of these patients suffering infection it is difficult to know whether much of these observed increased cytokine levels are a consequence of this, rather than a sterile proinflammatory state, as this was not taken into account in the largest cited observational cohort¹⁵. There is now a growing body of evidence which supports the

hypothesis that AD patients, particularly in their advanced stage of disease, have an inadequate response to a pathogenic stimulus and have a level of immunoparesis resulting in high levels of clinical infection. Cirrhosis-associated immune dysfunction (CAID) refers to both immunodeficiency and systemic inflammation that occur in cirrhosis. The previously conflicting reported phenotypes represent the extremes of a spectrum of reversible events that take place during the course of the patient's clinical pathway. Under constant challenge from bacterial product, the immune response in cirrhosis switches from a predominantly 'pro-inflammatory' phenotype in patients with 'stable' decompensated cirrhosis to a predominantly 'immunodeficient' one as disease progresses (figure 3.12).

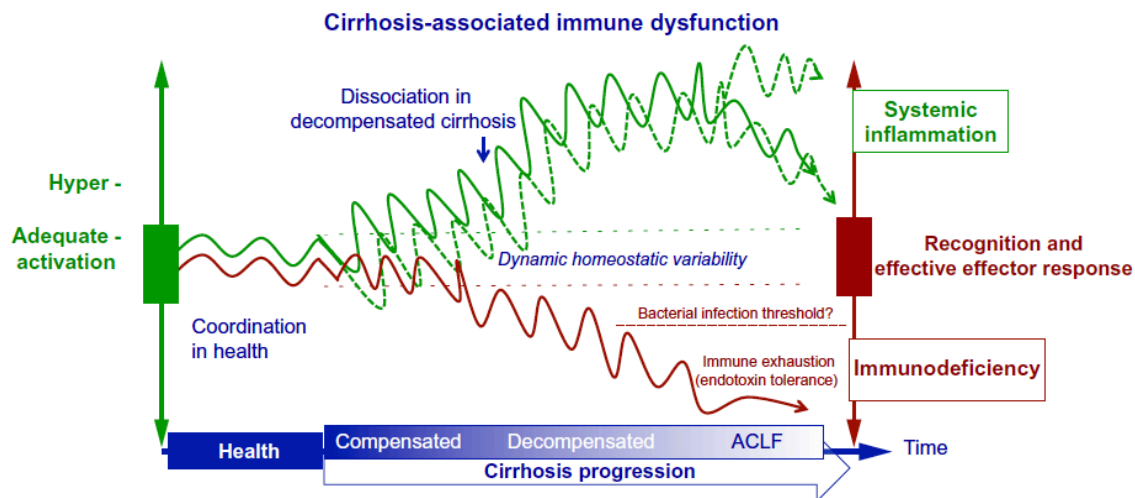


Figure 3.12 Cirrhosis-associated Immune Dysfunction. Taken from Albillos, et al. ¹²⁵

The previously reported conflicting cytokine profiles are reports from patients at different stages of their disease, possibly undergoing differing clinical events at the time of sampling. In this study patients with clinically diagnosed **baseline** infection did not have different **baseline** cytokine profiles, this was surprising and may reflect inaccuracy in the recording of an infection diagnosis at the time of study recruitment.

The small number of ACLF patients in this study were analysed as a subgroup. There were no overall differences in cytokine levels however endotoxin levels were higher and significantly decreased post treatment with HAS. Again, this could be due to a time effect and other treatments these very unwell patients received in hospital and needs to be explored with a control arm of patients who did not receive albumin.

3.5.5. In patients that develop infection there is a reversal in the initial improvement in plasma mediated MDM dysfunction

The LPS stimulated MDM assay was developed as a simple ex vivo measure of plasma mediated immune suppression and the impact that patient HAS treatment may have upon that. My hypothesis was that 20% HAS will reduce infection and its complications in AD patients. Nonetheless, as with any intervention, there will ultimately be a subgroup of patients who do not respond to HAS treatment. Therefore, samples from patients who developed infection, after at least 48hours of HAS treatment, in this single arm feasibility study were analysed. Plasma endotoxin and TNF α were higher at baseline in patients who went onto develop infection, although this was not statistically significant. sCD14, a marker of monocyte activation, and LPS binding protein were significantly higher in patients around the time of infection as compared to time matched AD patients without infection. These results demonstrate that these plasma markers may be a useful adjunct in the diagnosis of infection or identification of patients at high risk of developing infection.

It appears that although there is an initial improvement in plasma mediated suppression of TNF α production from LPS stimulated MDMs, this is lost over time in the patients who go onto develop infection. It is possible that there are other plasma mediators affecting the assay, however there was a definite improvement in MDM function when PGE₂ antagonists were applied suggesting PGE₂ is undeniably playing a role. Why this happens in these patients is uncertain, but one of the reasons may be the quality of their circulating albumin, which is evaluated in chapter 4 of this thesis. The finding was consistent with the use of a gram-positive stimulus (S.Aureus PTG) and a gram-negative stimulus (LPS), however the impact of PGE₂ antagonists was more pronounced with a gram positive stimulus. Even though not much is known about the downstream signaling of PGE₂, LPS acts via TLR4 where as PTG from gram-positive bacterial cell walls generally acts via TLR2¹²⁶ and this alternative pathway may provide some insight as to why the differential impact of PGE₂ antagonism on both receptors.

CHAPTER 4: INVESTIGATING THE BINDING AFFINITY OF ALBUMIN FOR PROSTAGLANDIN E2

Publications relating to this chapter:

Albumin Counteracts Immune-Suppressive Effects of Lipid Mediators in Patients With Advanced Liver Disease.

China L*, Maini A, Skene SS, Shabir Z, Sylvestre Y, Colas RA, Ly L, Becares Salles N, Belloti V, Dalli J, Gilroy DW, O'Brien A. *Clin Gastroenterol Hepatol*. 2018 May;16(5):738-747.

Presentations relating to this chapter:

Albumin Binding Capacity is Impaired in Decompensated Liver Cirrhosis and Dysfunction is Reversed by Targeted in vivo 20% Human Albumin Solution Infusions.

China L, Maini A, Gilroy D, O'Brien A. EASL 2017 (Amsterdam). JOURNAL OF HEPATOLOGY. ELSEVIER SCIENCE BV. 66: S390. Bursary awarded.

ATTIRE Stage 1 - Albumin To prevent Infection in chronic liver failure : a single-arm feasibility trial of targeted therapy with 20% Human Albumin Solution.

China L*, Skene S, Maini A, Shabir Z, Forrest E, O'Beirne J, Portal J, Ryder SD, Wright G, Gilroy D, O'Brien. *AASLD & EASL Masterclass, Florida. Poster presentation.*

Exploring treatment failures in a multicentre feasibility trial using human albumin solution to prevent infection in acute decompensation of liver cirrhosis.

China L, Becares N, Gilroy D, O'Brien A. Oral presentation (basic science) Vienna. Full bursary awarded. EASL 2019. JOURNAL OF HEPATOLOGY.

Contributions by other people to this chapter:

- R. Porcini & G. Verona (Amyloidosis Laboratory, Royal Free Hospital): Phosphoimaging of ³H-PGE₂ and plasma on agarose gel
- M. Rhead & A. Coker: technical work for HPLC

4.1 INTRODUCTION

It has previously been demonstrated that circulating concentrations of PGE₂ are significantly elevated in AD patients¹¹ and this was confirmed in analysis in chapter 3 (figure 3.8) of this thesis. My overarching hypothesis is that albumin binds to circulating PGE₂ and reduces its immune suppressive effect. In AD patients serum albumin concentrations fall by as much as 50%, as it is produced by the liver. Therefore administration of albumin intravenously to increase circulating levels should remove excess 'free' PGE₂ and improve immune function in AD patients.

However, even when levels of PGE₂ are raised in advanced liver disease patients, they are still in the low pg/mL (5-100pM) range¹²⁷. In comparison, albumin is present at 35-50g/L, (525-750µM) in healthy subjects or 20-30g/L (300-450µM) in patients with liver cirrhosis. Therefore there should, in theory, be considerably more than enough circulating albumin, even in AD patients, to bind and remove excess free PGE₂ which is a major challenge to my hypothesis to explain the possible immune restorative effect of albumin.

4.1.1. Background to the binding and breakdown of PGE₂ by Albumin

4.1.2.1. Albumin – ligand binding

Human Serum Albumin (HSA) is the most abundant protein in human blood plasma, accounting for approximately 60% of total plasma protein¹²⁸. It is synthesised in the liver and made of 585 amino acids with a molecular mass of 66,500 Da. Albumin consists of 3 domains: I, II and III. The first two loops (formed by disulphide bonds between adjacent cysteine residues) of each domain (loops 1-2, 4-5 and 7-8) are grouped together as subdomains IA, IIA and IIIA, while the third loop in each domain (loops 3, 6 and 9) are named subdomains IB, IIB and IIIB.

Sudlow's binding sites I and II, are located in subdomain IIA and IIIA respectively (figure 4.1). Many ligands, both endogenous and exogenous have been found to bind to Site I. Endogenous ligands include bilirubin, haematin, as well as prostaglandins, while exogenous ligands include warfarin, salicylate and indomethacin^{129:p103,130-135}. Ligands of Site I are typically bulky heterocyclic anions/dicarboxylic acids with the negative charge placed fairly central in the molecule^{129:p102,136}.

However, there is reason to believe that Site I is particularly flexible, in that it can bind ligands of very different chemical structures with very high affinity^{129:p102,135,137}. It has also been shown that single-residue mutations in Site I have very significant effects on the conformational and thermal stability of albumin, while similar mutations in Site II have much smaller effects^{138,139}. Therefore, the “cosmopolitan” reputation of albumin as a protein transporter is largely attributed to Site I, due to its ability to adapt to a wide variety of ligands, both endogenous and exogenous^{129:p104}.

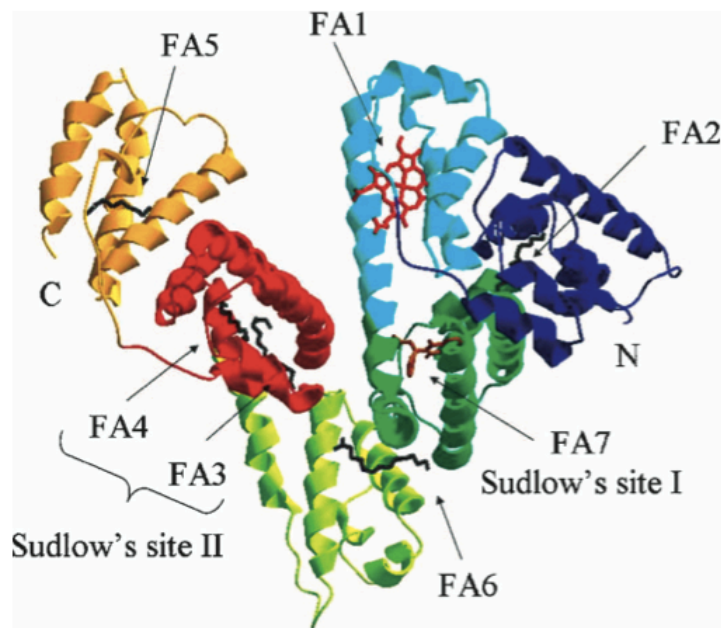


Figure 4.1. The Structure of Human Albumin. Taken from Fasano, et al. ¹⁴⁰.

The six subdomains of HSA are colored as follows: subdomain IA: blue; subdomain IB: cyan; subdomain IIA: dark green; subdomain IIB: light green; subdomain IIIA: red; subdomain IIIB: orange.

Site II is said to be less flexible compared to Site I^{136,141}. Ligands which bind to Site II are generally aromatic and can be neutral, should a charge be present. It is situated fairly peripherally on the molecule, away from the hydrophobic centre. Endogenous ligands which bind to Site II include L-tryptophan, L-thyroxine and chloride ions¹⁴¹⁻¹⁴⁴. Exogenous ligands include diazepam, ibuprofen, propofol and diclofenac^{129:p103,145-148}. HSA is also able to bind seven equivalents of long-chain fatty acids (FAs) at multiple binding sites with different affinities¹⁴⁰.

4.1.2.2. Modulation of binding sites in HSA

HSA undergoes pH and allosteric reversible conformational isomerization which can affect the capacity to bind ligands. At lower (acidic) pHs (4-7) HSA loses its α -helical

content and the resulting structural change causes alterations in its capacity to bind drugs and fatty acids. This is likely to have less of a consequence in vivo as physiological pH is tightly controlled; even extremely unwell patients who are termed 'acidotic' will rarely have a blood pH below 7.0.

There are many examples of endogenous and exogenous ligands causing allosteric modulation of Sudlow's binding sites in HSA. Perhaps one of the most relevant ones for my hypothesis is that of Nitric Oxide causing nitrosylation of the free Cys34 residue which causes a modification in the binding of anaesthetic agents at site I¹⁴⁹, which is where PGE₂ is thought to bind.

4.1.2.3. Albumin-prostaglandin binding

A series of binding studies using radiolabeled PGE₁, PGE₂, PGA₂, and PGF₂ found that the only plasma protein that significantly binds to the above prostaglandins is HSA¹⁵⁰.

Although the affinity of HSA for a variety of biologically active arachidonic acid metabolites is considered to be relatively low^{133,151}, the high serum albumin concentration (40 g/L) makes these interactions physiologically significant.

Competitive binding studies with warfarin and other site I ligands suggest that interactions of HSA with arachidonic acid metabolites¹⁵² occur at ligand binding site I on HSA. The effect of HSA on metabolism of the above arachidonic acid metabolites can be eliminated by adding high concentrations of ligands that compete for binding to site I, but not by ligands that bind to other sites on HSA.

Yang, et al.¹² conducted a study to obtain further insights into the above HSA/prostaglandin interaction by comparing the rate at which specific site-directed mutants of HSA with substitutions in subdomain IIA catalyze the breakdown of 15-keto-PGE₂ to the ketoenol tautomer intermediate and to the final reaction product PGB₂ (see figure 4.2). They chose to assay various subdomain IIA mutants for their ability to convert 15-keto-PGE₂ to the ketoenol tautomers.

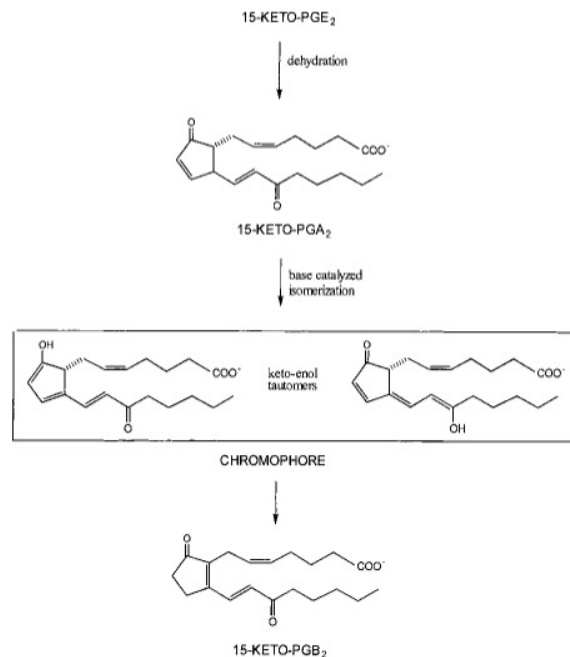


Figure 4.2. Taken from Yang, et al. ¹²showing the proposed mechanism by which 15-keto- PGE₂ is converted to 15-keto-PGB₂

They concluded that specific amino acid residues within site I were responsible for a 2 step catalytic process in the breakdown of 15-Keto-PGE₂ and altering the pH of the binding site effected this process, however these were not pHs that would be consistent with life (pH >10). Their results also suggested that around half of the PGE₂ added was metabolized by 4 hours. However this was a spectrophotometric assay and we do not know what the effects of 15-Keto-labelling on PGE₂ metabolism really are as it is not a substance that exists in vivo and 15-Keto labeling itself may well effect usual mechanisms of PGE₂ binding.

4.1.2.2. Methods of assessing binding capacity

When assessing the capacity for a protein such as albumin to bind to a ligand the simplest method is to mix known concentrations of each and then measure free and bound ligand to calculate the percentage of ligand bound. The equilibrium dissociation constant (K_d) is the concentration of a ligand that occupies half of a receptor population (e.g. Sudlow's site I on HSA). It is a measure of binding affinity for a ligand to a receptor, the higher the value the more ligand required to achieve 50% binding site saturation – and therefore the weaker the binding affinity.

Currently used methods for free ligand measurement in binding assays include equilibrium dialysis, ultrafiltration, microdialysis, ultracentrifugation, and fluorescence spectroscopy as well as chromatography and capillary electrophoresis. Each has both advantages and limitations. Independent of the specific method used to determine the free ligand fraction, factors that can impact protein binding should be maintained within physiologic conditions in order to mimic the *in vivo* situation.

4.1.2.4. Techniques to measure plasma protein binding in vitro

Although there is no standard method for measurement *in vitro*, equilibrium dialysis is often regarded as the gold standard (and therefore the reference when comparing other techniques) for determining the protein binding profile of a drug¹⁵³. Equilibrium dialysis is relatively labor intensive but precise¹⁵⁴. However a concern is that, depending on the membrane material and ligand concentration, a fraction of the ligand may be absorbed by the dialysis membrane. Therefore this should be taken into account when calculating the free ligand concentration in equilibrium dialysis experiments.

In ultrafiltration, another widely used method for determination of plasma protein binding, centrifugal forces are usually employed as the driving force for the passage of plasma across a filter membrane. Adsorption of ligand by ultrafiltrate membranes may be problematic but can be compensated for by taking into account measurements obtained from conducting preliminary experiments in protein free solute. Additionally as the protein concentration in the plasma sample is increased during the filtration of diluted plasma, only a small volume of ultrafiltrate should be collected, since the protein concentration in the upper reservoir rises during the filtration process.

Fluorescence spectroscopy, chromatography, and capillary electrophoresis are now rarely used in this field¹⁵⁵. Fluorophore labeling of PGE₂ is possible, however custom synthesis is expensive. The molecular weight and physicochemical properties of fluorophore-labelled PGE₂ are substantially different from the native molecule, and, as the only site of labeling is at the carboxyl group (which is believed to be essential for binding), the ligand may not exhibit full binding activity. Surrogate markers of binding (NMR shift, changes in fluorescence) would need to be validated by authentic binding assays.

4.1.2.5. *Techniques to measure plasma protein binding in vivo*

Microdialysis can be used *in vivo* to determine unbound drug concentrations in circulating blood vessels¹⁵⁶. Microdialysis examines the diffusion of substances along their concentration gradient from blood into the dialysate. It is an invasive procedure as a probe containing a dialysis membrane has to be surgically implanted into a blood vessel and then a dialysate is pumped through the probe. The unbound ligand in the plasma diffuses across the membrane into the probe. According to the molecular weight cutoff of the semipermeable membrane, large molecules like proteins will be retained by the membrane. Microdialysate samples can then be collected over time for subsequent analysis of the free fraction of a ligand. This offers the significant advantages of *in vivo* measurement however, due to the small volumes of dialysate, sensitive analytical techniques are required to measure ligand concentrations. This would be a significant problem with PGE₂ as we would have to implant an invasive devices, theoretically prone to infection at the site, into acutely unwell patients with decompensated liver cirrhosis.

4.1.2. **Albumin dysfunction in liver cirrhosis**

HSA is often present at low concentrations in liver cirrhosis, due to decreased production and, at times, increased catabolism (e.g. sepsis). In addition the albumin that is present may not function well, with regards to binding capacity, due to multiple post-transcriptional modifications when circulating in the unwell patient.

Post translational alterations to the albumin molecule have been observed in patients with AD cirrhosis, resulting from oxidation, enzymatic and non-enzymatic glycosylation and truncation of terminals, all of which are likely to affect its function⁶⁰. These post-translational changes result in the formation of different structural isoforms of HSA, the proportions of which vary within patients with liver cirrhosis. It has been found that the relative abundance of the native, unaltered HSA isoform is negatively correlated with Child-Pugh and Model for End-Stage Liver Disease (MELD) prognostic scores in liver cirrhosis patients¹⁵⁷.

Domenicali, et al.⁶⁰ found that cysteinylolation of the free Cys-34 residue was the most frequently observed alteration in liver cirrhosis patients, occurring alone or in combination with other post-transcriptional changes. Significant increases in relative abundances of altered HSA isoforms, namely the C-terminal truncated form (HSA-L) and N-terminal truncated form with cysteinylolation of the Cys-34 residue (HSA+CYS-DA), as

well as a significant reduction of native HSA isoform, are all observed in liver cirrhosis patients who develop bacterial infections¹⁵⁷.

In healthy adults, about 70– 80% of the Cys34 in albumin contains a free sulphhydryl group (human mercaptalbumin, HMA); 25% of the Cys34 forms a disulphide with small sulphhydryl compounds like another cysteine, homocysteine or glutathione (human nonmercaptalbumin1, HNA1); and a small fraction of the Cys34 is more highly oxidized to the sulphinic or sulphonic acid form (human nonmercaptalbumin2, HNA2)¹⁵⁸. Oettl, et al.¹⁵⁹ found increased HNA2 in patients with decompensated liver cirrhosis and in a subsequent study¹⁶⁰ found that this HNA2 had decreased binding capacity for dansylsarcosine and increased levels of HNA2 correlated with poor prognosis in liver cirrhosis.

Increases in relative abundances of various HSA isoforms have also been associated with complications common in patients with liver cirrhosis, such as ascites and renal impairment, as well as with diabetes mellitus¹⁶¹. Unsurprisingly, the residual proportion of the native HSA isoform present in circulation has been demonstrated to be a predictor of 1-year survival, as the altered HSA isoforms increase in parallel with the progression of liver cirrhosis. Additionally other studies have focused on albumin N-terminus binding function in AD patients and found a decreased binding capacity to cobalt may have prognostic significance¹⁴, however it is difficult to interpret the significance of this assay in vivo and whether the correlation with poor outcome is simply related to other prognostic factors that have not been corrected for in the analysis.

It may be that the amount of structurally-preserved, native HSA is far lower than the total serum albumin concentration in liver cirrhosis¹⁵⁷ which certainly has implications when considering giving these patients 'healthy albumin' in the form of 20% HAS infusions. There is little published work looking at whether administering albumin infusions actually changes levels of 'damaged' circulating albumin in patients and none which look at changes in binding capacity in liver cirrhosis patients. A recently published study in pig models, looking at the effects of using an extracorporeal liver assist device plus albumin infusions found that those animals which had albumin infusions in addition to 'toxin removal' with the device had lower levels of HNA2 as compared to animals not receiving albumin infusion¹⁶² it is unclear whether this has functional significance in vivo.

Finally AD patients will have increased endogenous (e.g. bilirubin) and exogenous (e.g. antibiotics) ligands that will compete for binding sites on circulating albumin. This is also likely to have an impact on the binding capacity of albumin for PGE₂ in vivo and has not been investigated to date.

4.1.3. Potential differences between Human Albumin Solutions manufactured by diverse commercial producers of albumin

In the UK there are multiple manufacturers of 20% HAS for infusion. Different hospitals will use different suppliers according to negotiated procurement rates. There are certain specifications required by the European medicines agency (purity, concentration, contamination) but different suppliers will have slightly differing manufacturing processing and stabilisers in their solutions.

6 commercially available albumin solutions for infusion were analysed by Bar-Or, et al.¹⁶³. Using positive electrospray ionization, time-of-flight mass spectrometry, various posttranslational modifications were identified within these solutions. They found high levels of oxidation at the Cys34 residue in the commercial preparations (57.2 +/-3.3%) as compared to albumin isolated from the plasma of healthy volunteers (22.9 +/-4.8%). In addition there were differences between suppliers and between different batches from the same supplier. They did not analyse any potential functional effects (such as binding) but theorized that this could have an impact on how well albumin functions as an 'anti-oxidant' in vivo.

Zenalb 20	Albunorm 20%
Sodium 50-120 mmol/L Potassium Chloride Citrate Sodium n-octanoate Zenalb® 20 contains not more than 200 µg/L of aluminium	Sodium chloride 5.7 g/l N-acetyl-DL-tryptophan 3.9 g/l Caprylic acid 2.3 g/l Sodium 144-160 mmol/l

Table 4.1. A comparison of excipients in 20% HAS for infusion from two different manufacturers.

In the UK there is one commercial supplier of recombinant human albumin for infusion (Albumedix Ltd). Comparisons of recombinant albumin for infusion and albumin for infusion from donated blood products have suggested that recombinant albumin contains a lower percentage of oxidized Cys34 residues and is more consistent between batches^{164,165}. In addition it has been evaluated in a small RCT (71 patients) in patients with cirrhotic ascites and found to be safe as compared to standard 20% HAS. However

the production of recombinant albumin is expensive and therefore this line of supply has not been taken forward by manufacturers of recombinant albumin for infusion.

To summarise:

- The binding efficacy of albumin-PGE₂ is likely to be low, the exact K_d is unknown
- Albumin is likely to be not only low but functionally deficient in AD patients
 - Biologically relevant investigation of this functional deficit is lacking in the literature
 - There are no studies evaluating whether administration of 20% HAS (pooled from healthy donors) to AD patients changes albumin function
- The 20% HAS from different manufacturers, or different batches, appear to have varying levels of post transcriptional modifications and it has not been established whether this has any functional consequence.

Chapter Aims:

- Establish an albumin-PGE₂ binding affinity assay in order to:
 - Compare different commercial preparations of 20% Human Albumin Solution (HAS) for infusion available in the UK
 - Evaluate whether this can assess PGE₂ binding to plasma proteins in healthy individuals' plasma and whether this differs in patients with acute decompensation of chronic liver disease
 - Determine if there is an improvement in plasma protein binding of PGE₂ in AD patients after infusion with 20% HAS, and explore whether this changes in patients who develop an infection

4.2. METHODS

4.2.1. Labelled PGE₂

PGE₂ labeled with tritium (³H-PGE₂) was chosen as the method of measuring ligand levels for these experiments (Figure 4.3). Tritium (³H) is a radioactive isotope of hydrogen which emits beta decay via the loss of an electron from the nucleus when a neutron transforms into a proton. It is very stable, having a long half life of approximately 12.3 years and emits very low energy in the process thus making it easy to work with and store. However this does mean it can only be measured using liquid scintillation counting. ³H-PGE₂ was supplied by Perkin Elmer (UK, product no. NET428025UC).

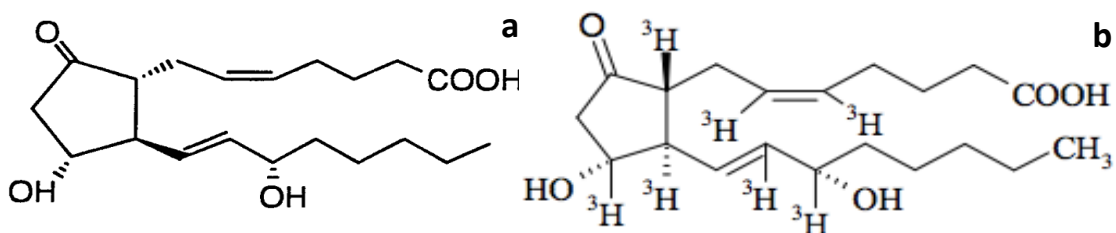


Figure 4.3. (a) Unlabeled Prostaglandin E₂ (b) Tritium labeled Prostaglandin E₂

³H-PGE₂ is expensive and that supplied contains around 340,000cpm/pmol. Therefore to work at nM or μ M concentrations of PGE₂ it has to be mixed with 'cold' unlabelled PGE₂. After the assay was established I used a consistent ratio of cold PGE₂: ³H-PGE₂ of 2727:1. An example of this is:

- 200 μ l cold PGE₂ at 25 μ g/mL or 70.92 μ M (14184.4 pmol PGE₂) with an added 8 μ l of supplied ³H-PGE₂ (which contains 5.2pmol PGE₂ and 1,776,000 counts).
- Therefore the final solution contains 68.22 μ M PGE₂ and 125.2cpm/pmol

In plasma equilibrium dialysis experiments 10 μ l of this 68.22 μ M PGE₂/³H-PGE₂ mix would be added to 240 μ l of plasma giving an end concentration of 2.73 μ M.

4.2.2. Biospin

Micro Bio-Spin 6 columns (Bio-rad, UK) were used in the initial attempt to evaluate albumin-PGE₂ K_d (Figure 4.4).

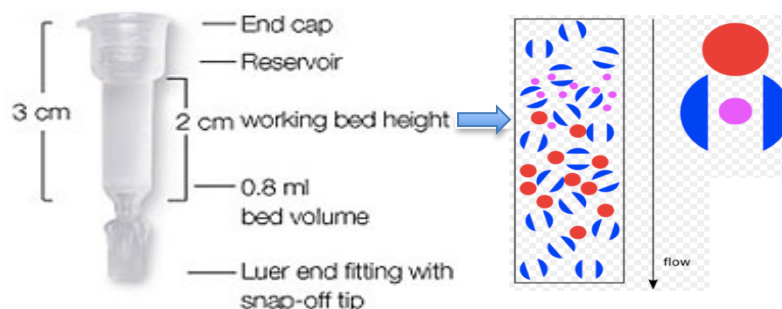


Figure 4.4. Biospin-6 (bio-rad) with cartoon illustrating albumin (red) passing through the column with unbound PGE₂ (pink) remaining on the column.

These columns contain 'Bio-Gel' hydrated in Tris buffer and remove compounds <6kD by size exclusion chromatography. 50µl of human defatted albumin (lyophilized powder, Fatty acid free, Globulin free, ≥99%, A3782, Sigma-Aldrich, USA) at 1.3µM and 0.13µM was incubated with varying concentrations of 10µl 3H-PGE₂ plus unlabelled PGE₂ (total PGE₂ concentrations used were 0-150µM) for 30 minutes. Following this all 60µl was added to the reservoir of a bio-spin. The biospin was sat within a 1.5mL eppendorf and centrifuged at 3000rpm for 4 minutes. In theory unbound PGE₂ would remain on the column and PGE₂ bound to albumin would pass through the column into the eppendorf. Subsequently all bound PGE₂ could be measured (see scintillation counting below) and the % bound calculated for each concentration of PGE₂. Analysis of multiple concentrations of PGE₂ would then generate a binding curve from which the K_d could be calculated.

4.2.3. ³H-E₂ equilibrium dialysis

Equilibrium dialysis using a Thermo Scientific™(USA) Single-Use RED (rapid equilibrium dialysis) Plate was used with varying concentrations of albumin and constant amounts of PGE₂/3H-PGE₂ (Perkin Elmer, UK), or vice versa, to establish a concentration of sigma defatted albumin at which 50% of PGE₂ would bind (see figure 4.5). 10µl of PGE₂/3H-PGE₂ was incubated with 240µl HAS, plasma or control for 30minutes (concentrations varied depending on the experiment and whether the protein or the PGE₂ was kept constant). Whenever possible 3 technical repeats for each sample were obtained. Samples were then dialysed against PBS in the red plate for 4 hours at 37°C. Counts from sample and buffer were then measured and %bound was calculated using: % Bound = 100 – ((cpm buffer chamber/cpm plasma chamber) × 100). Results

can be presented and plotted as % bound or concentration bound against concentration free (calculating back to concentrations as counts per pmol of total PGE₂ were known).

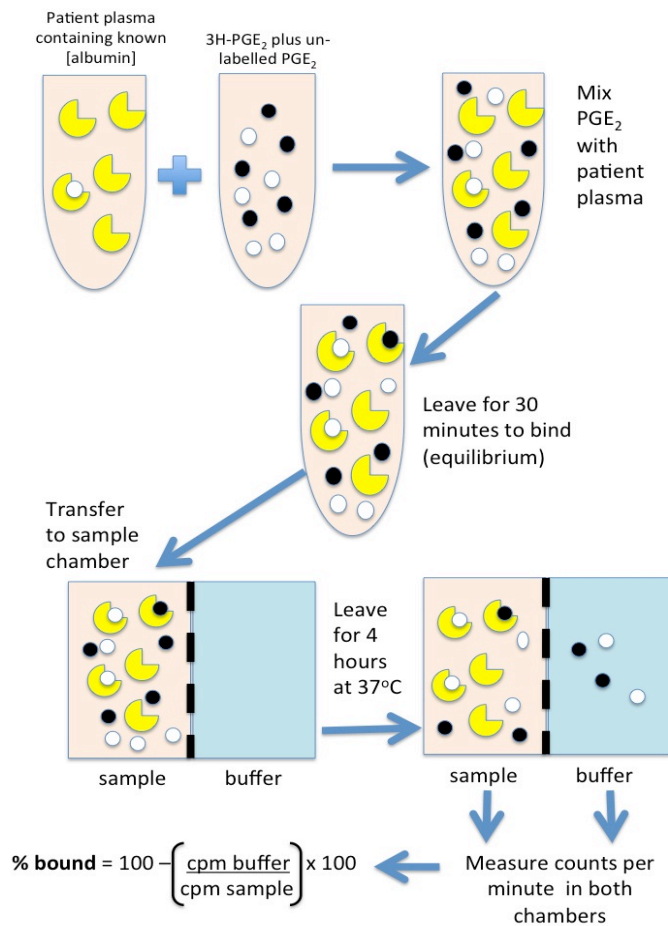


Figure 4.5. Illustration of equilibrium dialysis with RED plate.

Scintillation counting

This operates by detecting ‘scintillations’ produced when radiation interacts with certain chemicals called fluors within scintillation fluid. Usually, 150µl of sample to be counted was dissolved in 5mLs of scintillation fluid (EcoScint A, SLS Ltd, UK) in 20mL polypropylene counting vials (Thermo Fisher Scientific, S31). Vials were shaken and then placed in racks within a counter. Reference ranges were taken prior to counting of the samples.

4.2.4. Calculation of the concentration of albumin in commercial 20% HAS

4.2.3.1. UV spectrophotometer

A spectrophotometer was used to check the concentration of 20% HAS from different manufacturers. 20% HAS was diluted to 1mg/mL (assuming stated concentration of 20% was accurate) and placed in a 1mL cuvette. 3 readings from each sample was taken and compared to a known concentration of defatted human albumin (99% pure) in PBS (Sigma Aldrich, USA).

4.2.3.2. Bromocresol Green

BCG (Bromocresol Green) Albumin Assay Kit (MAK 124, Sigma Aldrich, USA) was used to measure albumin concentration in 20% HAS for infusion. 5 mL of diluted standards, blank, and diluted samples were added to appropriate wells of a clear bottom plate followed by 200 mL of supplied bromocresol green reagent and then tapped lightly to mix. This was incubated for 5 minutes at room temperature and absorbance at 570–670 nm (peak absorbance at 620 nm) measured.

4.2.5. Phosphoimaging: H³-PGE₂ and plasma

Conducted by R. Porcinni and G. Verona at The Royal Free Hospital Amyloidosis Laboratory (under supervision of Dr G Taylor).

4.2.6. Peripheral Blood Collection and Patient Samples

As described in section 3.2.1.

4.2.7. HPLC analysis of plasma

Albumin was fractionated by high performance liquid chromatography to give three peaks according to cysteine-34 in the free sulfhydryl form (HMA), as a mixed disulfide (HNA1) or in a higher oxidation state (HNA2). Plasma was diluted 1:4 with sample buffer: 0.2M dibasic sodium phosphate (49 parts), 0.2M monobasic sodium phosphate (51 parts) with 0.3M NaCl with a pH of 6.8. All solvents and solutions were filtered through a filter unit (0.22µm, Sterivex-GS, Millipore, Billerica, MA, USA) prior to use. 10µl of diluted plasma was injected into the HPLC system (AKTA pure™, GE Healthcare Life Sciences, UK) using a Shodex Asahipak ES-502N 7C anion exchange column (Showa Denko, Europe) and 0.2M sodium acetate, 0.4M sodium sulfate, pH 4.85 as mobile phase. For elution, a gradient of 0–6% ethanol and a flow rate of 0.6 mL/min were used. The column was kept at room temperature. Detection was carried out by

fluorescence at 280/340nm. The HPLC data were subjected to numerical curve fitting, and each albumin peak shape was approximated by a Gaussian function for calculation of the area under the peak. Quantification was based on peak heights determined by chromatography software (Unicorn 7.3 Evaluation Classic).

4.2.8. Statistical methods

Data is presented as mean +/- standard deviation (s.d). Differences were considered significant at $p < 0.05$ by a two tailed student t-test. Two-tailed (paired for pre/post treatment samples, unpaired for other comparisons) was used when comparing groups of values with a normal distribution whose means were not expected to be equal. For correlation studies the R value was calculated by Pearson's correlation coefficient to assess linear covariance of two variables. r^2 is presented with p value for the confidence interval of r.

For binding studies K_d (dissociation constant) was calculated from binding curves using non-linear regression and assuming a single binding site with no competition in Graphpad prism (version 8.0). For theoretical estimates of free ligand based on altering K_d with altering albumin concentrations the following formulas were used in excel (supervised by Dr G Taylor, RFH Amyloidosis Centre):

P=protein, Pt = total protein, Pf= free protein, L=ligand, Lt = total ligan, Lf = free ligand

$$K_d = \frac{[P] \cdot [L]}{[PL]}$$

$$K_d = \frac{(P_t - PL) \cdot (L_t - PL)}{PL}$$

$$K_d \cdot PL = P_t \cdot L_t - P_t \cdot PL - PL \cdot L_t + PL^2$$

$$PL^2 - k_d \cdot PL - P_t \cdot PL - L_t \cdot PL + P_t \cdot L_t = PL^2 - PL \cdot (k_d + P_t + L_t) + P_t \cdot L_t$$

$$aX^2 + bX + c \quad (a=1, b= K_d + P_t + L_t, C= P_t \cdot L_t)$$

$$\text{so: } x = \frac{-b \pm \sqrt{b^2 - 4Ac}}{2a}$$

4.3 RESULTS

4.3.1. Albumin binds to PGE₂ with a weak affinity

4.3.1.1. Establishing an estimated K_d of Sigma defatted albumin-PGE₂

Biospin method

Varying concentrations of PGE₂/³H-PGE₂ (referred to as PGE₂ in subsequent text) were incubated initially with 0.13μM albumin and run through biospin columns, as described. Even with very high concentrations of PGE₂, only tiny amounts of bound PGE₂ could be measured (figure 4.6Ai). It was possible that PGE₂ was so weakly bound to albumin that it was being removed in the centrifugation process and nearly all adhered to the biospin column (see figure 4.4 in methods for schematic).

The experiment was repeated using ten times the concentration of albumin initially used (a constant of 1.3μM) which produced the same binding curve (figure 4.6Aii) but with twice the amount of PGE₂ bound as previously. However, this was still a very small proportion of the total starting PGE₂ and at lower concentrations of PGE₂ (still considerably higher than physiological ranges) 'counts bound' were the same as measured background i.e. no PGE₂ bound.

Unfortunately using the biospin method, the material contained on the column could not be counted as it was solid. Therefore there was no certainty regarding the efficacy of the column in retaining free PGE₂ and multiple assumptions were necessary to calculate binding efficacy. This method was quick, low cost and had determined that the binding efficacy was likely to be low. However due to the assumptions that were required, it was abandoned as a poor method to use with a ligand that binds weakly.

Equilibrium dialysis

Equilibrium dialysis enabled the measurement of both free and bound PGE₂. Initially increasing concentrations of PGE₂ (1-250μM, specific activity 8.9cpm/pmol) were incubated with 100μM of albumin prior to dialysis. Results (figure 4.6) suggested that in order to get towards near 100% of PGE₂ bound, huge concentrations of PGE₂ would be required (i.e. the binding affinity was very low).

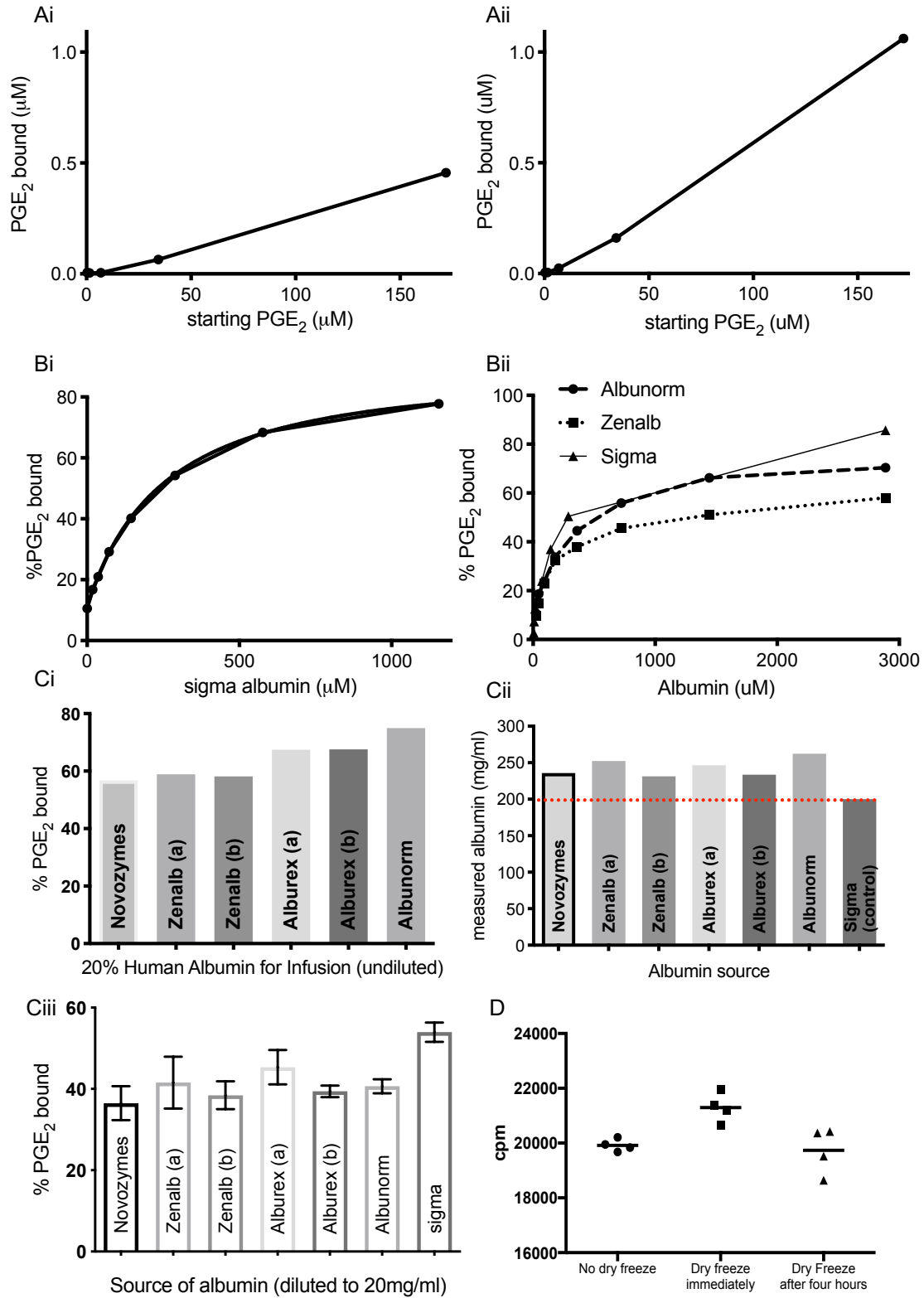


Figure 4.6. The binding affinity of Albumin to PGE₂ is low

[A] The biospin column could not confidently be used to estimate the amount of PGE₂ bound to albumin (i) using 0.13uM albumin (constant) very little of the total starting PGE₂ was measured as bound to the albumin

(ii) albumin concentration was increased tenfold to 1.3 μ M (constant) but only tiny amounts of PGE₂ were able to be measured as bound.

[B] (i) Equilibrium dialysis of sigma albumin (varying, 0-1200 μ M) with PGE₂ (2.73 μ M constant). Least squares fit of the resulting binding curve gave a K_d of 271.1 μ M (CI 210.6 μ M to 331.5 μ M), B_{max} 85.62, r² 0.998. (ii) Equilibrium dialysis of sigma albumin and two types of albumin for infusion: zenalb and alburnorm (varying, 0-3000 μ M) with PGE₂ (2.73 μ M constant). Less PGE₂ was bound to the albumin for infusion at all dilutions.

[C] (i) PGE₂ bound to undiluted 20% HAS for infusion from different manufacturers (a/b refer to different batches). No technical repeats due to the expense of the dialysis plate (ii) Albumin concentrations in HAS for infusion using the BCG assay, sigma albumin diluted to 200mg/mL used as a control. (iii). % PGE₂ bound using HAS from difference sources diluted to an albumin concentration of 20mg/mL or Sigma albumin made to a concentration of 20mg/mL in PBS. (*technical repeats =3, mean and s.d. shown*).

[D] Low B_{max} was not due to metabolism and formation of ³H water from ³H-PGE₂. ³H-PGE₂ was left for 4 hours (length of rapid dialysis) and compared to sample that had been freeze dried immediately. There was no significant or meaningful difference in cpm (n=4 technical repeats, line represents mean).

Unger ¹³³ evaluated binding of ³H-PGE₁ using equilibrium dialysis. In his study concentrations of the protein (albumin) were altered rather than that of the ligand (PGE₁). The author does not state why but it may have been to do with difficulties reaching near saturation due to the low binding affinity of PGE₁ to albumin. I therefore decided to adopt this approach and keep PGE₂ constant but alter concentrations of albumin using similar concentrations to those stated in this paper.

Using this method it was possible to produce binding curves and calculate the K_d of defatted sigma albumin – PGE₂ to be around 270 μ M (figure 4.6Bi). For this calculation a single site binding was assumed. B_{max} is the maximum binding in the same units as Y and can be estimated at the plateau of the binding curve (saturation). The B_{max} in figure 4.6Bi was lower than expected which could possibly have been secondary to formation of ³H water from ³H-PGE₂ during the dialysis time. ³H-PGE₂ in solution was freeze dried immediately and at 4 hours with meaningful differences in measured counts which suggests that ³H water formation was not having an impact on the assay.

When evaluating what in vivo significance this K_d value might have, it is useful to examine some estimated calculations (Table 4.2a and 4.2b). Depending on the total available PGE₂ and the K_d of albumin, the free (unbound) PGE₂ will alter. For example, decreasing the albumin from healthy adult range (40mg/mL or 600 μ M) to concentrations observed in AD patients (20mg/mL or 300 μ M) with a high K_d (poor affinity) will mean that increasing PGE₂ from low to high levels could theoretically result in a much higher fold increase in circulating free PGE₂ to values that have been demonstrated to be immune suppressive in cell culture experiments.

Albumin 40g/L (or 600μM) = lower range of healthy volunteer plasma				
	Kd = 0.02 μ M	Kd = 2 μ M	Kd = 200 μ M	Kd = 270 μ M
Total available PGE ₂ (pg/mL)	Free (unbound) PGE₂ (pg/mL)			
8.8125	0.0003	0.0293	2.2031	2.7349
17.625	0.0006	0.0586	4.4062	5.4698
35.25	0.0012	0.1171	8.8125	10.939
70.5	0.0023	0.2342	17.625	21.879
105.75	0.0035	0.3513	26.437	32.818

Table 4.2a. How change in available total PGE₂ and Kd may change circulating free PGE₂ in the presence of normal range serum albumin.

Albumin 20g/L (or 300μM) = value of a decompensated cirrhosis patient					
	Kd = 0.02 μ M	Kd = 2 μ M	Kd = 200 μ M	Kd = 270 μ M	Kd = 500 μ M
Total available PGE ₂ (pg/mL)	Free (unbound) PGE₂ (pg/mL)				
8.8125	0.0006	0.0584	3.5250	4.1743	5.5078
17.625	0.0012	0.1167	7.0500	8.3487	11.0156
35.25	0.0023	0.2334	14.1000	16.6974	22.0313
70.5	0.0047	0.4669	28.2000	33.3947	44.0625
105.75	0.0070	0.7003	42.3000	50.0921	66.0938

Table 4.2b. How change in available total PGE₂ and Kd may change circulating free PGE₂ in the presence of normal range serum albumin.

4.3.1.4. Comparison of PGE₂ binding using albumin for infusion

UV spectrophotometer to check solution concentrations found an exceptionally high absorbance spectrum of Alburnorm which is likely due to the stabilizer: N-acetyl-DL-tryptophan (3.9 g/l) as an additive to Alburnorm therefore only the bromocresol green (specific binding to albumin) method could be used to measure albumin in solution concentrations.

Two preparations of HAS for infusion (Zenalb and Alburnorm) were compared at varying concentrations to defatted albumin from Sigma (figure 4.6Bii). Alburnorm appeared to have better binding efficacy compared to Zenalb but not to that of sigma albumin. Due to these apparent differences between manufacturers the comparison was repeated using undiluted 20% HAS for infusion from three manufacturers of 20% HAS from

donated human blood (Zenalb (BPL), Alburnorm (Octapharm), Alburex (CSL Behring)) and recombinant albumin for infusion (Novozymes, Albumedix). 2 different batches (a,b) of Zenalb and Alburex were used. Differences in the % PGE₂ bound (2.73 μ M) ranged from 56.8% (Novozymes) to 74.9% (Alburnorm) (figure 4.6Ci).

It had been assumed for these calculations that all samples had 200mg/mL albumin present, however, when the concentrations of albumin in these solutions were measured there were marked variation in the supplied solutions (figure 4.6Cii). Using the actual measured concentrations the HAS samples were diluted to a concentration of 20mg/mL (300 μ M) albumin (figure 4.6Ciii) and, on this occasion, there were only small, non-significant differences between the HAS samples. Defatted Sigma Albumin bound more PGE₂ than all of the HAS or rHAS at equivalent concentrations.

4.3.2. Plasma protein binding to PGE₂ using equilibrium dialysis

Initial attempts to isolate albumin from plasma using ammonium sulphate precipitation (not described in this thesis) and subsequent PBS dialysis produced yields of albumin of approximately 10% of the starting plasma concentration. Therefore this was deemed to be a poor method to evaluate albumin present in plasma. Dr R.Porcini (Belloti lab) evaluated binding of healthy volunteer plasma (proteins) and ³H-PGE₂ using phosphoimaging. We found that ³H-PGE₂ was only bound at the 66.5KDa band (i.e. the size of HSA) and not elsewhere suggesting that ³H-PGE₂ was only binding to albumin within the plasma (figure 4.7). There have been similar reports within the literature to support this¹⁵⁰. Therefore I proceeded to focus on 'plasma protein binding' (likely all albumin) as it was likely to have more realistic in vivo comparisons.

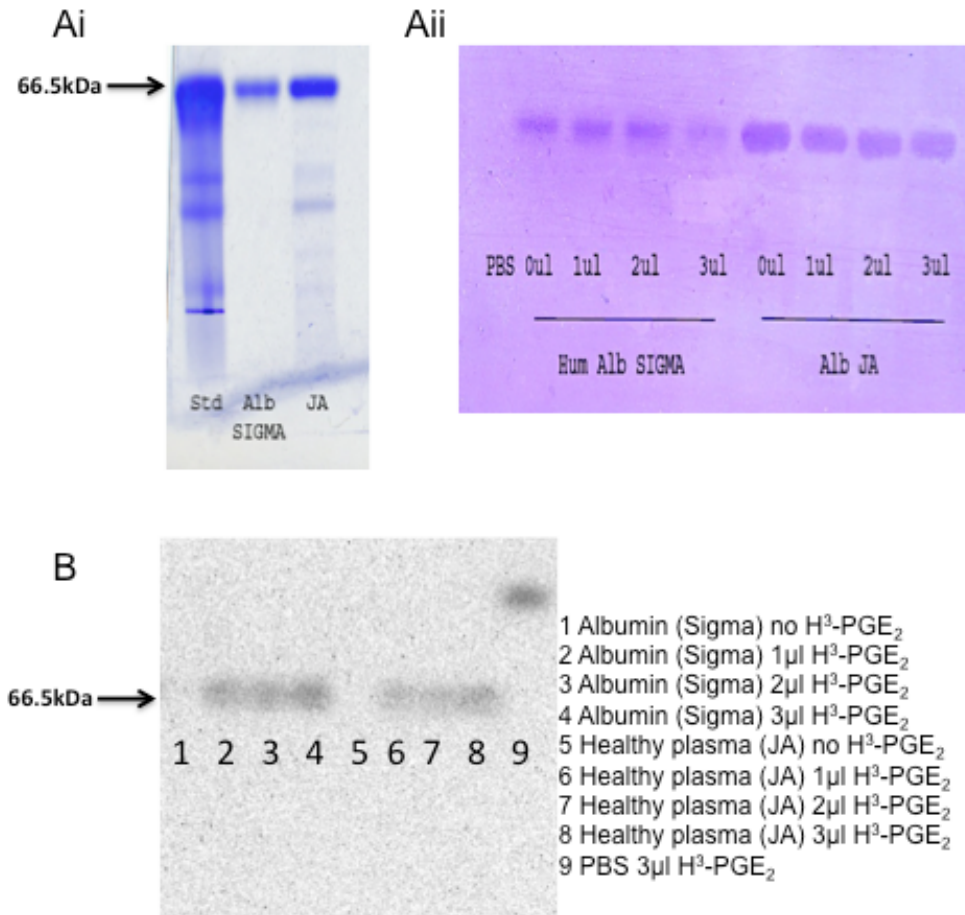


Figure 4.7. H³-PGE₂ binds to albumin in plasma but not other plasma proteins

[Ai] Agarose gel with Human Albumin from sigma and Healthy volunteer plasma (JA) showing a clear band at the 66.5kDa position (molecular weight of albumin) (ii) in the absence or presence of different volumes of H³-PGE₂. [B] phosphor plate radiography of radiolabelled (H³) PGE₂ and albumin .vs. plasma .vs. PBS after running through an agarose gel shows the H³-PGE₂ only appearing at the molecular weight of albumin in the plasma sample.

Approximately 50% of PGE₂ was bound using 2.73μM PGE₂ and 300μM albumin and the rapid equilibrium dialysis (RED) plate. Therefore I worked around these concentrations to begin a comparison of plasma protein (assumed albumin) binding to PGE₂. PGE₂ binding to 8 different healthy volunteers (HV) plasma was assessed initially at starting concentrations of albumin 692-842μM (mean 750μM) and then diluted to 217μM. Inter-volunteer variability was small and slightly higher when plasma was diluted to the same albumin concentration (figure 4.8Ai).

Comparing these 8 HV plasma to 8 acutely decompensated (AD) patient plasma samples, undiluted AD plasma bound a mean of 15.2% less PGE₂ as compared to HV

(77.1% v 61.9%, CI -23.3 to -7.15, $p=0.0012$) (figure 4.8Aii). These AD patients had a mean albumin of 30.25g/L (454.8 μ M) compared to 49.9g/L (750 μ M) in the HV therefore this may have simply been due a concentration difference in albumin. However when plasma was diluted to the same concentration of albumin (217 μ M) a non-significant decrease in binding with AD plasma of -7.1% (CI -22.09 to 7.944) was still present with a much wider spread between patient samples supporting AD plasma binding PGE₂ less efficaciously than HV (Figure 4.8. Aii).

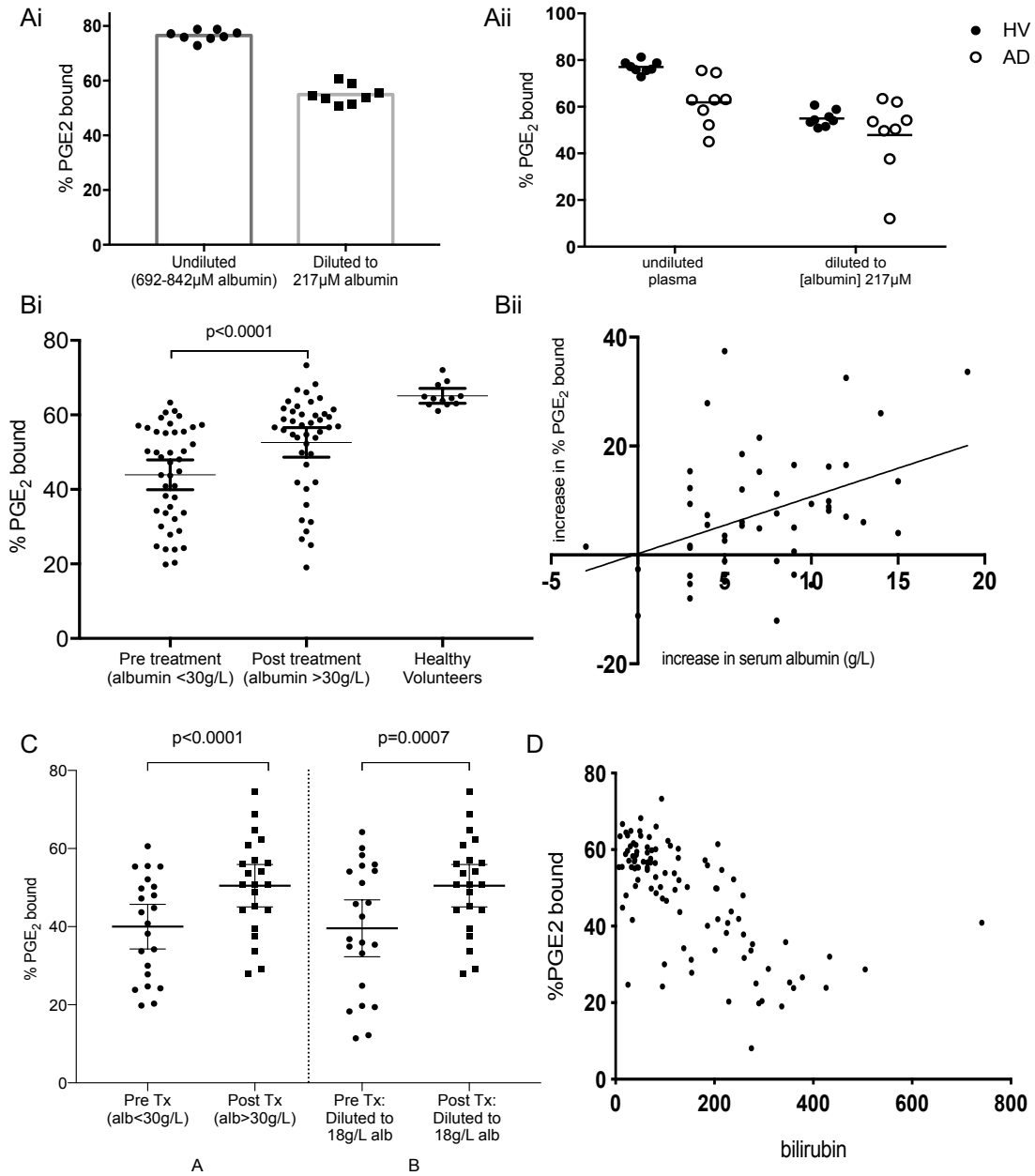


Figure 4.8. Targeted 20% Human Albumin Solution Infusions Improved AD Plasma Ability to Bind Prostaglandin E₂ by Increasing Albumin Concentration and Functional Binding Capacity
[A](i) Healthy volunteers (n=8) plasma bound to PGE₂. Albumin concentrations varied from 692-842μM (mean 750μM), each sample was diluted to the same albumin concentration of 217μM albumin. **(ii)** Plasma from n=8 AD patients bound less PGE₂ than healthy volunteer plasma (n=8). The difference was smaller when all plasma was diluted to the same albumin concentration. **[B](i)** Post-HAS treatment plasma binds more PGE₂ than pre-HAS (mean increase, 8.7%; CI, 5.2%–12.1%; p < .0001; n=45). **(ii)** Increment in serum albumin correlates with increase in %PGE₂ bound (n=52). $r^2 = 0.17$, $p=0.0038$. **[C]** Percentage of PGE₂ bound to patient plasma protein using equilibrium dialysis, comparing patient plasma pretreatment and post-treatment with 20% HAS (n=23 patient samples were selected that had shown at least a 8% improvement (mean 16.1%, CI 6.0 - 15.0%, p<0.0001)). Data shown with undiluted samples and when all samples had been diluted to the same albumin concentration (18 g/L). Despite the same albumin concentration post treatment plasma still bound significantly more PGE₂ than pre treatment plasma (mean

increase 10.9%, CI 5.2 -16.7%, $p=0.0007$). [D] PGE₂ bound to plasma albumin decreases as bilirubin levels in plasma increase. $r^2=0.44$, $p<0.0001$

4.3.3. Targeted 20% Human Albumin Solution Infusions Improved AD Plasma Ability to Bind Prostaglandin E₂ by Increasing Albumin Concentration and Functional Binding Capacity

52 patients samples from the ATTIRE feasibility study were analysed, blindly, pre and post treatment with daily 20% HAS infusions. All pre treatment samples had a serum albumin <30g/L and 45/52 post treatment samples had a serum albumin >30g/L. The remaining 7/52 patients did not increment their albumin level to >30g/L therefore the analysed sample was the sample from the day in which their serum albumin was at its highest. Mean albumin in this group was 25.3g/L and mean day of treatment was day 3 as opposed to mean albumin of 32.1g/L and day 4 in the other 45 patients.

In patients who incremented their serum albumin from <30g/L to >30g/L, mean increase in %PGE₂ bound post treatment with IV HAS was 8.7% (CI, 5.2%–12.1%; $p < 0.0001$; $n=45$, figure 4.8Bi) but the increased % bound PGE₂ was not to that of healthy volunteer plasma. Interestingly, in the 7 patients who did not increment their serum to >30g/L there were no overall differences in post treatment PGE₂ binding as compared to pre treatment. Total increase in albumin concentration in post treatment samples (g/L) was directly proportional to the increase in PGE₂ binding in all samples analysed ($r^2 = 0.17$, $p=0.0038$, $n=52$, figure 4.8Bii). The r^2 is lower than expected and suggests another factor influencing results than simply increasing albumin concentration.

23 patient samples that had shown at least a 8% improvement (mean 16.1%, CI 6.0 - 15.0%, $p<0.0001$) in binding post treatment, were selected for further exploration of factors which may have resulted in an improvement in post treatment binding. The most obvious explanation for an improvement in PGE₂ binding post treatment is that there is an increase in plasma albumin concentration. However, when pre/post treatment samples are diluted down to the same albumin concentration, post treatment plasma still bound significantly more PGE₂ than pre treatment plasma (mean increase 10.9%, CI 5.2 -16.7%, $p=0.0007$). This suggests the increased ability to bind PGE₂ is linked to an improvement in the function of the post treatment albumin, and not purely an increased quantity of available albumin.

4.3.3.2. Serum bilirubin and PGE₂-Albumin Binding using radiolabelled PGE₂

There appeared to be a significant relationship between bilirubin and PGE₂ binding capacity when other biochemical parameters in the analysed 52 patient samples were explored. PGE₂ bound to plasma albumin decreases as bilirubin levels in plasma increase ($r^2=0.44$, $p<0.0001$, figure 4.8D).

It is possible that bilirubin competes for the same site as PGE₂ to bind. Moreover, in radioassays color quenching is a source of error when liquid-scintillation methods are used in samples that contain haemolysed blood and samples from jaundiced patients¹⁶⁶. These compounds absorb highly in the same region in which fluors emit light and in which the photomultiplier tube detector is most sensitive. This results in a lower number of counts being recorded than is actually present. Counters can correct for this when converting counts per minute (cpm) to disintegrations per minute (dpm) using a calibrated quench curve and efficiency correction.

Therefore an AD plasma sample from a patient with a bilirubin of 453 μ mol/L was incubated with a constant volume and concentration of PGE₂/³H-PGE₂ with 1:2 dilutions of plasma (with PBS). These serial dilutions were counted in scintillation fluid (table 4.3).

Diluted level of bilirubin (μ mol/L)	CPM	DPM	% efficiency
420	4948	34244	14.4
210	12909	55668	23.2
155	22680	68692	33.0
77.5	28474	67945	41.9
38.8	34003	73138	46.5
19.4	36873	73799	50.0
9.7	38306	74761	51.2
PBS	38564	72433	53.2

Table 4.3. Effect of high bilirubin levels on counting scintillation efficiency

These results suggest that elevated bilirubin levels may be a significant confounder in the evaluation of this assay, particularly when the bilirubin in the sample is $>77.5\mu$ mol/L.

In the samples analysed the mean bilirubin on day 1 was 154.5 μ mol/L (s.d. 145.1 μ mol/L) and mean bilirubin in post treatment samples was 141.2 μ mol/L (s.d. 119.7 μ mol/L). This was likely to have decreased the counting efficiency as compared to

healthy volunteers with a normal bilirubin level but the values are not different enough to have an impact on counting efficiency post treatment.

4.3.3.3. Functional binding capacity of albumin initially improved following HAS treatment in patients who go on to develop infection but this improvement was reversed just prior to infection, despite continued HAS infusions

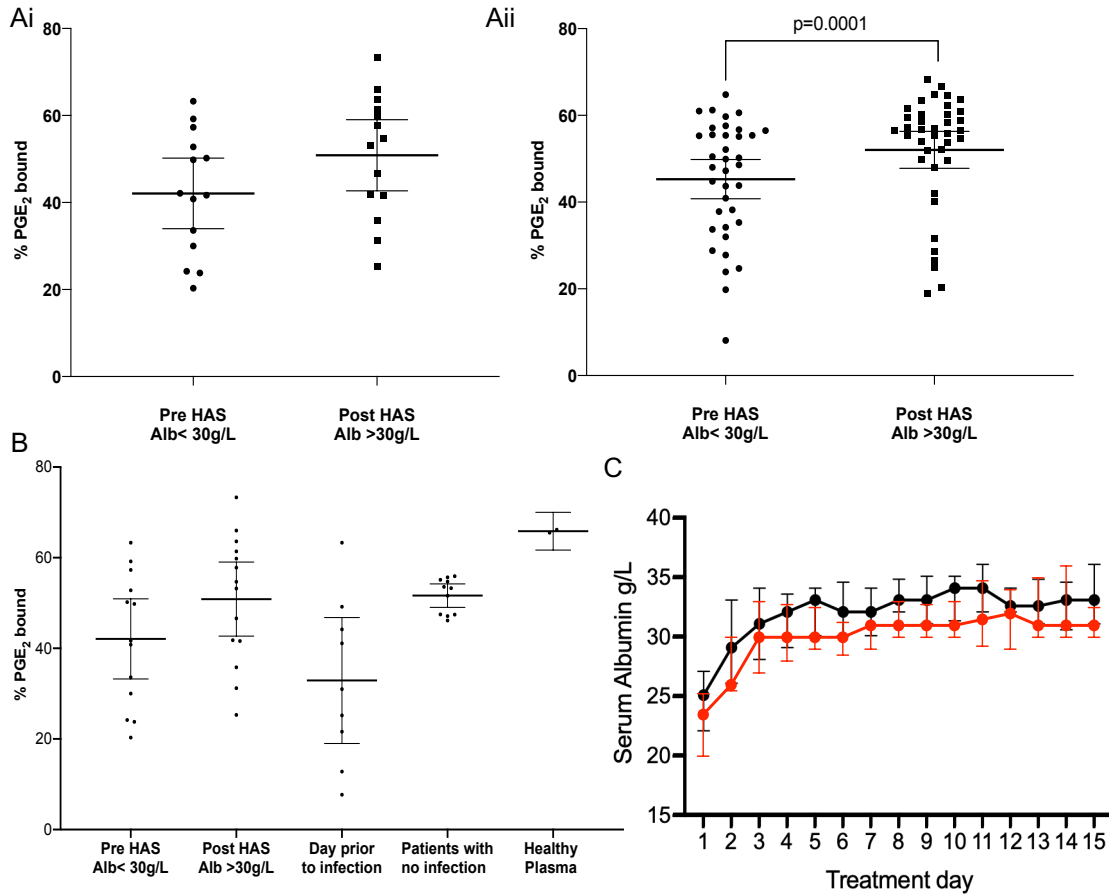


Figure 4.9. Functional binding capacity of albumin initially improves post HAS treatment in patients who develop infection but this improvement is lost over time

[A]. PGE₂ binding to plasma increases post HAS treatment in (i) patients who go onto develop infection (n=14, mean improvement 8.8%, CI -1.8%-19.4%) and in (ii) those who do not go onto to develop infection (n=37, mean improvement 6.7%, CI 3.6% - 9.9%, p=0.0001). **[B].** Infection patients show a significant decrease in PGE₂-albumin binding function of 17.96% the day prior to infection as compared to the initial post HAS treatment samples (p=0.014, CI -31.97 to -3.954). Time matched samples from patients without infection (n=11, mean 52.6% bound) remain at the level of the initial post HAS treatment samples. **[C].** Serum albumin concentration over treatment days in all patients (n=79, median and IQR). Black line represents patients who did not develop a new infection and red line represents patients who did develop a new infection.

Samples were re-evaluated according to whether patients had developed a new infection during the trial treatment period. Clinical characteristics are described in

chapter 2 (section 2.3.5). There was an initial increase in PGE₂ binding capacity in both groups of patients post treatment with 20% HAS (figure 4.9Ai & Aii), although this increase did not reach significance in the patients who went onto develop infection (likely as they were a smaller group). Available samples were analysed over subsequent treatment days. There was a mean 17.96% decrease in the capacity of plasma albumin to bind PGE₂ the day prior to infection, in patients that developed a new infection (figure 4.9B, p=0.014, CI -31.97% to -3.954%) despite no overall change in the serum albumin concentration (figure 4.9C, red line). Time matched samples from patients who did not develop infection showed PGE₂-albumin binding capacity to remain at that of the initial post treatment levels (figure 4.9B), however the binding capacity was still not near healthy volunteer levels.

	Infection (n=21) <i>Mean (s.d)</i>	No Infection (n=59) <i>Mean (s.d)</i>
% protocol deviations	26%	38%
HAS/day	131mLs	113mLs
Time to increment albumin >30g/L	3.7 days	1.2 days
Death (30/7)	8/21 (38%)	5/59 (8%)

Table 4.4. Summarising variations in HAS treatment, time to serum albumin increment and death in patients who developed a new infection during the trial versus those who did not

When HAS treatment throughout the trial was examined, protocol deviations were similar in the nosocomial infection patients compared to those who did not develop infection excluding this as a potential confounder. There were also similar volumes of HAS per treatment day in both groups, with slightly more being given in the patients that developed infection (table 4.4). However patients who developed infection took longer to increment their serum albumin to the targeted value of >30g/L (table 4.4) and median levels were lower throughout the trial treatment period (figure 4.9C). As expected there were higher mortality rates in the new infection patients.

4.3.3.4. Non-oxidised albumin is present in higher proportions in patients who do not develop infection and this correlates with an increased PGE₂ binding capacity

A small number of the post HAS treatment samples were analysed to evaluate proportions of non oxidised (human mercaptalbumin, HMA), reversibly oxidised (human

nonmercaptalbumin-1, HNA1) and irreversibly oxidised (human non mercaptalbumin-2, HNA2) albumin present in the plasma (figure 4.10). Patients who did not develop an infection had higher proportions of non-oxidized albumin present in their plasma (figure 4.10Ai) as compared to patient plasma taken from patients the day prior to diagnosis of infection (figure 4.10Aii).

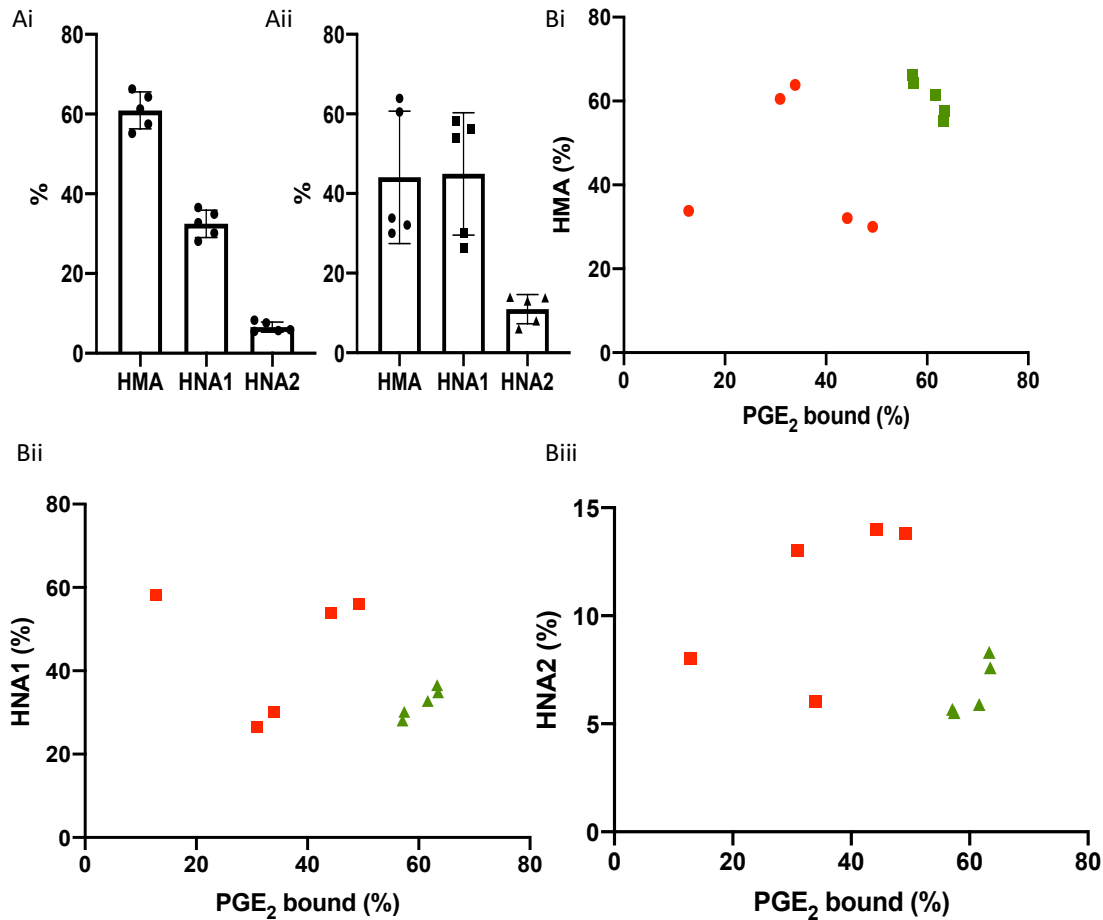


Figure 4.10. Oxidised albumin from patient plasma in patients treated with targeted 20% HAS infusions: plasma from patients that develop infection (n=5) versus those who do not (n=5).
 [A] Plasma the day prior to infection (Aii) has more reversibly oxidized (HNA1) and permanently oxidized (HNA2) albumin with less healthy non oxidized albumin (HMA) as compared to time matched plasma from patients that did not develop infection (Ai)
 [B] (i) Higher PGE₂ binding capacity is associated with higher percentage non oxidized albumin in patients who do not develop infection (green). In patients who develop infection there is a larger spread of results (red). (ii, iii). Higher PGE₂ binding capacity is associated with lower levels of oxidized albumin in patients who do not develop infection

In the patients who did not develop infection a higher albumin-PGE₂ binding capacity was associated with higher levels of healthy, non-oxidised albumin and consequently lower levels of reversibly and non-reversibly oxidized albumin Figure 4.10Bi-iii). There

was a larger spread of results from the infection patient plasma which bound less PGE₂ with higher proportions of oxidized albumin present.

4.4 SUMMARY

- *The binding affinity of albumin – PGE₂ is low (Kd approximately 270µM) which suggests that physiological decreases in circulating albumin and increases in PGE₂ concentration could result in significant increases in free circulating PGE₂ to pathophysiological levels.*
- *There is slight variability in the PGE₂ binding capacity of different commercial preparations of 20% Human Albumin Solution (HAS) for infusion that are available in the UK.*
 - This is small and not significant difference in samples that were assessed.
 - There appears to be albumin concentration differences between samples of 20% HAS.
 - Recombinant 20% HAS was no more efficacious at binding PGE₂ than HAS from pooled human plasma.
- *Using rapid equilibrium dialysis with ³H-PGE₂ there is decreased binding capacity of healthy volunteer plasma proteins as opposed to acutely decompensated cirrhosis plasma proteins.*
 - ³H-PGE₂ appears only to bind to albumin in the plasma of healthy individuals.
- *There is a significant improvement of ex vivo assessment of plasma protein binding of ³H-PGE₂ in AD patients after infusion with 20% HAS when serum albumin >30g/L*
 - This appears to be caused by a functional improvement in the circulating albumin rather than increased levels.
 - It is possible that confounding factors, such a general improvement in patient's clinical condition, could account for the observed effect.
- *AD patients who develop a new infection whilst receiving targeted HAS therapy have a decrease in PGE₂ binding capacity the day prior to infection despite no decrease in plasma albumin concentration*
 - The concentration of oxidised albumin (HNA1) increases at this time point.
 - This could result in an increase in bioavailable PGE₂ and may therefore be responsible for the 'immunosuppressive' plasma effect observed at the same time point in chapter 3.

4.5. CONCLUSIONS

4.5.1. The binding affinity of albumin–PGE₂ is very low (K_d around 270μM)

This supports my hypothesis that physiological decreases in circulating albumin and increases in PGE₂ concentration could result in significantly raised free circulating (immune suppressive) PGE₂ as explained in tables 4.2a&b which use physiological range PGE₂ and albumin.

Further experiments with higher concentrations of albumin (with the aim of getting as close to 100% PGE₂ bound as possible i.e. the B_{max}) could not be completed as it was becoming more difficult to dissolve albumin at higher concentrations. Nonetheless, sufficient data points were obtained to estimate a K_d. In addition the assay was reproducible making it usable for further analysis in different settings (with plasma and 20% HAS); therefore I achieved my set aims.

Limitations in interpretation

Non-specific binding of PGE₂ to other albumin binding sites (for example at fatty acid binding sites) was not explored in this thesis. In theory this could have been evaluated by using a ligand with high affinity and specificity for Sudlow's site 1 which would therefore block the binding of PGE₂ at this site and then binding at other locations could be measured. However albumin can undergo conformational change when bound to a ligand which affects binding at other sites, therefore I would not be certain that any additional binding I saw with a competitive ligand would happen in the absence of that additional ligand.

I made a number of assumptions in my calculations:

- Only one binding site was available
- Equilibrium had been met with my dialysis times
- There was no significant loss of PGE₂ by attachment to the dialysis membrane
- Binding was reversible
- PGE₂ was not catabolized during the incubation time

This is an estimated K_d and the binding curves were reproducible in different experiments. In addition I used defatted albumin in these experiments and it is possible

that albumin with present fatty acids (FA) at FA binding sites may bind to PGE₂ with different efficacy. In fact binding data with HAS for infusion suggested an even higher K_d (>300µM). It isn't known if this is because it is not 'de fattened' or some other ligands within the solution (e.g. stabilisers) are effecting the binding capacity.

Finally this ex vivo binding assay will not fully reflect in vivo mechanisms of binding and is simply an indication of potential binding efficacy. Best attempts were made to keep pH within physiological range (pH 7.4) and incubation and dialysis were at 37°C. However, in vivo changes are likely to occur at a microenvironment level and there will be huge heterogeneity in an AD patient population (e.g. physiological parameters, competing endogenous and exogenous ligands) so we must interpret these results as a guide only.

4.5.2. There is some variability in the binding capacity of different commercial preparations of 20% Human Albumin Solution (HAS) for infusion that are available in the UK

20% HAS for infusion was assessed from 3 suppliers, including 2 batch variations. There was a small and non-significant difference in these samples. It is unlikely that these differences would have an impact in vivo and a very large number of samples would need to be evaluated in order to detect a difference between suppliers (which is also likely to vary between batches – due to the variable source of healthy donors).

Surprisingly there was albumin concentration differences between samples of 20% HAS from difference suppliers and batch to batch variability within the same supplier's HAS. It was also surprising to discover the variations in stabilisers in the solution, for example Alburnorm contains N-acetyl-DL-tryptophan 3.9 g/L. Reine, et al. ¹⁶⁷ found that free fraction of naproxen increased after infusion of only 100mL 20% HAS containing these levels however this was transient and brief (30 minutes – the ½ life of N-acetyl-DL-tryptophan).

Recombinant 20% HAS for infusion was no more efficacious at binding PGE₂ than HAS from pooled human plasma despite claims that it has less post transcriptional modification and was of better quality ¹⁶⁴.

4.5.3. Albumin in healthy volunteer plasma is more efficacious at binding PGE₂ than plasma from patients with acute decompensation of cirrhosis

Tritium labeled PGE₂ seemed to selectively bind albumin in plasma and not other plasma proteins, therefore whole plasma was used in the tritiated PGE₂ equilibrium dialysis assay to evaluate how efficacious albumin-PGE₂ binding was in ex vivo samples.

PGE₂ binding capacity was significantly decreased in patients with decompensated cirrhosis with a much larger variation in binding capacity between patients, likely reflecting the heterogeneity in the clinical characteristics of this population. Plasma albumin concentration is higher in HVs however decreased albumin binding function in ADs was maintained when the HV and AD plasma was diluted to the same concentration of albumin. This could, in part, be due to a decrease in the functional quality of the albumin.

Limitations in interpretation

When interpreting these changes in binding of PGE₂ in plasma samples I am making the assumption that albumin is the only plasma protein binding to PGE₂. This was supported by evaluation of ³H-PGE₂ binding to healthy patient plasma using a size exclusion gel and phosphoimaging. However in liver cirrhosis hypergammaglobulinaemia is common¹⁶⁸. Therefore it may be that in the AD samples there is additional binding of PGE₂ by immunoglobulins. This could also happen in HV plasma and it may have been that, if the binding was very weak, the complex would become unbound when a plasma sample was run through a size exclusion gel.

If more definitive conclusions about albumin binding specifically are to be made then a technique to isolate albumin from plasma, causing no interference with function and concentration, would be required.

4.5.4. Plasma albumin - PGE₂ binding capacity improves in AD patients after infusion with 20% HAS, but not to the level of healthy volunteers

Blinded analysis of 45 patient plasma samples pre and post treatment (serum albumin <30g/L versus >30g/L) with 20% HAS showed an improved PGE₂ binding efficacy post treatment. These samples were the same set analysed in chapter 3 and are from the clinical trial described in chapter 2. This analysis supports the hypothesis that giving albumin infusions will improve the capacity to remove high circulating free levels of PGE₂. Correlating improvement in binding function with increase in albumin

concentration suggested that a rise in the amount of circulating albumin was not entirely responsible for the improvement in binding capacity. In fact when all samples were diluted to the same albumin concentration there was still a significantly improved functional binding capacity in the post treatment samples suggesting that the quality of the circulating albumin had changed. All of the patient samples evaluated in this study were from a single arm study so it is possible that other clinical factors occurring over time as patients were hospitalized and treated for infection or hypovolaemia could have led to the improvement seen, rather than the albumin concentration being directly causal. For example a decrease in other ligands, such as drugs or bilirubin, may have meant less competition for available sites for PGE₂ to bind.

High bilirubin did appear to be a confounder in this assay. This is likely due to a combination of its impact as an additional ligand competing for the binding site (biologically relevant) and in the scintillation counting process (biologically irrelevant). Further work should focus on removing bilirubin further by chemical decolorisation, for example with tetramethylammonium hydroxide or hydrogen peroxide¹⁶⁶ prior to scintillation counting.

4.5.5. There may be a deterioration in albumin binding function in patients who develop infection

The aim of administering 20% HAS infusions in this study is to prevent infection and its complications. The patient samples analysed in this chapter were from the single arm feasibility study (described in chapter 2) with the primary aim of evaluating efficacy of the infusion protocol to increase serum albumin levels from <30g/L to >30g/L. However clinical outcome data were analysed and 21/79 of these patients went onto develop infection after being treated with the 20% HAS infusions. Splitting patients into those who did and those who did not go onto develop a new infection showed that both groups had an initial improvement in PGE₂ binding capacity (at mean day 4.1) in this ex vivo analysis. This was consistent with the improvements seen in the effect of patient plasma in suppressing an appropriate inflammatory response to an infectious stimulus in chapter 3. The mean day of new infection diagnosis was day 6.7. PGE₂ binding function decreased by nearly 18% (worse than pre treatment plasma) the day prior to infection, this was not seen in time-matched analysis from patients who did not develop new infection.

Although patients who developed infection had lower albumin levels, the median albumin level was maintained above 30g/L over time therefore it is unlikely that this is purely due to a drop in the concentration of the patient's albumin due to increased consumption around the time of infection.

It is possible that a functional deficit, resulting from allosteric transformation of binding site 1, may develop secondary to posttranslational modification of the circulating albumin. This could increase the K_D of albumin to PGE_2 , therefore making dissociation more likely. Alternatively there may be other circulating ligands (e.g. drugs, bilirubin) which have a higher affinity for the binding site, displacing PGE_2 resulting in increased bioavailable PGE_2 . This would explain the observed outcomes from analysis in chapter 3 when plasma becomes more immunosuppressive to monocyte derived macrophages the day prior to infection (figure 3.10Bi). There were not HAS administration differences which could have explained this effect.

Using an anion exchange column the proportions of modified albumin in the plasma samples the day prior to infection (or time matched patient controls) were evaluated. There were higher amounts of oxidized albumin in the samples from infection patients and higher amounts of 'unaltered' healthy albumin in the non-infection patients which correlated with an improved functional capacity to bind PGE_2 . These analyses were somewhat limited by sample availability as we were unable to evaluate pre treatment samples.

My hypothesis proposes that a deficit in albumin results in an inability to modulate circulating plasma mediators of immunosuppression. However, Alcaraz-Quiles, et al.¹⁶⁹ recently proposed that oxidized albumin directly acts to activate peripheral leucocytes, contributing to the systemic inflammation sometimes observed in AD patients. In their study, albumin from healthy volunteers was artificially oxidized and incubated with PBMCs and isolated neutrophils alone. They found that PBMCs became 'hyperactivated' in the presence of HNA1. In addition it resulted in high PGE_2 release from cells (see figure 4.11).

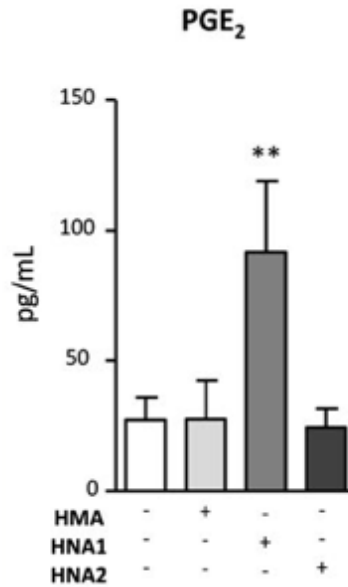


Figure 4.11 taken from Alcaraz-Quiles, et al. ¹⁶⁹. HNA1 incubation with healthy PBMCs results in high production of PGE₂.

This is an interesting proposition for a mechanism of effect in these patients although it is difficult to define what would come first – oxidized albumin or a deficit in immune response resulting in oxidized albumin when infection occurs. It is possible that altered forms of albumin may contribute to an inevitable cycle of decline in these patients.

CHAPTER 5: TARGETED HUMAN ALBUMIN INFUSIONS DO NOT REDUCE INFECTION IN PATIENTS WITH ACUTE DECOMPENSATION OF LIVER CIRRHOSIS

Publications in relation to this chapter

ATTIRE: Albumin To prevent Infection in chronic liver failure: study protocol for an interventional randomised controlled trial.

China L*, Skene SS, Bennett K, Shabir Z, Hamilton R, Bevan S, Chandler T, Maini AA, Becares N, Gilroy D, Forrest EH, O'Brien A. *BMJ Open*. 2018 Oct 21;8(10):e023754.

Presentations in relation to this chapter

ATTIRE: Albumin To prevent Infection in chronic liver failure: an interventional randomised controlled trial

Louise China, Simon S Skene, Nicholas Freemantle, Kate Bennett, Natalia Becares, Jim Portal, Yiannis Kallis, Gavin Wright, Derek Gilroy, Ewan H Forrest, Alastair O'Brien
Oral presentation (Late Breakers) London. Full bursary awarded. EASL 2020.

Contributions by others to this chapter:

- UCL Clinical Trials Unit: led ethics approval, site management, data entry
- Statistical analysis: power calculations by Simon Skene, main clinical outcome analysis by Nick Freemantle, plasma sub study analysis unguided
- Recruiting Hospitals: 32. Responsible for screening, recruitment and patient management
- Trial Supervision Committees: IDMC and TSC responsible for oversight of the trial (all attended and contributed to by myself)
- Isolation of monocytes and plasma PGE₂ EIA: N.Becares

5.1 INTRODUCTION

5.1.1. Challenges of interpreting outcomes in single arm studies using albumin as an intervention

In chapters 2- 4 the hypothesis that prophylactic HAS infusions increase serum albumin and subsequently prevent AD patients from developing infection was explored in a single arm study with no comparator patient group. Patients acted as their own controls by evaluating clinical characteristics and samples in ex vivo assays at specified time points, pre and post HAS treatment.

AD patients are an unwell and heterogeneous patient group receiving multiple supportive interventions on admission to hospital. Therefore there are several confounding factors to consider, such as:

- Antibiotics
- Fluid and electrolyte resuscitation
- Nutrition
- Abstinence from alcohol
- Organ support (RRT, artificial ventilation and oxygen)
- Treatment of portal hypertensive bleeding and ascites

The complicated nature of many AD patients' care make these potential confounders impossible to control. Therefore, to make meaningful conclusions regarding the capacity of 20% HAS to reduce the development of infection, or evaluated ex vivo measures, a control arm of patients not receiving serum targeted HAS as an intervention is required i.e. a randomised control trial. Effective randomisation will balance the incidence of confounding differences in baseline patient characteristics in each arm (serum targeted HAS treatment or no serum targeted HAS treatment).

My thesis has focused upon the role HAS may play in the reversal of PGE₂ mediated innate immune dysfunction. As discussed in chapter 1 there are multiple other components of the immune response which HAS may modulate leading to decreased clinical rates of infection or improvements in laboratory assays. In addition, there is evidence to support fluid resuscitation with HAS prevents further renal dysfunction in LVP, SBP and HRS. Improving circulating volume, when required, is considered to reduce subsequent organ failure(s) that increase the risk of nosocomial infection.

Therefore, aside from potentially improving the immune response, the beneficial oncotic effects of HAS treatment may reduce infection rates. Conversely, large volumes of HAS may increase the risk of fluid overload, leading to pulmonary oedema which could increase the risk of respiratory tract infection. Evaluating serum targeted HAS infusions as a randomly allocated intervention with an additional 'non-treatment arm' offers the opportunity to examine the impact of these factors on pre-defined endpoints.

5.1.1.1. Comparator fluids in RCTs evaluating IV HAS

HAS is extensively used as a volume expander in cirrhosis patients. Many of the original studies evaluating the efficacy of HAS use plasma expanders such as hydroxyethyl starch or saline as a comparator fluid^{62,170-172}. However, in the last 10 years only three HAS studies^{74,173,174} have used a comparator fluid, perhaps because of concerns about these fluids causing excessive salt load and precipitating fluid overload in unwell cirrhosis patients. Although it seems highly unlikely that such low volumes (100mL, as in each 20g HAS vial) of these fluids would be harmful for the majority of patients. There have been no published HAS studies in decompensated liver disease in the last 20 years which administer HAS in an attempt to increase serum albumin levels, although post-hoc analyses of the ANSWER study⁷³ found that when serum albumin levels increased in response to 20% HAS administration mortality decreased¹⁷⁵. We considered a blinded trial unsafe as investigators in standard care using 'non-albumin' placebo fluid to increase serum albumin might administer potentially harmful large volumes of fluid. Furthermore, in chapter 2 I described that it took 2-3 days for most patients to increment their albumin to >30 g/L with targeted HAS infusions. Thus attempts to blind such a study would be also be futile as it would become rapidly apparent to investigators which study arm the patient was in.

5.1.2. Alternative therapeutic mechanisms for HAS in AD patients and possibilities of measuring their impact in a multi-centre study

The focus of my thesis is to investigate how IV HAS may improve the innate immune response and prevent AD patients presenting with low serum albumin from developing infection via a reduction in the immunosuppressive effects of PGE₂.

However the potential beneficial impact of improved vascular filling and endothelial function on the risk of infection needs to be considered when interpreting the results.

Here I will discuss exploratory measures of extra cellular volume, ex vivo, and endothelial function that are feasible using samples from a multicenter study.

5.1.2.1. Atrial Natriuretic Peptide (ANP) and Renin:

When considering how ANP and renin may change in response to albumin therapy it is useful to consider the basic homeostatic regulatory mechanisms of extracellular volume. Renin is secreted in response to low sodium in the distal tubule and converts angiotensinogen to angiotensin 1, down-stream this leads to aldosterone release, upregulation of sodium channels in the ascending loop and subsequent sodium reabsorption and volume expansion. Plasma renin has historically been used as a neurohumoral marker of adequate extracellular filling in liver cirrhosis studies^{54,66}. Studies using HAS as a volume expander post LVP and SBP to treat patients with infection have consistently shown a decrease in plasma renin levels^{54,55,74}, although the control group in SBP study(s) received no fluid. However, renin levels do not always correlate with improvement in renal function or mortality.

Natriuretic peptides (NPs) are peptide hormones predominantly synthesised by the heart and brain. Atrial natriuretic peptide (ANP) is a small peptide that is synthesised, stored, and released by atrial myocytes in response to atrial distension, angiotensin II stimulation, endothelin, and sympathetic stimulation. Therefore, high levels of ANP are found during hypervolaemic states, such as heart failure. ANP is first synthesised and stored in cardiac myocytes as pre-pro-ANP, which is then cleaved to pro-ANP and finally to ANP. ANP is the biologically active peptide but is rapidly removed from circulation and therefore difficult to measure. NT-pro ANP (the cleaved N-terminal) does not bind clearance receptor and therefore has a long half-life (60–120mins) and so serves as an excellent marker of ANP secretion. Natriuretic peptides act to counter balance the renin-angiotensin system in high volume states as summarised in figure 5.1.

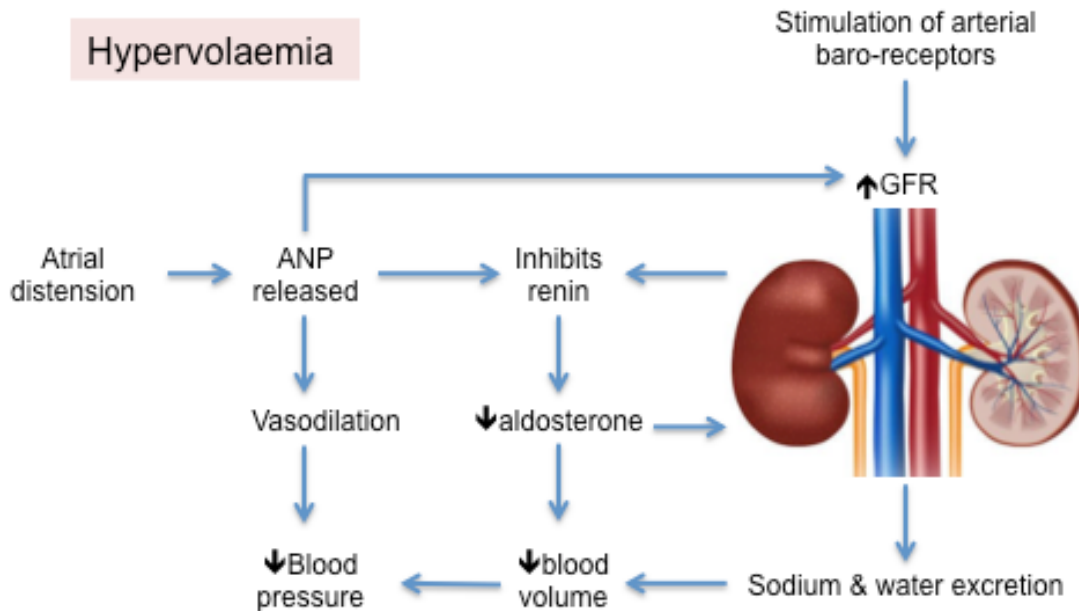


Figure 5.1. Regulation of hypervolaemia: impact of ANP and renin

Albumin infusion would be predicted to increase vascular filling leading to an increased glomerular filtration rate (GFR) and subsequent decrease in renin. However if albumin contributes to excess filling this would lead to ANP release, as well as renin inhibition. Therefore if infused albumin is having a positive impact, one would expect to see lower levels of plasma renin in the albumin group without excessive high ANP.

There are significant limitations to using renin and ANP as biomarkers which have prevented widespread use in clinical practice. Haemodynamics change in advancing liver disease and this has an impact on the renin angiotensin system¹⁷⁶ potentially confounding interpretation. Liver and renal function impact upon clearance of these hormones, so changes in overall levels might be independent of an intravascular filling effect. Most importantly the biological significance of changes in ANP and renin in advanced liver disease are uncertain as there are inconsistencies in reporting of correlations with clinically important patient outcomes such as mortality. Therefore use is limited to research studies investigating fluid resuscitation.

5.1.2.2. Endothelial dysfunction and Syndecan-1

The glycocalyx is a gel-like layer covering the luminal surface of vascular endothelial cells. It is comprised of membrane-attached proteoglycans, glycosaminoglycan chains, glycoproteins, and adherent plasma proteins¹⁷⁷. It functions to maintain homeostasis of

the vasculature which includes controlling permeability, tone, preventing microvascular thrombosis and regulating leukocyte adhesion.

During sepsis, sheddases (e.g. metalloproteinases - MMPs) are activated by reactive oxygen species and pro-inflammatory cytokines such as TNF α and IL-1 β . This leads to inflammation-mediated glycocalyx degradation and subsequent vascular hyper permeability, unregulated vasodilation, microvessel thrombosis and increased leukocyte adhesion.

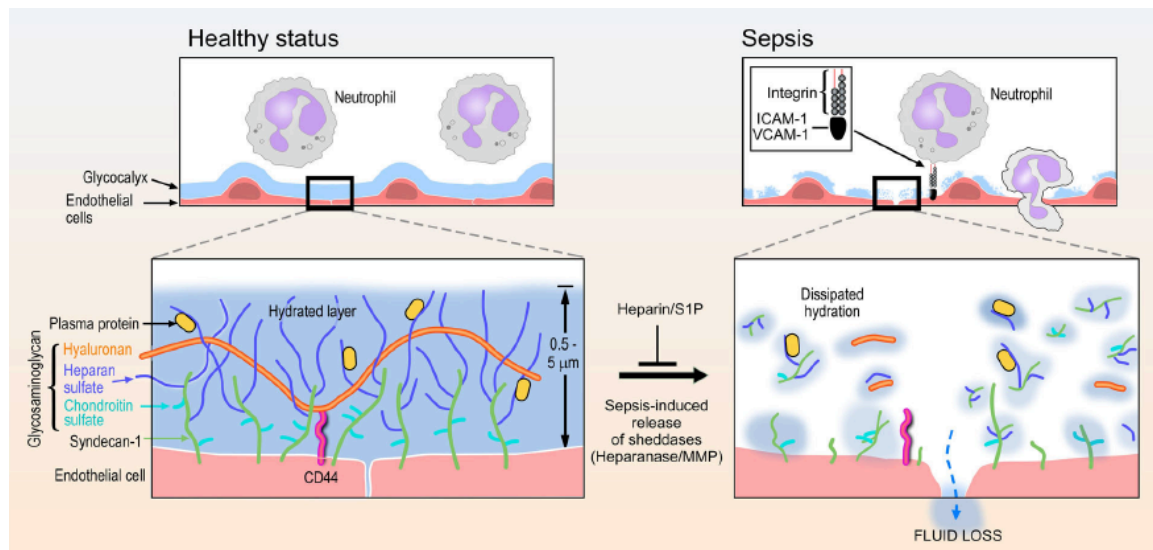


Figure 5.2. Endothelial glycocalyx structure during health and degradation during sepsis.

Taken from Uchimido, et al. ¹⁷⁷.

MMP metalloproteinase, S1P sphingosine-1-phosphate, ICAM-1 intercellular adhesion molecule 1, VCAM-1 vascular cell adhesion molecule 1

Clinical studies have demonstrated a positive correlation between blood levels of glycocalyx components with sepsis-related organ dysfunction, severity and mortality^{177,178} in non-cirrhosis patients. Syndecan-1 is released during glycocalyx breakdown and represents an easily measured circulating biomarker of this (figure 5.2). Nelson, et al. ¹⁷⁹ studied in septic shock patients admitted to ICU (n = 18) and found they had a significantly higher median levels of syndecan-1 compared to healthy controls (n = 18; 246 [interquartile range (IQR) 180–496] ng/mL vs 26 [IQR 23–31] ng/mL, p < 0.001). There was also a correlation between syndecan-1 level and Sequential Organ Failure Assessment (SOFA) score (r = 0.48, p < 0.05) and Cardiovascular SOFA score (r = 0.69, p < 0.01) during the first 24 h of admission. Authors reported no association between the median level of syndecan-1 and mortality, however the study was underpowered to accurately evaluate this endpoint.

Pro-inflammatory cytokines, particularly TNF α , are postulated to have a direct effect on glycocalyx breakdown, however, mechanisms are unclear. Nieuwdorp, et al.¹⁸⁰ administered low dose endotoxin to 8 healthy volunteers and found an expected reduction in the microvascular glycocalyx as measured by orthogonal polarization spectroscopy (OPS) imaging of the sublingual microcirculation. Administration of the TNF α inhibitor entanercept attenuated the reduction in glycocalyx thickness and decreased biomarkers of glycocalyx breakdown. In a 'chicken and egg' type scenario, it is thought that although inflammatory stimuli can initiate glycocalyx degradation, glycocalyx integrity can also feed-back on the inflammatory process itself. Pro inflammatory cytokines adhere to components of the glycocalyx, such as syndecan-1, and during breakdown of the glycocalyx they are again released^{181,182}. The effect after shedding remains unclear.

In sepsis, excessive fluid resuscitation is thought to contribute to glycocalyx breakdown. There was an association between high levels of ANP, indicating atrial stretch secondary to fluid overload, and high levels of syndecan-1 and it has been assumed that ANP is somehow initiating glycocalyx breakdown¹⁸³. Most studies evaluating this have examined levels of ANP and levels of glycocalyx breakdown biomarkers such as syndecan-1¹⁸⁴ in the context of volume loading during cardiac surgery.

It has been suggested that IV human albumin solution may be protective to glycocalyx and prevent breakdown. S1P (sphingosine-1-phosphate) is a sphingolipid that may improve glycocalyx integrity by inhibiting syndecan-1 shedding¹⁸⁵. S1P activates the S1p receptor which suppresses the activity of MMPs which cause syndecan-1 shedding. Albumin carries erythrocyte derived S1P to the endothelium. Animal models have assessed the use of albumin as a protective perfusate in explanted hearts and found lower levels of biomarkers of glycocalyx breakdown when albumin is used as opposed to other colloids¹⁸⁶. However, there have been no studies of the impact of albumin infusion on glycocalyx breakdown in patients who have infection or in patients with decompensated cirrhosis.

Therefore in combination ANP, renin and syndecan-1 may provide additional mechanistic information about adequate or excess vascular and extravascular filling following IV HAS infusions.

5.1.3. The challenges of accurate infection diagnosis and the changing spectrum of infection in chronic liver disease

In chapter 2, I discussed the problems of diagnostic accuracy of infection in clinical practice and as an endpoint in clinical trials, particularly in patients with end stage liver disease. It is widely perceived that culture negative infection is common¹⁸⁷, but this may relate to patient samples not being taken prior to initiation of antibiotics. Clinicians often rely on biomarkers of infection such as CRP, a protein produced by the liver and therefore in liver failure may not rise appropriately when an infection is present. There is currently no reliable biomarker for infection in patients with chronic liver disease, which also makes validating a clinical diagnosis of infection in clinical trials a challenge.

In this study I attempted to collect comprehensive clinical, biochemical and microbiological data when a trial patient had a new infection diagnosis. New biomarkers of infection have been developed that are not produced in the liver and this study posed an opportunity to examine these markers in patients that received an infection diagnosis.

5.1.3.1. Procalcitonin

Procalcitonin (PCT) is a widely used biomarker for the diagnosis of bacterial infections outside the UK. It is produced by thyroid C cells, with very low concentration (< 0.05 ng/mL) in the blood of healthy individuals. During an inflammatory response PCT is produced ubiquitously in response to endotoxin or mediators released in response to bacterial infection (e.g. IL-1 β , TNF α , and IL-6).

There is conflicting data with regards to the diagnostic value of PCT in bacterial infection in advanced liver disease. In an inflammatory response the liver is the main source of PCT production¹⁸⁸, hence theoretically one may expect levels to be low in cirrhosis. However Bota, et al.¹⁸⁹ reported that PCT levels are not different between patients with and without cirrhosis and did not correlate with the severity of cirrhosis. PCT levels were observed to be higher in cirrhotics with infection (mean 0.89 ng/mL) than without (mean 0.35 ng/mL). The cut-off value to rule out infections was 0.25 ng/mL. In an alcoholic hepatitis study authors found that a cut-off value of 0.57 ng/mL performed well (with a sensitivity of 79% and specificity 82%) in the diagnosis of sepsis¹⁹⁰. In a meta-analysis that included 1144 patients and 435 bacterial infection episodes, the authors concluded that the positive likelihood ratio for PCT was sufficient to use the test as a 'rule in' diagnostic test for infection in cirrhosis, but that CRP should not be used¹⁹¹. Conversely

other groups have found CRP to be more sensitive and specific in cirrhosis infection diagnosis^{192,193} with authors concluding it was an acceptable 'rule out' test when patients had no clinical features of infection. The diagnostic accuracy of PCT had been shown to be improved, in cirrhosis patients, when combined with other markers such as serum albumin or IL-6^{194,195}.

5.1.3.2. Soluble CD14

As a glycoprotein expressed on monocytes and macrophages, cluster of differentiation 14 (CD14) serves as a receptor of the LPS binding protein-LPS complexes and activates a series of signal transduction pathways and inflammatory cascades that finally lead to an inflammatory response. CD14 has two forms: a membrane-bound CD14 (mCD14) and soluble CD14 (sCD14). sCD14 plays an important role in mediating the immune responses to LPS of CD14-negative cells, such as endothelial and epithelial cells.

After production sCD14 is cleaved and the soluble N-terminal fragment is formed and circulates. In recent years this has been marketed as 'presepsin' (sCD14 subtype) - a biomarker of infection. An interesting study looking at immunosuppressed rheumatoid arthritis (RA) patients compared presepsin and PCT to CRP and WCC in the diagnosis of infection¹⁹⁶. Many RA patients have elevated CRP related to inflamed joints. Patients were split into those with infection and those without infection (without infection was subdivided into 'CRP positive' and 'CRP negative'). Levels of PCT and presepsin were significantly higher in the infection group as compared to the CRP positive non-infection group. According to receiver operating characteristic curve (ROC) analysis, presepsin and PCT appeared to have a higher diagnostic accuracy for infection than CRP or WBC in RA patients. When assessing severity of infection presepsin was superior to PCT. A meta-analysis of over 2000 non cirrhosis patients with infection found that presepsin was potentially a valuable biomarker in the early diagnosis of sepsis¹⁹⁷. However, it showed only a moderate diagnostic accuracy in differentiating sepsis from non-sepsis which prevented it from being recommended as a definitive test for diagnosing sepsis in isolation. Pre-sepsin (sCD14 subtype) has not progressed to routine clinical use.

An additional problem with using sCD14 as a biomarker for infection is the confounding impact of renal dysfunction (decreased removal from the circulation), which is common in acute decompensation patients¹⁹⁸.

5.1.3.3. CD163

CD163 is expressed by activated macrophages and continuous shedding of the extracellular domain leads to elevated plasma levels. It has various proposed roles including the clearance of free haemoglobin from the circulation and as a regulator of erythropoiesis. Fabriek, et al.¹⁹⁹ identified CD163 as a macrophage surface receptor for gram negative and positive bacteria. Recognition of bacteria by CD163 potently enhanced inflammatory cytokine production in a monocytic cell line (THP-1) and cytokine production by freshly isolated human monocytes was strongly suppressed by novel agonistic mAb against CD163. Feng, et al.²⁰⁰ went on to prospectively assess over 100 sepsis patients of differing severity (but all admitted to ICU) and found sCD163, with a cut of above 1.49µg/mL, differentiated between patients who had no infection but SIRS (systemic inflammatory response) and moderate to severe sepsis (diagnosed using clinical criteria, not culture positivity). They also proposed that sCD163 was superior to PCT and CRP not only in the diagnosis of sepsis but better at determining sepsis prognosis due to dynamic changes in the levels during the patients' hospital admission. However, all published studies evaluating sCD163 as a biomarker are in intensive care unit patients and its utility as an early biomarker for infection, in a ward based setting, has not been explored. It is possible that sCD163 is raised in other chronic inflammatory diseases, post blood transfusion²⁰¹ and in some forms of hepatological disease and cirrhosis²⁰¹⁻²⁰³ making confounding factors too significant to use it as a more subtle early biomarker in ward based patients.

5.1.3.4. Plasma calprotectin

Calprotectin is a calcium-binding protein that belongs to a group of danger-associated molecular patterns (DAMPs) known as alarmins. Calprotectin is a highly abundant protein in neutrophils, accounting for approximately half of the cytosolic content. It consists of a complex of 2 intracellular proteins: calgranulin A and calgranulin B. This complex is translocated from the cytosol to the neutrophil cell membrane following calcium mobilization.

Calprotectin, measured in the faeces, is an established investigation in the diagnosis and monitoring of inflammatory bowel disease²⁰⁴. It is a highly sensitive marker of neutrophil migration to the bowel although it is not specific to any one condition and can be raised in anything from coeliac disease to bacterial infection.

Recently plasma calprotectin has been evaluated as a diagnostic marker of infection²⁰⁵. In 66 patients with sepsis (the majority were culture positive with E.coli infection) plasma calprotectin was significantly raised compared to patients with viral infections and healthy volunteers. The calprotectin results were comparable with CRP and PCT although values took longer to return to near normal as the infection was treated over time.

There has been no investigation into the utility of plasma calprotectin in early infection or established infection not defined as 'sepsis'²⁰⁶. There is limited investigation in liver disease. In 1995 Homann, et al.²⁰⁷ linked raised plasma calprotectin with worse outcomes in patients with alcohol induced liver cirrhosis (compensated and decompensated patients). Some patients had infection, but not all. It is possible alcoholic hepatitis, now known to be an acute pro inflammatory condition, could have been present in some patients causing neutrophil activation. Alternatively the worse outcomes could have been caused by undiagnosed infection or neutrophil activation by increased translocation of bacterial products. Ascitic calprotectin has been used to aid the diagnosis of SBP¹⁹⁵ in one small study. There has been no other or recent investigation into plasma calprotectin in patients with advanced liver disease who are at risk of infection.

5.1.3.5. Lipopolysaccharide binding protein

Lipopolysaccharide-binding-protein (LBP) is a soluble acute phase protein with a long half-life, produced by hepatocytes. LBP enhances the binding of bacterial LPS to CD14 cell membrane molecule and Toll-like receptor 4. This activates a cascade that leads to cytokine production and an inflammatory response. LBP levels are considered to reflect the long-term exposure to bacteria and endotoxins. Serum and plasma LBP levels have been used as a surrogate marker of bacterial translocation. Measurement of LBP in cirrhosis research has become more common place than direct measurement of LPS (as described in chapter 3) as LPS measurement is complicated by its short half-life and concerns about the accuracy of some of the assays used for measurement (e.g. HEK cells).

Agiastotelli, et al.²⁰⁸ measured LBP in 88 cirrhotic patients (serum and ascites). 18 of these patients had clinical evidence of infection and they found LBP had a good negative-predictive value (90% for serum and 95.1% for ascites) to rule out infection.

Patients who had a high LBP without clinical evidence of infection were followed up over time (90 days) and found to have a mortality rate of 48% versus 24.4% in patients with low LBP. Albillos, et al.²⁰⁹ followed 84 patients with ascites and cirrhosis who were 'infection free' at recruitment. They found baseline serum LBP levels were the only factor in the multivariate analysis predictive of the development of bacterial infection (relative risk 4.49, 95% confidence interval 1.42-14.1) during a 46-week follow-up period. It is unclear whether these patients had undiagnosed infection at recruitment or whether LBP is a marker of increased gut bacterial translocation with subsequent increased risk of infection.

A problem with LBP as a biomarker for infection diagnosis (or prediction) is that it has only been shown to be associated with Gram-negative, but not Gram-positive bacteria²¹⁰. With the growing burden of gram positive infection, particularly in hospitalized cirrhotics, this is a significant limitation.

5.1.3.6. Summary

Of all the biomarkers discussed above, none have proven reliable in the diagnosis (rule in) or exclusion (rule out) of an infection. It is believed that bacterial infections in cirrhosis are often subclinical (e.g. no fever or raised traditionally used inflammatory markers) due to a defective pro inflammatory response. However, the majority of the current surrogate markers do not allow the discrimination of sterile inflammation due to non-viable bacterial translocation from infections by viable bacterial translocation. The above markers have been detected in plasma, serum, ascitic fluid and stool. However, the optimal detection site has not been determined and the threshold levels where bacterial translocation becomes pathologic have not been defined for each of the parameters.

Chapter Aims:

Using samples and data from the ATTIRE RCT that compared a daily 20% HAS IV treatment protocol targeted to increasing serum albumin levels to >30g/L with standard medical care, I aimed to determine:

1. If HAS infusion versus standard medical care reduced the incidence of infection diagnosis in patients hospitalized with acute decompensation of cirrhosis
2. Whether IV HAS has an immunomodulatory effect using ex vivo assays
 - a. Does IV HAS impact on PGE₂ or other markers of immune dysfunction?
 - b. Do infusions improve the functional properties of circulating albumin?
 - c. Do infusions improve markers of vascular filling and does this correspond to improvements in outcome?
2. Whether we can improve the way infection is recorded in liver cirrhosis studies
 - a. Does an external review process of infection diagnosis support site clinician diagnosis?
 - b. Are exploratory laboratory biomarkers of infection increased around the time of infection diagnosis?
 - c. What types of infection are UK hospitalised cirrhosis patients diagnosed with?
 - d. Do patients in the HAS arm develop different types of infection to those in standard care?
3. Can we identify patients at a higher risk of infection at baseline?
 - a. Do these patients benefit from HAS treatment?

5.2 METHODS

5.2.1. Clinical Study Design

5.2.1.1 Trial Design

This was a multicentre, open-label, RCT in which patients were either treated with 20% HAS to raise and maintain serum albumin above 30 g/L or received their usual standard of care treatment. Sequential patients admitted to 35 UK participating hospitals with a clinical diagnosis of cirrhosis and decompensation were screened using the inclusion and exclusion criteria (table 5.1). Decompensation included: jaundice, ascites, hepatic encephalopathy, variceal bleeding, coagulopathy and hepatorenal syndrome (HRS).

As advanced liver disease requires frequent hospital readmissions, patients could be enrolled in the RCT more than once, following a 30-day 'washout period' after discharge from the previous enrolment. The washout period was to account for albumin's half-life of 18–21 days⁷⁷. Patients re-entering the trial in this way were re-randomised, so that each enrolment was considered an independent patient 'presentation'²¹¹.

To ensure homogeneity in the approach to patient recruitment, intervention and data collection, all sites received introduction training and regular follow-up re-training plus monitoring and support visits from the sponsor (UCL clinical trials unit).

Patient Inclusion Criteria	Patient Exclusion Criteria
All patients admitted to hospital with acute onset or worsening of complications of cirrhosis	Advanced hepatocellular carcinoma with life expectancy of less than 8 weeks
Over 18 years of age	Patients who will receive palliative treatment only during their hospital admission
Predicted hospital admission ≥ 5 days at trial enrolment, which must be within 72 hours of admission	Patients who are pregnant
Serum albumin $<30\text{g/l}$ at screening	Known or suspected severe cardiac dysfunction
Documented informed consent to participate (or consent given by a legal representative)	Any clinical condition which the investigator considers would make the patient unsuitable for the trial
	The patient has been involved in a clinical trial of Investigational Medicinal Products (IMPs) within the previous 30 days that would impact on their participation in this study
	Trial investigators unable to identify the patient (by NHS number)

Table 5.1. RCT patient inclusion and exclusion criteria

5.2.1.2. Endpoints

Primary Endpoint

A composite endpoint comprising incidence of infection, renal dysfunction and mortality within the treatment period (for a maximum of 14 days OR when the patient was considered fit for discharge if <14 days).

The three components of the composite endpoint were:

1. New infection: indicated by clinician diagnosis. The clinical evidence underlying diagnosis was entered onto an infection case report form (CRF). These data were scrutinised blindly to validate the clinical diagnosis according to peer reviewed criteria⁸⁹ (table 5.2) by a clinical trial endpoint review committee. The committee was led by a consultant microbiologist.
2. Renal dysfunction: defined as a serum creatinine increase of $\geq 50\%$ as compared with serum creatinine at randomisation OR the patient is initiated on renal replacement support (either haemodialysis or haemofiltration) OR a rise in serum creatinine of ≥ 26.5 $\mu\text{mol/L}$ within 48 hours.

3. Mortality

Primary endpoint data was recorded throughout the treatment period; however, only contributing events captured on the treatment day 3 case report form (CRF) through to the day 15 CRF (the end of treatment examination day) contributed to the primary outcome. This was the day at which serum albumin had incremented to the desired >30g/L in the majority of patients in the feasibility study²¹² (chapter 2) and therefore allowed any putative biological effect of albumin to be established prior to assessing clinical effects

If the participant was discharged or deemed medically fit for discharge prior to day 15, no further primary outcome data will be measured after this date, as this will signal the end of the participant's treatment period.

1. Spontaneous bacteraemia: positive blood cultures without a source of infection.
2. SBP: ascitic fluid polymorphonuclear cells >250 cells/mm³
3. Lower respiratory tract infections: new pulmonary infiltrates in the presence of: i) at least one respiratory symptom (cough, sputum production, dyspnoea, pleuritic pain) with ii) at least one finding on auscultation (rales or crepitation) or one sign of infection (core body temperature >38°C or less than 36°C, shivering, or leukocyte count >10,000/mm³ or <4,000/mm³) in the absence of antibiotics.
4. Clostridium difficile Infection: diarrhoea with a positive C. difficile assay.
5. Bacterial entero-colitis: diarrhoea or dysentery with a positive stool culture for Salmonella, Shigella, Yersinia, Campylobacter, or pathogenic E. coli.
6. Soft-tissue/skin Infection: fever with cellulitis.
7. Urinary tract infection (UTI): urine white blood cell >15/high-power field with either positive urine gram stain or culture.
8. Intra-abdominal infections: diverticulitis, appendicitis, cholangitis, etc.
9. Other infections not covered above.
10. Fungal infections as a separate category.

Table 5.2. Classification and diagnosis of infection: pre defined criteria

5.2.1.3. Patient Population

This included all patients admitted to hospital with complications of decompensated liver cirrhosis and serum albumin < 30 g/L, aged over 18 years with anticipated hospital length of stay of 5 or more days at trial enrolment, which was no later than 72 hours from admission. This was subject to exclusion criteria as detailed in table 5.1. The diagnosis of cirrhosis was made by the clinical team as per standard UK practice and did not require liver biopsy or imaging. Acute decompensation of liver cirrhosis associated with organ failures is termed ACLF (Acute on Chronic Liver Failure). ACLF has a number of definitions^{97,213} based on the SOFA (sequential organ failure assessment) score, all are associated with a poor prognosis. This study included patients with decompensated cirrhosis with and without ACLF and recorded the development of ACLF during the study treatment period.

5.2.1.4. Consent

Patient information sheets were given to and discussed with potential patients before consent was sought. Informed consent was obtained from each participant or his or her legal representative. Patients who lacked mental capacity, for any reason, were not excluded from the trial. An important subgroup of patients with hepatic encephalopathy would lack capacity to consent and were amongst those considered to receive maximum benefit from HAS prior to the trial^{97,99,100}. In these cases consent was sought from an appropriate legal representative independent of the research team as per current UK clinical trials regulations¹⁰¹.

5.2.1.5. Intervention

After randomisation (when serum albumin is <30g/L) patients received either daily dose of 20% HAS intravenously if their serum albumin level was less than 35g/L (at approximately 100mLs/hour) or standard medical care (which may include 20% HAS infusions for indications listed in established guidelines only – see below) for a maximum of 14 days or discharge (if < 14 days). The volume of HAS each day will be determined by the patient's serum albumin level on that day (or the closest previous measurement if there are no results from that day available).

Table 5.3 shows the suggested dosing protocol for albumin administration in the treatment arm group. Responsible clinicians were given flexibility to alter this depending on the clinical situation. The effectiveness of this protocol, and approach, was verified in

the ATTIRE feasibility study²¹² (see Chapter 2). Differing regimens could be used to cover large volume paracentesis (8g of albumin per litre of ascites drained) or treat Hepatorenal syndrome (1g of albumin per kilogram of body weight) as per international guidelines^{98,102} but HAS *must* be prescribed and given if serum albumin <35g/L unless there were any safety concerns. All variations were be recorded in the patient’s daily Case Report Form (CRF).

Patient’s Serum Albumin Level	Amount of 20% HAS to be administered
≥35 g/L	none
30-34 g/L	100mLs
26-29 g/L	200mLs
20-25 g/L	300mLs
<20 g/L	400mLs

Table 5.3. Treatment arm dosing protocol for 20% HAS administration (amounts per day) as advised by measured serum albumin level on that day.

20% HAS was only permitted in the Standard of Care arm if the patient requires large volume paracentesis or has SBP or HRS (as per established guidelines^{53,102,214}). This was recorded in the patient’s CRF and if HAS was given for any other indication in the Standard of Care arm this was considered a protocol deviation. The administration of HAS in the Standard of Care arm was closely monitored by the Independent Data Monitoring Committee (IDMC) to ensure adherence to protocol.

Randomisation used a minimisation algorithm incorporating a random element, stratifying by centre, MELD score, and number of organ dysfunctions, serum albumin level and if antibiotics were currently prescribed. To ensure maximum balance was achieved across the stratification factors, minimisation was carried out on these factors separately.

5.2.1.6. Evaluations during and after treatment

Clinical, biochemical and microbiological data were collected during the trial treatment period using information from hospital notes. Blood samples for plasma storage were taken at day 1, 5, 10 and follow up only if the patient was also having standard of care blood tests.

5.2.2. Ethics

This trial involved a potentially vulnerable patient group that have hepatic encephalopathy and therefore lack the capacity to consent. Patients with encephalopathy are at high risk of infection and could be those that potentially receive maximal benefit from the intervention and therefore should not be denied access to the trial treatment. We have undertaken steps to ensure these patients are appropriately recruited to the trial and provided individual site training.

Research Ethics positive opinion was given by the London-Brent Research Ethics Committee (ref: 15/LO/0104) which specialise in trials involving patients who lack the capacity to consent. The Clinical Trials Authorisation was issued by the Medicines and Healthcare products Regulatory Agency (MHRA, ref: 20363/0350/001-0001). The trial is registered with the European Medicines Agency (EudraCT 2014-002300-24) and has been adopted by the NIHR. Recruitment commenced in April 2016 and finished in June 2019.

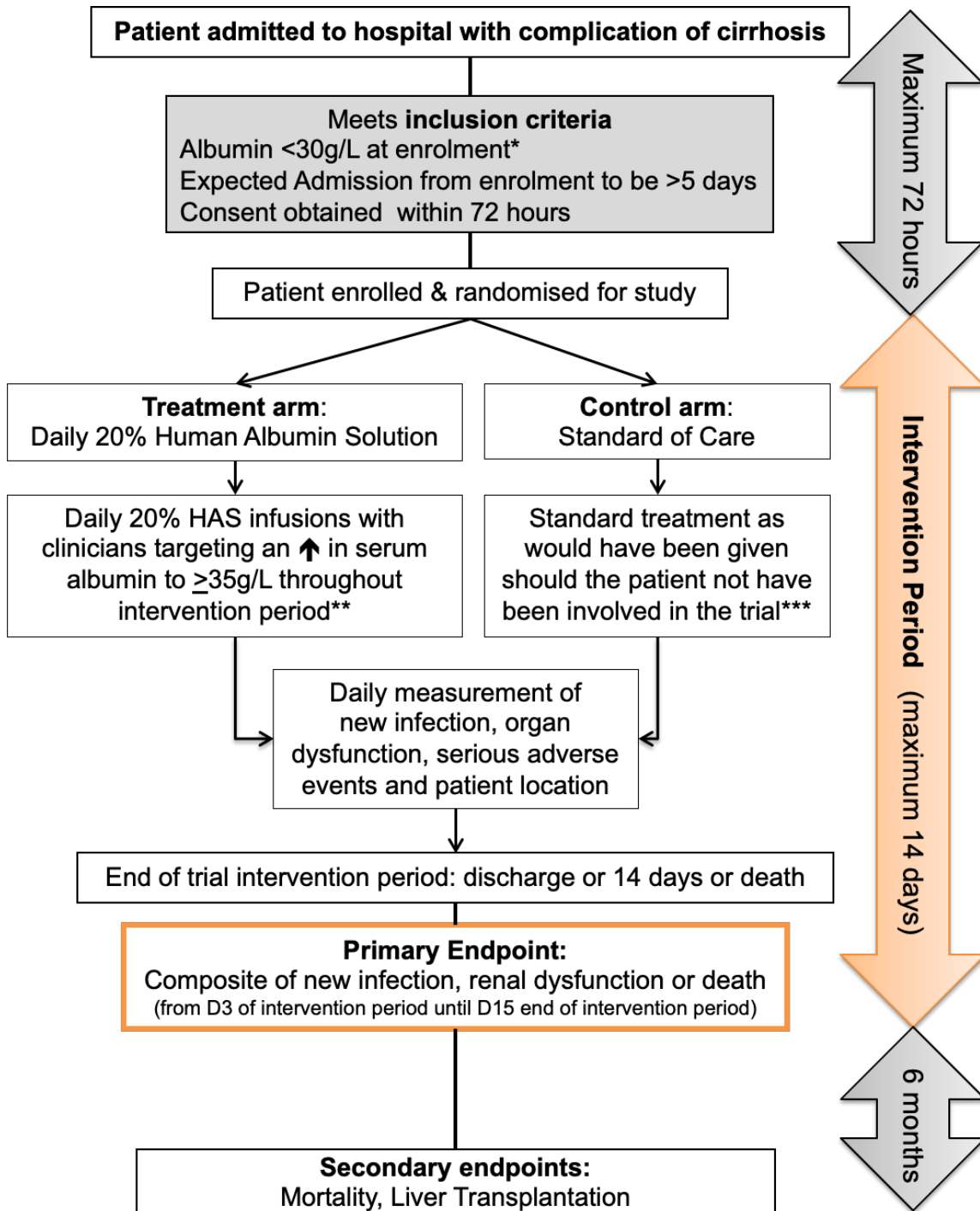


Figure 5.3. Overview of the clinical study protocol

5.2.3. Statistical considerations

Sample Size

A 20-30% incidence of nosocomial infection in decompensated cirrhosis patients is well documented with up to 30% of these patients developing organ dysfunction^{88,97} and an overall mortality of 38% at 1 month^{88,89,97}. These figures are supportive of 30% as a conservative estimate for the primary endpoint of incidence of new infection up to 14 days from randomisation.

We have assumed that the “immune-restorative” albumin treatment would reduce this rate by 30% to a rate of 21%, which would be considered clinically relevant. 389 patients per arm would be sufficient to detect such a difference with 80% power at a significance level of 0.05. Allowing for loss-to-follow-up/withdrawal of 10% from the trial, we aimed to recruit 433 to each arm (866 in total).

Statistical Evaluation

Baseline characteristics were summarised by treatment using appropriate descriptive statistics; means and standard deviations for approximately normally distributed variables, medians and interquartile ranges for non-normally distributed variables and counts (percentages) for categorical variables.

Primary outcome

The primary outcome was the difference in event rates, according to treatment, of the composite endpoint of infection, renal dysfunction and mortality within the intervention period (from ≥ 24 hours from the start of treatment/randomisation up to a maximum of 14 days or up to discharge if this is prior to 14 days).

Since the primary outcome has a binary classification, logistic regression was used to determine whether there was any difference in rates due to treatment, by inclusion of a binary covariate indicating treatment. The results were adjusted for pre-determined prognostic factors used as stratifying variables in the randomisation, which were included as additional covariates in the model. The model coefficient due to treatment gave an estimate of the difference in log odds, or equivalently to give an estimate of the odds multiplier, i.e. the change in odds of a negative outcome on the composite endpoint due to treatment with albumin. It is expected that this effect will be negative, so that treatment with albumin is seen to reduce the odds of a negative outcome. A reduction

from 30% to 21% would be associated with a reduction in odds of around 38%. Predicted probabilities will be presented for the composite outcome for each of the treatment arms, adjusted for the model covariates.

All statistical tests will use a 2-sided p-value of 0.05, unless otherwise specified, and all confidence intervals presented will be 95% and two-sided. All statistical analysis will be performed using Stata (StataCorp, College Station, TX, USA) and Prism.

5.2.4. Ex vivo analyses of the impact of 20% HAS treatment on plasma mediated immune dysfunction, albumin binding capacity and markers of vascular filling

Based on the clinical study two groups of exploratory, ex vivo analyses were conducted:

Study 1: Evaluating plasma mediated immune dysfunction, albumin-PGE₂ binding capacity and markers of vascular filling in patients treated with serum targeted 20% HAS versus those who were not.

Study 2: Evaluating plasma mediated immune dysfunction, albumin-PGE₂ binding capacity and markers of vascular filling in patients diagnosed with infection versus those who were not.

These analyses were conducted blinded to treatment arm with the assistance of UCL CTU.

5.2.4.1. Peripheral Blood Collection

5.2.4.1.1. Healthy Volunteer Plasma

For healthy volunteer plasma collection, blood was collected in Lithium Heparin (17 IU/mL) vacutainers (*Becton Dickinson*, UK). Tubes were inverted repeatedly and immediately centrifuged at 1300x *g*, 10 min at room temperature. Plasma was aliquoted and stored at -80 °C.

5.2.4.1.2. Patient Plasma

Samples were obtained at the time points described in figure 5.4. Patient's blood samples were taken using 9mL lithium heparin tubes prior to treatment with albumin, at day 5 and day 10 thereafter when usual standard of care blood was taken. These were then labeled with the patient's trial ID and the day of sample collection. Full lithium heparin tubes were transferred to site's hospital laboratories where samples were spun at 1300x *g* at 20°C. The plasma layer was removed and frozen at -80°C in 2mL cryovials

with the corresponding trial identifier. Samples were collected from 829 patients at 33 UK hospital sites. They were transferred to UCL (Rayne Building, O'Brien Lab) at the end of the recruitment period in 2019.

Sample Collection

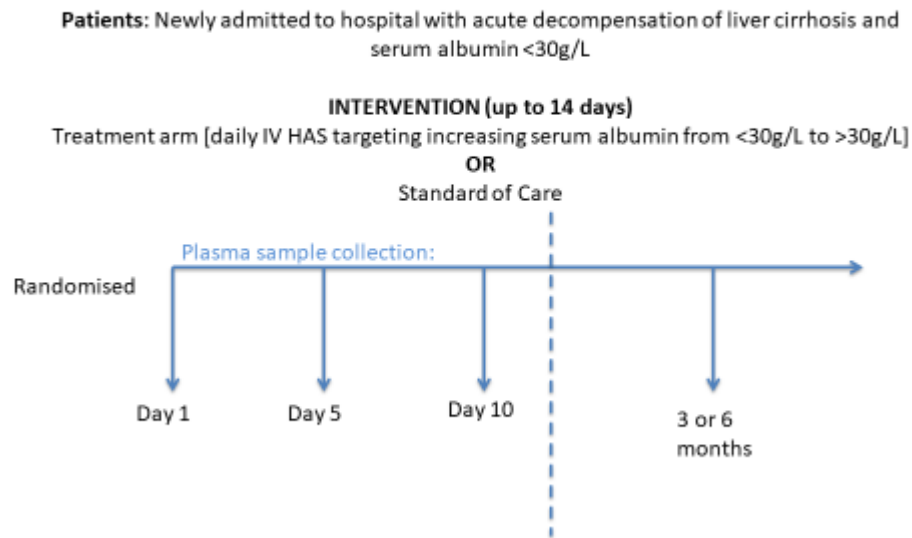


Figure 5.4. RCT Sample collection timeline.

5.2.4.2. Sample selection for analysis

The primary outcome measure of the ex vivo analysis was improvement post treatment in the bioassay developed in chapter 2 measuring plasma mediated immune dysfunction as measured by TNF α production from LPS stimulated monocyte derived macrophages (MDMs). The minimum number of patient samples selected for analysis was based on these previous measurements:

Based on the post HAS treatment improvement in LPS stimulated MDM TNF α production observed previously (17.7ng/mL pre-treatment, versus 19.5ng/mL post treatment) with a known sample size of 866 patients, estimated sample size for a two sample paired means test (with a power of 0.8 and 2 sided p value of 0.05) a sample size of 47 patients in each treatment arm was required. Therefore pre and post treatment planned analysis was for a total of at least 94 patients.

After CRF data entry of daily albumin levels at UCL Clinical Trials Unit (CTU) the trial statistician identified sample numbers for analysis corresponding to:

Study 1: Patients that had samples collected at day 1 and day 5 with 50% of patients in the albumin treatment arm (achieving a serum albumin >30g/L by day 5) and 50% of patients in the standard of care arm. The sample was stratified by starting albumin level (aiming to achieve a spread of starting albumins in the following groups: <20g/L, 20-25g/L and 26-29g/L).

Study 2: Patients who had been diagnosed with infection at any point in the trial with sample collected at day 1 and day 5 or 10. Plus a group of control patients who had not been diagnosed with infection at any point in the trial.

A list of trial ID numbers was provided for analyses in pairs (2 samples for each patient). It was not known by the analyser (myself) which treatment arm the patient was in, the baseline serum albumin or clinical information aside from the infection CRF data for those diagnosed with infection. After analyses were completed all results were sent to UCL CTU trial statistician and I was unblinded (3 months after recruitment of final patient) enabling me to process the results by treatment group and albumin level.

5.2.4.3. In vitro differentiation of blood-borne monocytes into macrophages

Isolation of monocytes from cones from the NHS plateletpheresis service

Due to donor-donor monocyte variation and the amount of MDMs required for this analysis pooled white cells in leukoreduction system chambers were obtained from the NHS blood donation service Collingdale. These were from anonymous healthy platelet donors (plateletpheresis), these cones contain concentration proportion of white cells obtained during the platelet phoresis process (roughly 10-15 x that from 110mL donated blood). LPS stimulation of MDMs sourced in this way prior to the analysis showed that the resulting TNF α production was comparable to the cells isolated as described in chapter 2.

Approximately 10mL of concentrated cells were provided from one platelet donor. This volume was diluted up to 150mL HBSS divided into 3 falcons. 25mL of this dilution was then layered over 15mL of Ficoll Paque (6 falcons) and spun at 1000x g, 30 min, 25 °C, brake off, low acceleration. The interface layer containing the monocytes was removed and placed with 2mL of ACK lysis buffer per falcon (6 tubes). Cells were then washed with

HBSS and counted. EasySep™ negative selection human monocyte isolation kit (Stemcell, France) was used to isolate the monocytes from this stage (rosette sep could not be used as there were an inadequate number of red cells compared to the very high number of white cells present for the unwanted white cells to bind to). EasySep™ labels unwanted cells (non monocytes and CD16+ monocytes) with a magnetic isolation cocktail and a magnet is subsequently used to retain unwanted cells whilst monocytes are poured into a separate falcon for use. The protocol was used as per manufacturers instructions. After cells were counted 100µl of isolation cocktail was added to 10 x10⁷ cells in 2mL HBSS and left at room temperature for 5 minutes. 100µl of magnetic particles were then added and again left for 5minutes at room temperature prior to the total volume of the sample being topped up to 2.5mL and placed in the magnet for 2.5minutes. The enriched cell suspension containing CD14+ monocytes is subsequently removed and place in a new falcon.

Culture of monocyte derived macrophages

After isolation monocytes (either from a cone or direct blood donor) were counted and then re-suspended at 4x10⁶ cells/3mLs media in polystyrene plates (Corning®Costar®) and placed in an incubator at 37°C, 5% CO₂. After one hour media with any non adherent cells was removed and replaced with fresh media which was then supplemented with 20ng/mL of M-CSF.

After 3 days media was changed and re supplemented with 20ng/mL M-CSF.

On day 6 media was aspirated and 1mL of lifting buffer (PBS plus 10mM EDTA and 4mg/mL lidocaine) at 10°C was added to each well and left for 20 minutes. Wells were then scrapped and suspended cells removed within the lifting buffer and placed in a 50mL falcon which was topped up to 50mLs with PBS and spun at 300x g at 20°C for 5 minutes. The supernatant was again removed and pellet was washed once more in 30mLs PBS and centrifuged at 300x g at 20°C for 10 minutes. The pellet was then resuspended in 1mL of media and cells were counted and then plated in a 96 well tissue culture treated plate (Corning®Costar®) at 50,000 cells/well in 100µl of media containing 20ng/mL M-CSF. Plates incubated for 24 hours prior to experiments to allow cells to re-adhere.

5.2.4.4. LPS Stimulation

MDMs were treated sequentially as follows:

1. 50µl media removed per well so all wells contained 50µl plus cells
2. 25µl of each PGE₂ receptor antagonist: MF498 8µM (EP4), PF-04418948 8µM (EP2 antagonist) (*end well concentrations were 1µM*)
3. 50µl (25% v/v) healthy volunteer or patient plasma
4. 50µl Lipopolysaccharide 400ng/mL (LPS; *Salmonella abortus equi* S-form, [TLRgrade™], Enzo Life Science, 1ng/mL) (*end well concentration was 100ng/mL*)

MF498 was obtained from Cayman Chemicals (MI, USA), reconstituted in DMSO (<0.01%) to form a stock solution, and working concentrations made in appropriate culture media. PF-04418948 (Sigma Aldrich, USA) was reconstituted in DMF (<0.01%). 15 minutes was allowed between each addition step, described above, to allow receptor binding/activation. After addition of LPS, cells were incubated for 4 hours (37°C/5% CO₂) and 50µl of supernatant removed and stored at -80°C prior to analysis. At 24 hours a further 50µl of supernatant was removed and stored at -80°C.

These experiments were conducted to characterise:

- Impact of healthy volunteer or patient plasma (anti-coagulated with Lithium Heparin) on cytokine release.
- A reversal of possible plasma PGE₂ effect by selective EP-receptor antagonists:
 - EP2: PF-04418948
 - EP4: MF498

Due to well – well variation in this assay all samples were evaluated with 3 technical repeats (this included 3 technical repeats when cells were pretreated with EP receptor antagonists) and mean of technical repeats reported in the results section.

5.2.4.4. Single-Analyte Enzyme Linked Immunosorbent Assay

Method described in section 3.2.6. ELISA was used to measure TNFα in 4 hour supernatants and IL-10 in 24 hour supernatants as described in section 5.2.5.

5.2.4.5. *R&D Systems Luminex® Assay*

Evaluation of 14 plasma cytokines, chemokines and small proteins known to be involved with the inflammatory response and immune regulation was undertaken via Luminex assay® (R&D Systems, USA) according to the manufacturer's instructions. This is a bead based multiplex assay allowing accurate, concurrent measurement of multiple analytes in a small volume of sample. They utilize color-coded superparamagnetic beads coated with analyte-specific antibodies. Beads recognizing different target analytes are mixed together and incubated with the sample. Captured analytes are subsequently detected using a cocktail of biotinylated detection antibodies and a streptavidin-phycoerythrin conjugate. The Bio-Rad Bio-Plex then uses a laser to excite dyes in each microparticle to identify the microparticle region and a second laser to excite the PE and measure the amount of analyte bound to the microparticle. All fluorescence emissions from each microparticle are then analysed as they pass through a flow cell giving different emission levels as measured by a photomultiplier tube and an avalanche photodiode.

Briefly, after defrosting, samples were centrifuged at 16000g for 6 minutes and then diluted in calibrator diluent RD6-52 (1:2 for all analytes apart from sCD14 and LBP in which assays plasma was diluted to 1:200). Standards were made up as per the specific product sheet and diluted 1:3 serially to produce a standard curve with the range of detection detailed in table 5.4. Samples and standards were plated using the supplied opaque plate and the microparticle cocktail was added as per instruction. The plate was then sealed with foil and left overnight (14-16 hours) at 4°C on an orbital shaker at 900rpm. Plates were washed with the addition of a plate magnet and antibody cocktail for the same analytes was added with the plate left on the orbital shaker at 900rpm for 1 hour at room temperature. Plates were washed again, with the use of a magnetic plate, and streptavidin-phycoerythrin conjugate was added and the plates placed on the orbital shaker at 900rpm for 30 minutes. The plate then underwent a final wash procedure and the remaining particles were then re suspended in wash buffer, placed on the orbital shaker at 900rpm for 5 mins and finally read on a Bio-rad Bio-plex reader (Department of psychobiology, Torrington place) to determine individual cytokine concentrations interpolated from a standard curve of known concentrations.

Cytokine	<i>Detection Range (pg/mL)</i>	Marker of vascular filling	<i>Detection Range (pg/mL)</i>	Proteins elevated in acute inflammatory response	<i>Detection Range (pg/mL)</i>
IL-1 β	7,900 - 10.8	NT-Pro Atrial natriuretic peptide	129,860 - 178	LPS binding protein	32,990,000 - 45,254
IL-6	1,460 - 2.0	Syndecan-1	126,840 - 174	Pro calcitonin	4,160 - 5.7
IL-8	1,440 - 2.0	Renin	52,660 - 72.2	Soluble CD14	11,344,000 - 15,561
IL-10	1,800 - 2.5			CD163	2,648,800 - 3,633
TNF α	4,100 - 5.6				
IL-4	6,760 - 9.3				
CCL8/ MCP-2	6,800 - 9.3				

Table 5.4. Measured analytes with luminex and range of detection

5.2.4.6. PGE₂ Enzymeimmunoassay (EIA)

PGE₂ concentration in plasma samples from the ATTIRE RCT was determined using the Amersham Prostaglandin E2 Biotrak Enzymeimmunoassay (EIA) System (GE Healthcare) as per the manufacturer's instructions. In brief, this assay relies on the forward sequential competitive binding technique whereby PGE₂ in a sample competes with Peroxidase-labelled PGE₂ for a limited number of binding sites on a mouse monoclonal antibody. Samples were first lysed to dissociate PGE₂ from soluble receptors or interfering binding proteins in plasma, leaving total PGE₂ to be analysed. Sample and labelled PGE₂ are added to the pre-coated wells absorbance simultaneously leading to direct competition for binding. After several washes, quantification of peroxidase labelled-PGE₂ was performed by monitoring the enzymatic activity of peroxidase in the presence of the substrate 3,3',5,5'-tetramethylbenzidine; which was measured spectrophotometrically by the increased absorbency at 450 nm. Therefore, absorbance intensity was inversely proportional to the concentration of PGE₂ in the sample. Unknown concentrations were determined via interpolation to a reference curve generated from a series of known PGE₂ concentrations.

5.2.4.7. Plasma Calprotectin (measured at Gentian laboratories, Sweden)

Plasma calprotectin levels were measured using the Gentian Calprotectin turbidimetric immunoassay GCAL® (Gentian, Norway) and measured in duplicate on a Cobas c501

analyser (Roche, Switzerland). The samples were stored at -80°C , and were measured within two hours after thawing.

5.2.4.8. $^3\text{H-E}_2$ equilibrium dialysis with plasma

As described in 4.2.3

5.2.4.9. HPLC analysis of plasma

As described in 4.2.7.

5.3. RESULTS

5.3.1. Infection is not reduced in acute decompensation patients treated with IV 20% HAS to target a serum albumin of 30g/L

5.3.1.1. Recruitment

Over 3-years, there were 9,273 patients screened, 1,563 considered eligible and 828 randomisations with evaluable data (one withdrew permission to use data), involving 778 independent patients and 50 re-randomisations to targeted albumin therapy or standard care (see figure 5.5). We initially estimated a loss to follow-up/trial withdrawal of 10% and calculated 433 per arm, however, with lower than anticipated loss to follow up/withdrawal, we completed the trial following 828 randomisations.

5.3.1.2. Baseline Characteristics

The majority of patients were male, in their early 50s and had alcohol as the aetiology of cirrhosis (table 5.5). Ascites, hepatic encephalopathy (any grade) and possible infection were the most common reasons for hospital admission. There was a spread of serum albumin levels, with most patients having a serum albumin of <25g/L. Physiological variables were well balanced between treatment arms.

CONSORT Flow Diagram

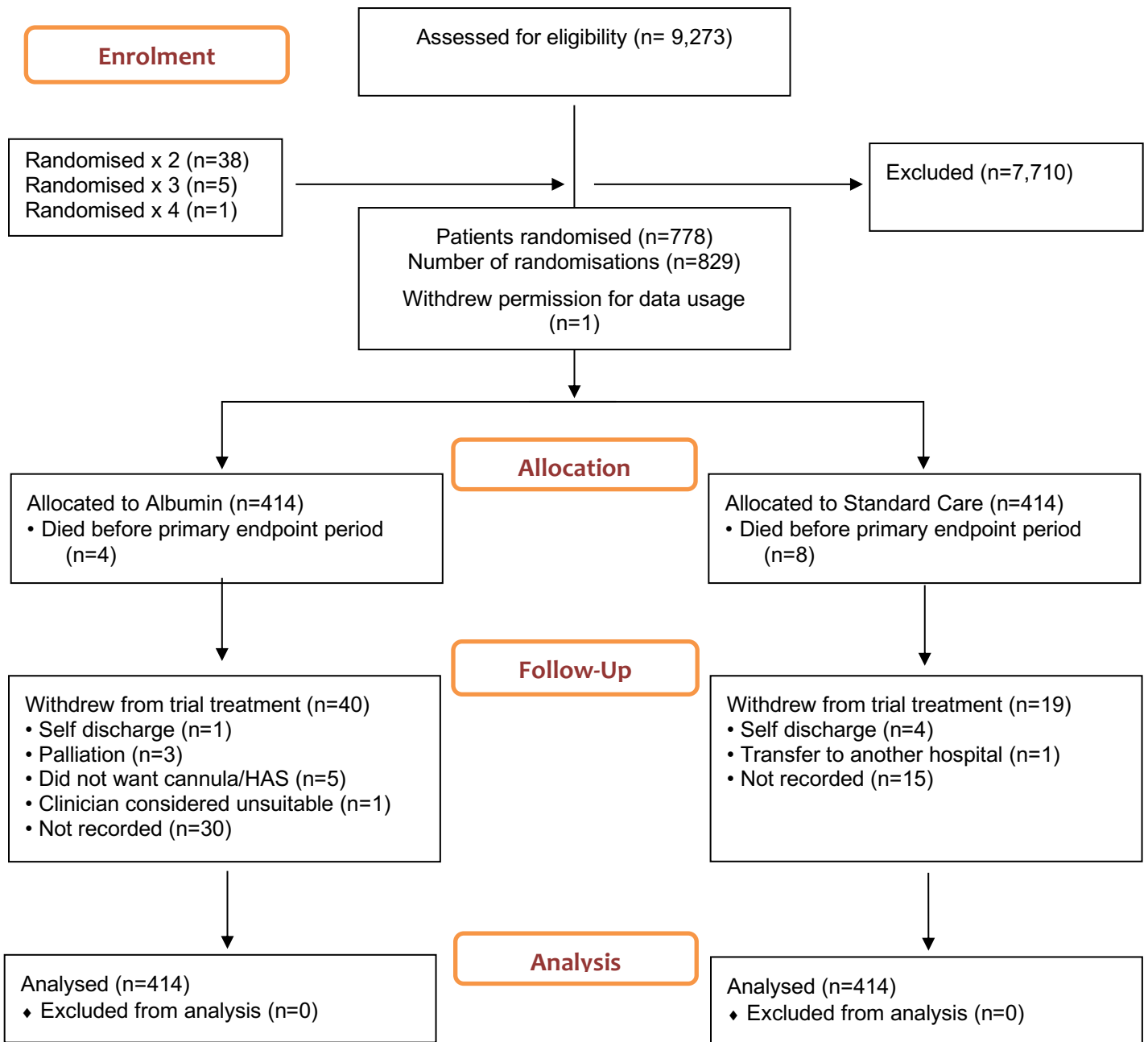


Figure 5.5 Consort Diagram

Characteristics of the Patients at Baseline.*			
	Albumin (N=414)	Standard Care (N=414)	Total (N=828)
Characteristic			
Age Mean (s.d.)	53.7 (10.5)	53.7 (10.6)	53.7 (10.5)
Female sex – no. (%)	109 (26.3)	133 (32.1)	242 (29.2)
Admitted to ward – no. (%)	402 (97.1)	404 (97.6)	806 (97.3)
Admitted to Intensive Care Unit – no. (%)	10 (2.4)	8 (1.9)	18 (2.2)
Aetiology of cirrhosis[†] - no. (%)			
Alcohol	379 (91.5)	364 (87.9)	743 (89.7)
Hepatitis C	31 (7.5)	42 (10.1)	73 (8.8)
NAFLD	27 (6.5)	31 (7.5)	58 (7.0)
Reason for decompensation admission[†] - no. (%)			
Encephalopathy	89 (21.5)	72 (17.4)	161 (19.4)
Suspected variceal Bleed	56 (13.5)	63 (15.2)	119 (14.4)
New onset or worsening ascites	259 (62.5)	296 (71.5)	555 (67.0)
Infection - no. (%)			
Diagnosed with infection [‡]	110 (26.6)	123 (29.7)	233 (28.1)
Prescribed antibiotics	210 (50.7)	215 (51.9)	425 (51.3)
Serum albumin level – no. (%)			
<20 g/L	120 (29.0)	121 (29.2)	241 (29.1)
20-25 g/L	234 (56.5)	227 (54.8)	461 (55.7)
26-29 g/L	60 (14.5)	66 (15.9)	126 (15.2)
Physiological variable – median (IQR)			
Creatinine (umol/L) [§]	66.32 (52.2-88.5)	68.97 (56.6-92.8)	68.1 (53.9-90.2)
Bilirubin (umol/L)	95.08 (46.0-174.1)	94.05 (46.0-165.0)	94.05 (46.0-171)
INR	2 (1-2)	2 (1-2)	2 (1-2)
MELD Score[¶] – no. (%)			
<20	222 (53.6)	221 (53.4)	443 (53.5)
>=20	192 (46.4)	193 (46.6)	385 (46.5)
Number of organ dysfunctions – no. (%)			
0-1	401 (96.9)	403 (97.3)	804 (97.1)
2-4	13 (3.1)	11 (2.7)	24 (2.9)

Table 5.5. Characteristics of the Patients at Baseline.*

* There were no significant differences between the two groups.

† Etiology and reason for admission was reported by the patient or taken from the clinical notes. Patients could have >1 cirrhosis aetiology (e.g. Hepatitis C and Alcohol) or reason for admission.

‡ Clinical diagnosis of infection at randomization by site medical team.

§ Creatinine measurement available for n=407 in HAS group and n=413 in SOC.

¶ Model for end stage liver disease <https://optn.transplant.hrsa.gov/resources/allocation-calculators/meld-calculator/>

|| Organ dysfunctions at baseline were defined as previously described in the ATTIRE feasibility study²² based on components of CLIF-SOFA score.

5.3.1.3. Intervention and protocol compliance

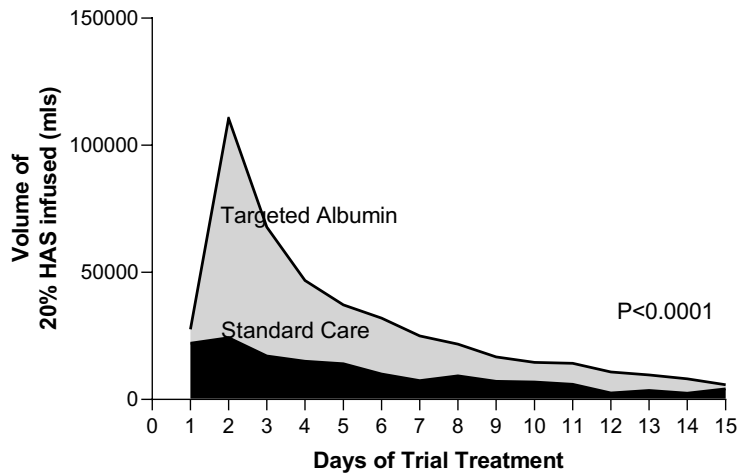
There were 58 major protocol deviations, however most of these were related to the timeliness of SAE reporting. Only 12 were related to under prescription of HAS in the treatment arm or prescription of HAS when not clinically indicated in the standard of care arm.

The total amount of HAS administered was significantly different between treatment arms with the median amount being 1000mLs 20% HAS in the treatment arm (Figure 5.6.A). The 20% HAS administration guidance protocol was adequately adhered to as demonstrated by the successful incrementation of serum albumin to >30g/L in the treatment arm as compared to no overall change in serum albumin in the standard of care arm (Figure 5.6.B).

5.3.1.4. Clinical Endpoints

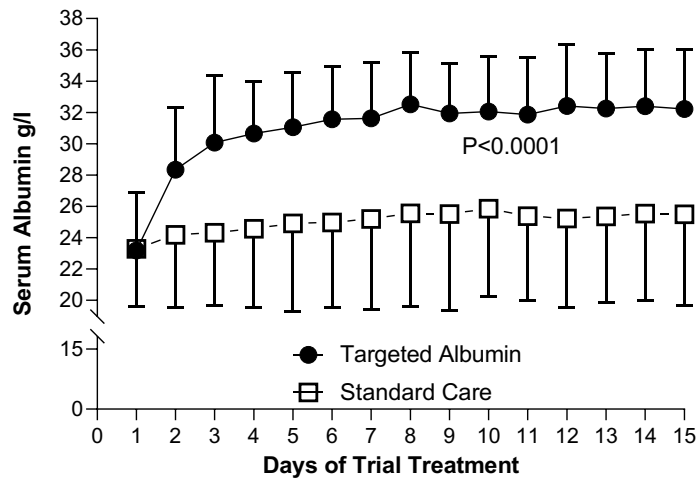
There was no difference in composite primary endpoint between targeted albumin (n=125/414; 30.2%) and standard care (n=128/414, 30.9%, Odds Ratio 0.968 (95%CI 0.716-1.307, p=0.830. Table 5.6). In addition there were no differences in components of the primary endpoint, extended mortality time periods, adverse events, use of terlipressin, or length of hospital stay (Table 5.6).

A. Total 20% HAS Infused



Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
TA	401	404	391	364	344	312	265	235	196	177	162	151	145	132	122
SC	400	402	380	360	342	305	266	233	205	183	169	152	134	124	108

B. Serum Albumin



Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
TA	414	373	342	315	296	264	239	194	171	139	140	131	115	108	85
SC	413	340	301	283	250	230	198	182	155	128	116	115	107	98	82

Figure 5.6. (A) Volumes of 20% HAS infused and (B) Median serum albumin levels during trial treatment period

	Albumin	Standard Care	Adjusted Odds Ratio (95% CI)	P-value
Outcome	(N=414)	(N=414)		
Primary outcome – no. (%)				
Protocol defined	125 (30.2%)	128 (30.9%)	0.968 (0.716-1.307)	0.830
Including all reported deaths†	128 (30.9%)	127 (30.7%)	1.011 (0.749-1.364)	0.944
Secondary Outcomes: no. (%)				
Composite endpoint components‡:				
Incidence of new Infection	87 (21.0%)	76 (18.4%)	1.196 (0.845-1.693)	0.313
Incidence of renal dysfunction	45 (10.9%)	62 (15.0%)	0.674 (0.445-1.022)	0.063
Incidence of death	32 (7.7%)	34 (8.2%)	0.936 (0.566-1.550)	0.798
Mortality at 28 days	56 (13.5%)	65 (15.7%)	0.834 (0.559-1.245)	0.374
Mortality at 3 months	98 (23.7%)	98 (23.7%)	1.014 (0.727-1.414)	0.935
Mortality at 6 months	140 (33.8%)	125 (30.2%)	1.212 (0.895-1.639)	0.213
Incidence of liver transplant§	3 (0.8%)	1 (0.2%)	-	0.6241
One or more SAEs¶	53 (12.8%)	50 (12.1%)	1.057 (0.694-1.611)	0.795
Use of terlipressin for:				
a) Renal dysfunction	12 (2.9%)	12 (2.9%)	-	1.00
b) Hypotension	4 (1.0%)	1 (0.24%)	-	0.374
c) Variceal bleeding	32 (7.7%)	32 (7.7%)	-	1.00
Time to death (days)+	147 (34.5%)	133 (32.1%)		0.398
Secondary Outcomes - median (IQR)				
Total 20% HAS administered (mLs)	1000 (700–1500)	100 (0-600)	710.4 (631.9-788.8)	<.0001
MELD at end of treatment period	18.39 (14.6-22.7)	17.35 (13.7-21.3)	0.621 (0.029-1.270)	0.061
Duration of hospital stay (days)	8 (6 – 15)	9 (6 – 15)	1.005 (0.961-1.052)	0.814
Days in ICU during treatment period	153	118	1.337 (1.042-1.715)	0.023

Table 5.6. Outcomes Unless stated time is given the measurement is during the trial treatment period (15 days from randomization).

‡ Outcomes are defined in the protocol paper²². ^Analysed without adjustment because of small number of events. . § As reported by sites at 6 months post randomisation. ¶ SAE = serious adverse events.

Subgroup analyses including baseline organ dysfunction, infection, MELD score, albumin level or reason for admission showed results consistent with the primary outcome. (Figure 5.7).

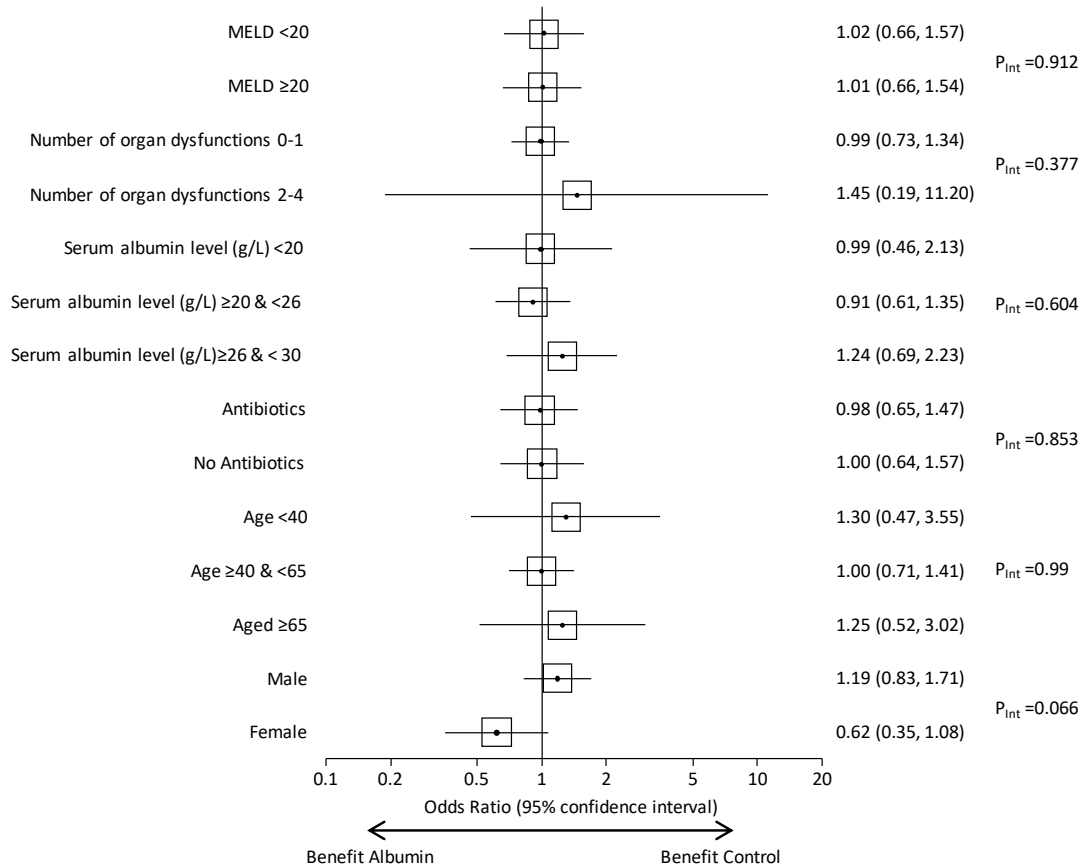


Figure 5.7. Primary outcome subgroup analysis.
P_{int} = Interaction test P value (nb figure produced by N.Freemantle).

5.3.1.5. Serious Adverse Events

There were more SAEs graded 'severe' in the albumin treatment arm (table 5.7). There were 21 serious adverse events reported in the albumin arm of the trial with evidence of pulmonary oedema or fluid overload and six in the standard care arm. There were no other differences in commonly occurring serious adverse events between treatment arms.

Serious Adverse Events*			
	Albumin (N=414)	Standard Care (N=414)	Total (N=828)
Event			
Serious Adverse Event			
Grade 3: Severe	47	20	55
Grade 4: Life threatening	32	20	52
Grade 5: Death	43	47	90
Most Common Serious Adverse Events*			
Respiratory tract infection	20	20	
Pulmonary oedema / fluid overload	20	7	
Gastrointestinal haemorrhage	12	12	
Hepatic Encephalopathy	6	5	

Table 5.7. Serious Adverse Events during the trial treatment period

*Possible to have greater than 1 clinical diagnosis per SAE.

5.3.2. Ex vivo analyses of the impact of 20% HAS treatment on plasma mediated immune dysfunction, albumin binding capacity and markers of vascular filling

5.3.2.1. Baseline characteristics and clinical outcomes of samples analysed in this sub-study reflected the overall study population

143/828 patients from the main clinical study had plasma samples analysed. Table 5.8 summarises the baseline clinical characteristics of the patients in this sub study as compared to the overall study (table 5.5). Characteristics were similar although slightly less well balanced between study arms, as expected with a smaller number of participants. Overall mean MELD score was 20.2 (SD 7.0) with mean baseline serum albumin of 19.8g/L (SD 7.6) in the HAS treatment arm and 20.6g/L (SD 6.2) in the standard of care arm.

Baseline characteristics of the patients in plasma analysis sub study			
Characteristic	Albumin (N=71)	Standard Care (N=72)	Total (N=143)
Age - yr			
Median	54.3	55.3	53.9
Interquartile range	12.7	13.7	14.0
Female sex – no. (%)	12 (16.9)	22 (30.5)	34 (23.8)
Aetiology of cirrhosis - no. (%)			
Alcohol	63 (88.7)	60 (83.3)	123 (86.0)
Hepatitis C	5 (7.0)	8 (11.1)	13 (9.1)
NAFLD*	5 (7.0)	10 (13.9)	15 (10.5)
Reasons for admission - no.			
Encephalopathy	15 (21.1)	12 (16.7)	27 (18.9)
Suspected variceal Bleed	8 (11.3)	6 (8.3)	14 (9.8)
Ascites management	48 (67.6)	45 (62.5)	93 (65.0)
Suspected infection	14 (19.7)	9 (12.5)	23 (16.1)
Jaundice	35 (49.3)	45 (62.5)	80 (55.9)
Serum albumin level – no. (%)			
<20 g/L	9 (12.7)	12 (16.7)	21 (14.7)
20-25 g/L	44 (62.0)	44 (61.1)	88 (61.5)
26-29 g/L	18 (25.4)	16 (22.2)	34 (23.8)
Physiological variable – median (IQR)			
Creatinine (µmol/L)	65.0 (28.5)	71.5 (31.0)	68.0 (31.0)
Bilirubin (µmol/L)	106 (190.5)	102.5 (164.5)	103 (183.0)
MELD Score – no. (%)			
<20	35 (49.3)	35 (48.6)	70 (49.0)
≥20	36 (50.7)	37 (51.4)	73 (51.0)
Diagnosed with infection – no. (%)	19 (26.8)	21 (29.2)	40 (28.0)
Prescribed antibiotics – no. (%)	39 (54.9)	34 (47.2)	73 (51.1)

Table 5.8. Baseline characteristics of the patients in plasma analysis sub study

** NAFLD = non alcoholic fatty liver disease

As in the larger clinical study, patients analysed in this sub study who were treated in the 20% HAS arm incremented their serum albumin to >30g/L by day 3 and this was maintained through the study treatment period (Figure 5.8i). A median of 1000mLs of 20% HAS was administered in the treatment arm and the majority of this fluid was given in treatment days 1-3 (Figure 5.8ii). 49% of the standard of care patients received 20% HAS at some point during the trial treatment period for established indications although this did not impact on the serum albumin levels overall in the standard of care group (Figure 5.8i) and median volume administered was 0mLs (IQR 600mLs).

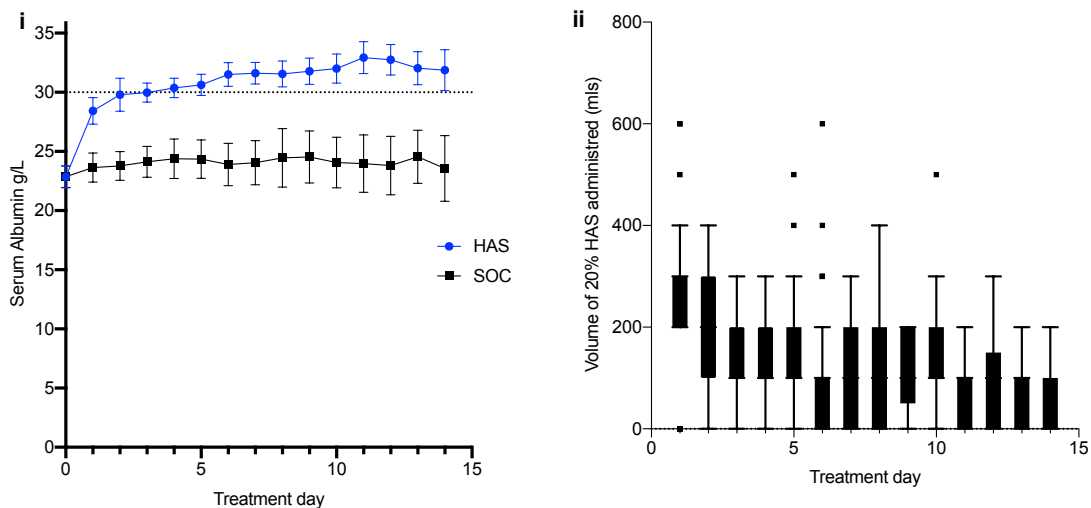


Figure 5.8. Serum albumin levels and amount of 20% HAS administered in plasma analysis patients
 (i). Daily mean serum albumin levels in HAS treatment arm patients (blue, n=71) versus standard of care patients (black, n=72) over the 14 day treatment period. (ii). Volume of 20% HAS infused per day in the HAS treatment arm. Box = IQR with error bars representing the range and dots the outliers.

Percentage incidence of the primary composite outcome was slightly higher in patients in the sample analysis study (36-39% as compared to 30%, table 5.9). This was due to higher percentage incidence of infection and renal dysfunction in both groups although numbers were a lot smaller so a change in 1-2 patient event rates would have a higher impact on percentages.

Clinical outcomes of the patients in plasma analysis sub study		
	Albumin (N=71)	Standard Care (N=72)
Outcome		
Primary outcome – no. (%)		
Protocol defined	28 (39.4%)	26 (36.1%)
Secondary Outcomes - no. (%)		
Composite endpoint components**:		
Incidence of new Infection	21 (29.6)	17 (23.6)
Incidence of renal dysfunction	9 (12.7)	14 (19.4)
Incidence of death	4 (5.6)	3 (4.2)
Mean duration of hospital stay (days)	11.2	11.3

Table 5.9. Clinical outcomes of the patients in plasma analysis sub study

5.3.2.2. HAS infusions have no immune modulatory effects which is consistent with lack of impact on clinical rates of infection:

5.3.2.2.1. There was no difference in patient plasma mediated monocyte derived macrophage dysfunction with 20% HAS treatment as compared to standard of care
Healthy volunteer monocyte derived macrophages (MDMs), in the presence of patient plasma, did not show any changes in the amount of TNF α produced 4 hours after LPS stimulation when patients had been treated with targeted 20% HAS therapy as compared to patients treated as per standard of care (figure 5.9i&ii). In the same assay IL-10 was measured 24hours after LPS stimulation. Again there was no differences post treatment (figure5.9iii&iv).

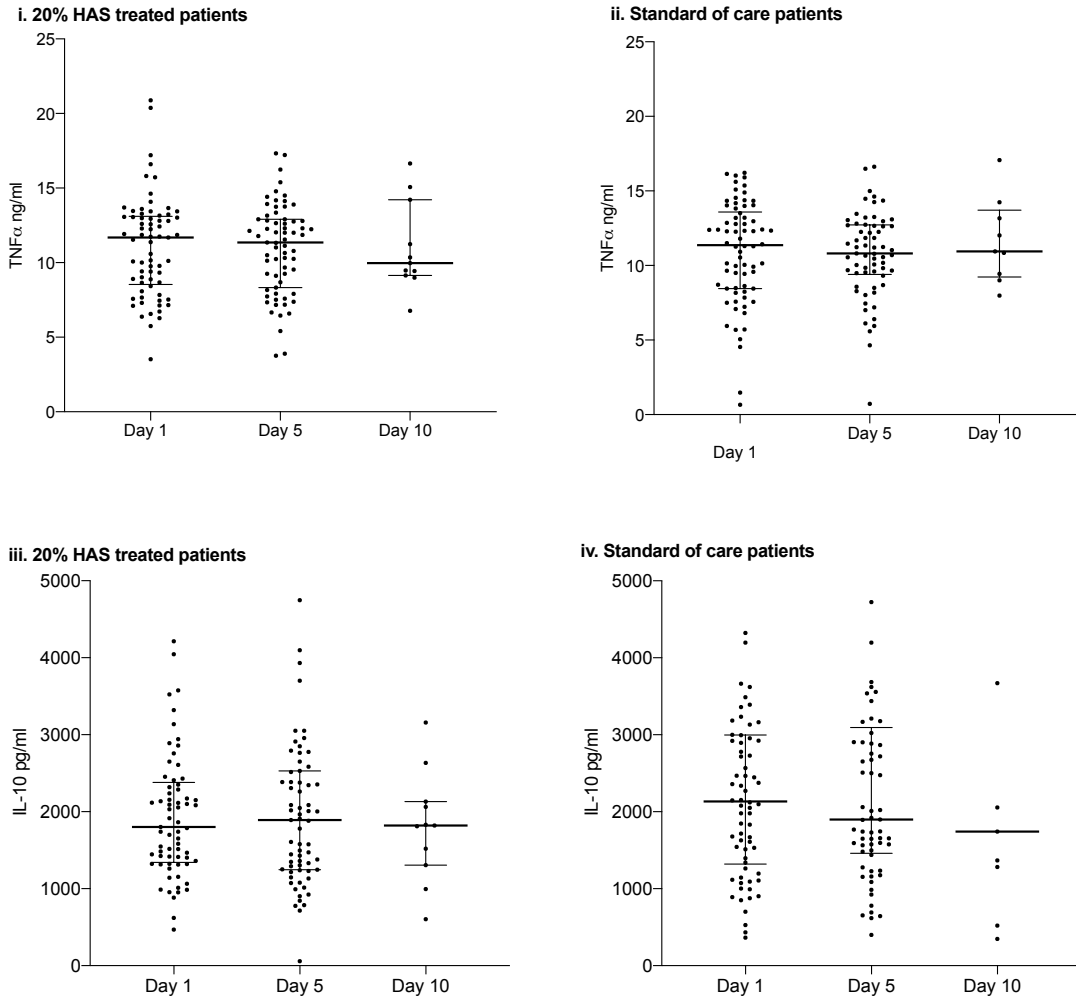


Figure 5.9. LPS stimulated TNF α (4 hours) and IL-10 (24 hours) production from MDMs in the presence of patient plasma at either day 1 or day 5 of the trial

There were no differences in TNF α production between day 1 and day 5 of the trial in both the (i) HAS treatment arm (n=67 patients with paired sample) and the (ii) standard of care arm (n=65 patients with paired sample). There were no differences in IL-10 production between day 1 and day 5 of the trial in both the (iii) HAS treatment arm (n=67 patients with paired sample) and the (iv) standard of care arm (n=65 patients with paired sample). *Horizontal bars represent mean, error bars 95% CI.*

5.3.2.2.2. There were no differences in plasma cytokines in patients treated with 20% HAS treatment as compared to standard of care

Plasma cytokines were measured at baseline, day 5 and day 10 in both treatment arms (figure 5.10 and table 5.10). There were no significant differences between treatment arms or over the course of the trial treatment period. Follow up sample numbers were small but plasma TNF α , IL-6 and IL-8 were lower at 3 month follow up than during the inpatient treatment period.

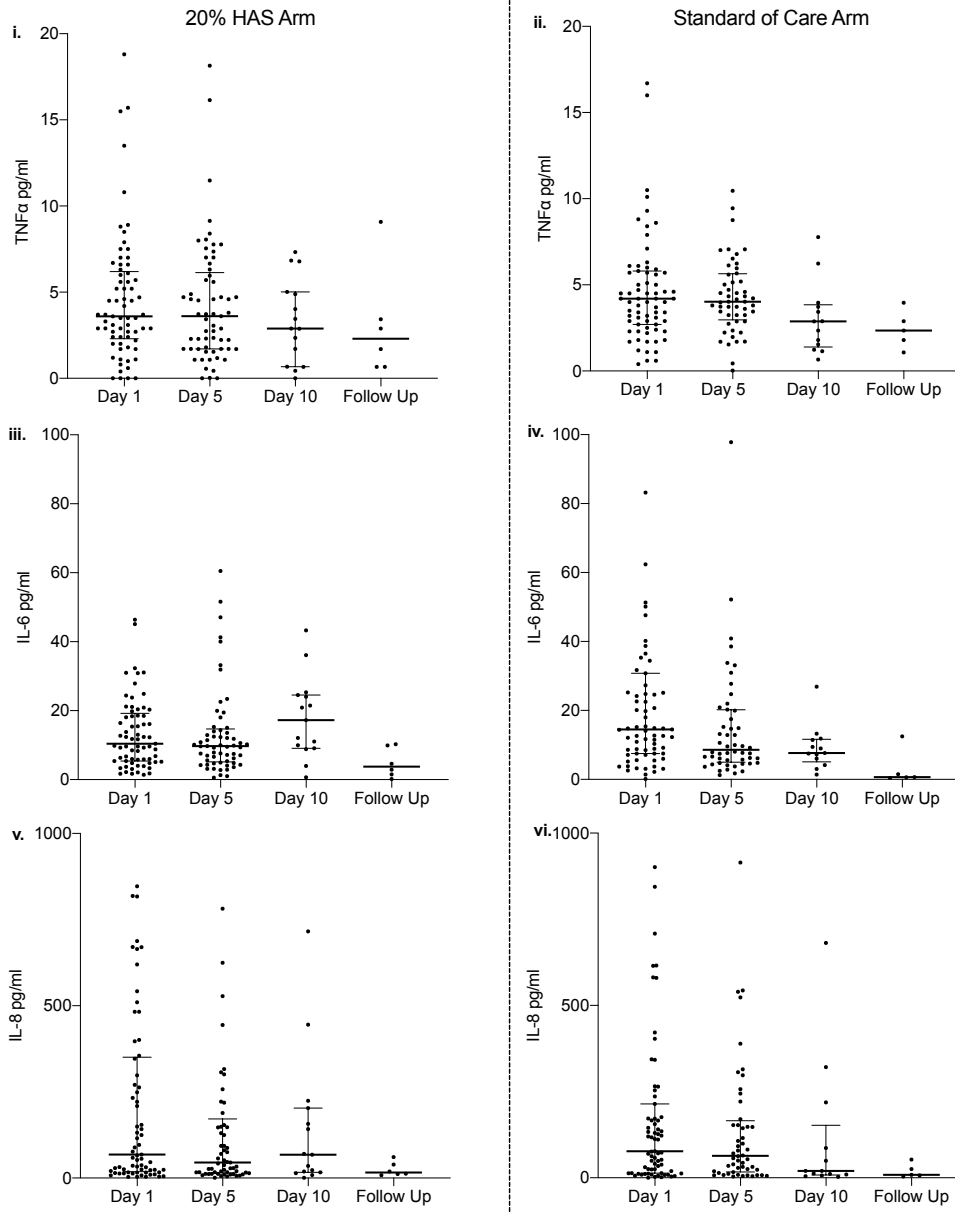


Figure 5.10. Plasma TNF α , IL-6 and IL-8 Levels at day 1,5, 10 and follow up in both treatment arms (I, iii, v) HAS arm patients at day 1 (n=69), day 5 (n=61), day 10 (n=15) and follow up (n=6). (ii, iv, vi) Standard of care arm patients at day 1 (n=71), day 5 (n=52), day 10 (n=13) and follow up (n=5). *Median and IQR shown (data not normally distributed)*

		Day 1 Median (IQR, range) pg/mL	Day 5 Median (IQR, range) pg/mL	Day 10 Median (IQR, range) pg/mL	Follow up Median (IQR, range) pg/mL
<i>n</i>		69	61	15	6
20% HAS Arm	IL-1 β	0 (1.30, 2.00)	0 (1.33, 2.72)	0 (0, 1.33)	0 (0,0)
	IL-4	0 (0, 29.80)	0 (0, 11.72)	0 (4.99, 14.03)	0 (0,0)
	IL-10	0 (0.35, 373.4)	0 (0.56, 314.5)	0 (0.67, 3.57)	0 (0,0)
<i>n</i>		71	52	13	5
Standard of care arm	IL-1 β	0 (1.30, 6.50)	0 (1.33, 5.24)	0 (0.12, 1.33)	0 (0,0)
	IL-4	0 (4.2, 298.7)	0 (4.19, 20.04)	0 (0, 10.40)	0 (0,0)
	IL-10	0 (0.90, 137.2)	0 (1.29, 96.87)	0 (1.82, 64.71)	0 (0,0)

Table 5.10. Median IL-1 β , IL-4 and IL-10 in plasma at days 1,5,10 and follow up in HAS and standard of care patients

5.3.2.3. Exploration of reasons for treatment failure – was it PGE₂ related?

5.3.2.3.1. There was a decrease in total plasma PGE₂ in patients treated with targeted 20% HAS as compared to patients managed as per current standard of care

Sample analysis from 62 patients with available day 1 and 5 paired samples from the HAS treatment arm showed a significant overall reduction in total plasma PGE₂ at day 5 (figure 5.11i) with no reduction in the standard of care arm post treatment (figure 5.11ii). In HAS treated patients PGE₂ decreased from a mean of 1072pg/mL (SD 1239) on day 1 to 805.9pg/mL (SD 742.2) on day 5. Healthy volunteer plasma PGE₂ using this assay in our lab measured 2.46pg/mL (SD 0.26, n=4)²¹⁵.

5.3.2.3.2. Plasma albumin-PGE₂ binding capacity improves in both treatment arms

Patients in the HAS treatment arm showed a similar percentage improvement in plasma albumin PGE₂ binding capacity as seen in the single arm study (chapter 4, figure 4.8Bi) with a mean increase of 8.3% more PGE₂ bound post treatment at day 5 (figure 5.11iii). However standard of care patients also had a significantly improved PGE₂ binding capacity at day 5 with a mean increase of 4.7% (figure 5.11iv).

5.3.2.3.3. Inhibition of PGE₂ improves patient plasma mediated MDM dysfunction in both treatment arms, but not to that of healthy volunteer plasma

When MDMs were stimulated with LPS, pan PGE₂ receptor blockade in the presence of day 1 (baseline) plasma consistently caused an increase in TNF α production as previously seen in chapter 3. There was a significant improvement with EP receptor blockade in baseline sample from both treatment arms, but not to levels of healthy volunteer plasma (figure 5.11v).

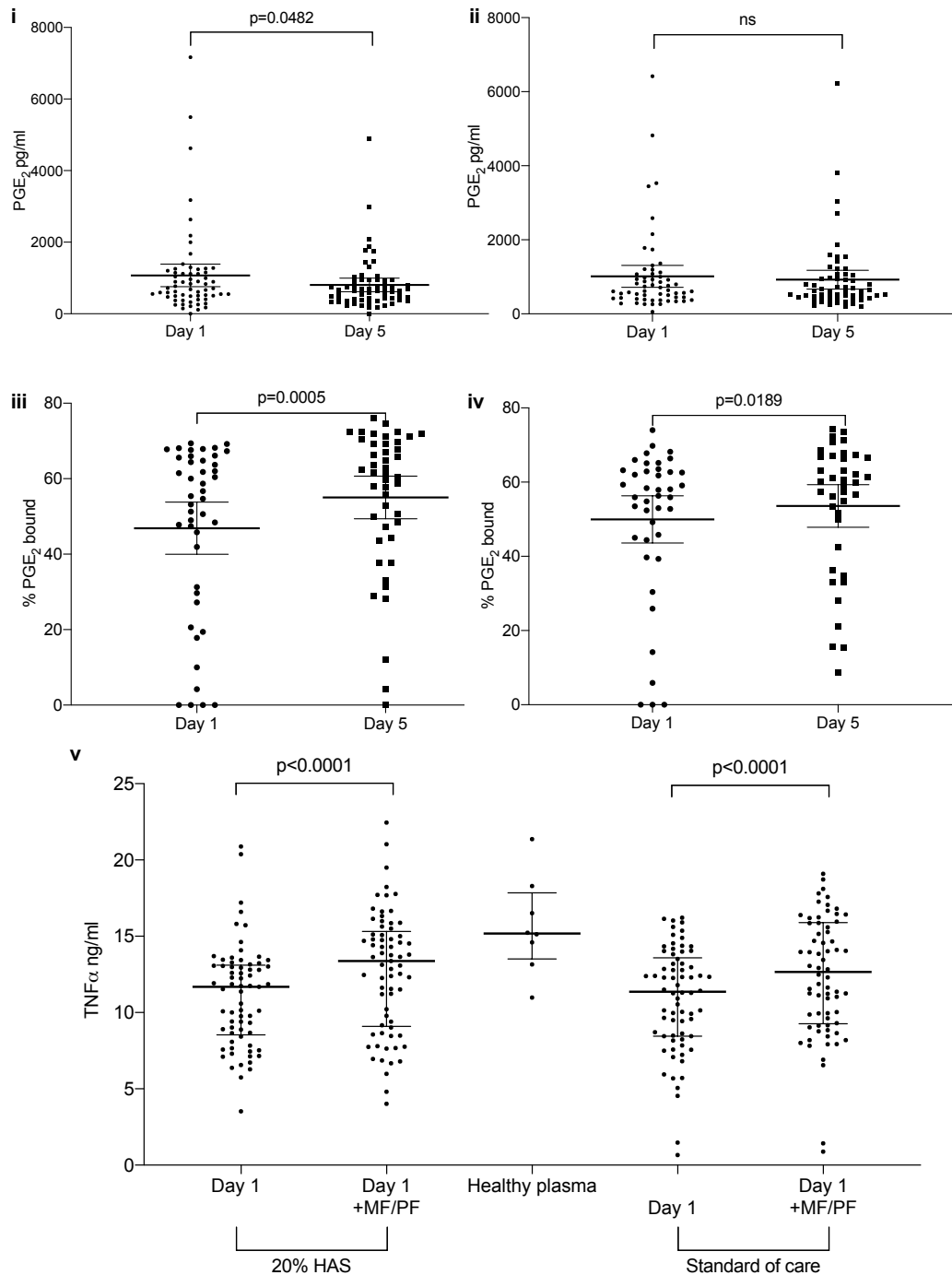


Figure 5.11. 20% HAS treated patients have a decrease in plasma PGE₂ at day 5 of treatment with an increase in albumin-PGE₂ binding capacity. PGE₂ receptor antagonism improves plasma MDM suppression at baseline in both groups.

(i.) Plasma PGE₂ decreases by a mean 266.2pg/mL (95% CI -530.3 to -2.133) by day 5 in n=62 patients with paired sample at day 1 and day 5. (ii.) There is no significant difference in plasma PGE₂ in 58 control arm patients (mean decrease 86.88pg/mL, -231.2 to 57.42, p=0.2329). (iii) %PGE₂ binding capacity significantly improves in patients treated with serum targeted HAS infusions by a mean of 8.317% (CI 3.898% to 12.74%, p=0.0005, n=42 patient paired samples). (iv) %PGE₂ binding capacity significantly improves in patients in the standard of care arm by a mean of 4.733% (CI 0.8314% to 8.635%, p=0.0189, n=42 patient paired samples). (v) EP receptor antagonism with MF/PF reverses the immunosuppressive effect of day 1 plasma in both trial treatment arms, but not to the level of healthy volunteer plasma.

5.3.2.3.4. There is no PGE₂ mediated difference between patients who develop infection versus those who do not

When patients were split into groups (those who developed infection and those who did not) there were no differences in total plasma PGE₂ between pre and post treatment samples (figure 5.12.i and ii). In patients who had sample available for analysis, baseline plasma PGE₂ was higher in patients who went onto develop infection D3-15 (n=37) as opposed to those who did not (n=74), however this difference was not significant (1310pg/mL vs 1012pg/mL, p=0.286, CI -257.2 to 852.2).

There were non-significant improvements in albumin-PGE₂ binding at day 5 in both HAS and standard of care patients who did not develop a new infection (figure 5.12iii). In the small number of patients who developed infection there is a smaller improvement (non-significant) in PGE₂ binding in the HAS treated patients at day 5 and a non-significant decrease in PGE₂ binding in the standard of care patients (figure 5.12iv).

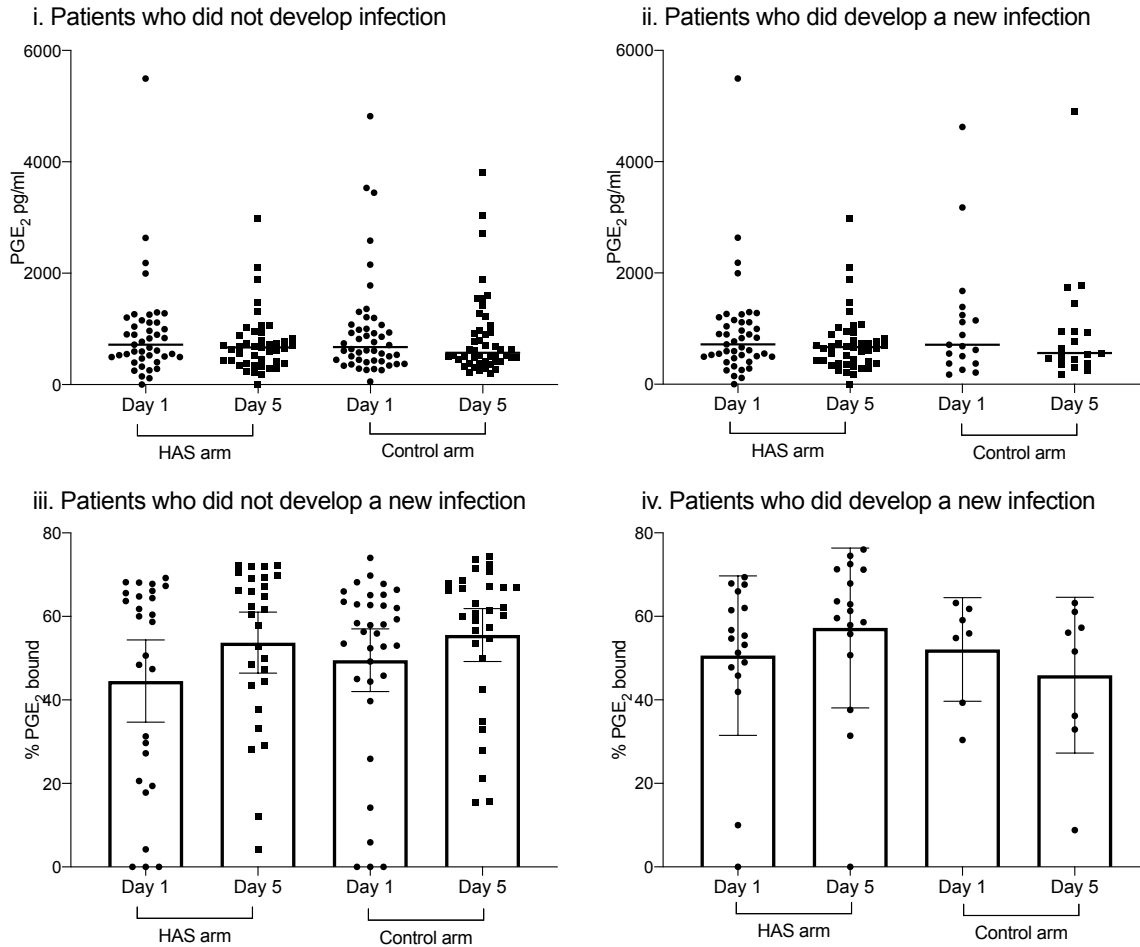
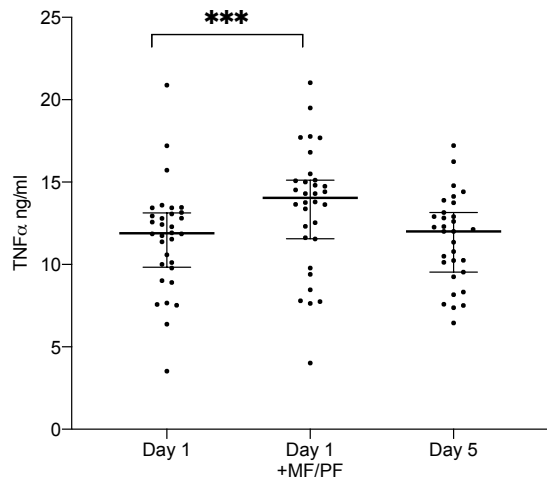


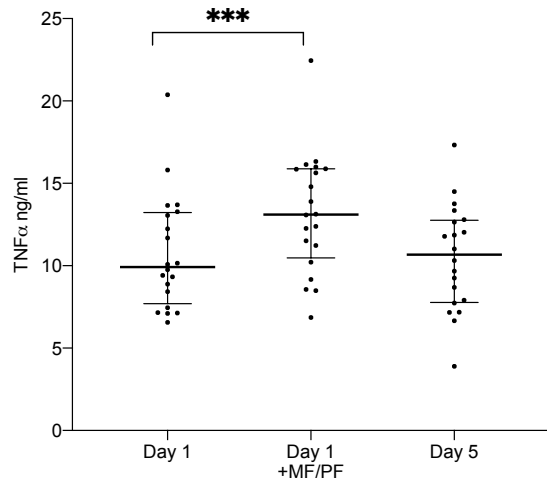
Figure 5.12. Infection subgroup analysis: total plasma PGE₂ and plasma albumin-PGE₂ binding capacity in those who did and did not develop a new infection (divided into trial treatment arm). When patients were split into those that (i) did not develop infection (day 1 no infection, day 5 no infection) and those that (ii) did develop infection (day 1 pre infection, day 5 infection) there were no changes between groups. (iii) %PGE₂ binding capacity in patients with no infection (n=29 HAS arm patients, n=35 standard of care patients). (iv) %PGE₂ binding capacity in patients who develop infection after day 3 of the trial (n=18 HAS arm patients, n=10 standard of care patients). Median and IQR shown (data not normally distributed).

There was no significant improvement in LPS stimulated TNF α production between day 1 and 5 in the patients who did not develop infection (figure 5.13i,iii). Pre treating MDMs with EP antagonists led to a consistent improvement in patient plasma mediated suppression of TNF α LPS stimulation, whether patients went on to develop infection or not (figure 5.13i-iv). Percentage improvement with EP receptor antagonism appeared larger in patients who went onto develop infection (both treatment arms, figure 5.13 ii, iv) however numbers of patients were too small in the infection group for this to reach significance.

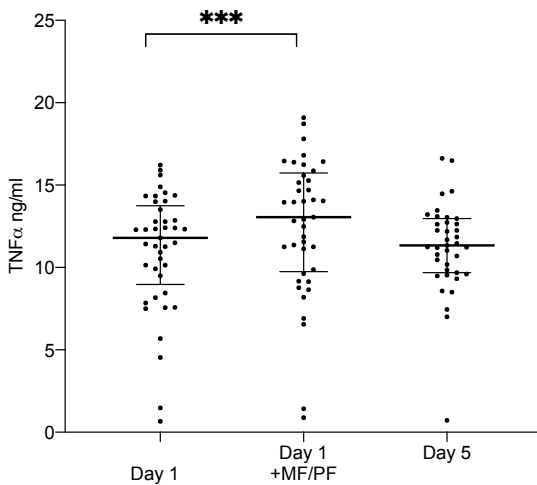
i. 20% HAS treated patients who did not develop infection



ii. 20% HAS treated patients who did develop infection



iii. Standard of care patients who did not develop infection



iv. Standard of care patients who did develop infection

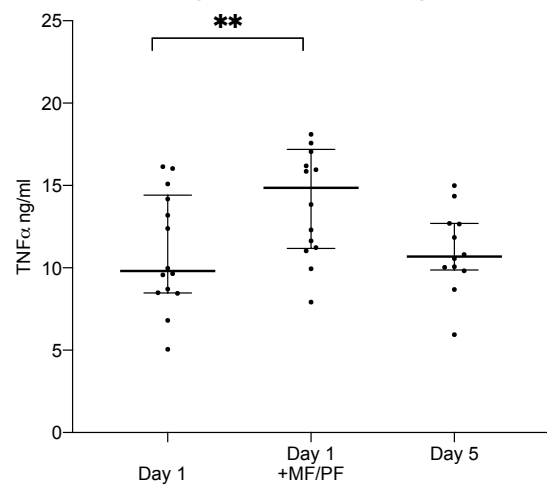


Figure 5.13. Infection subgroup analysis: LPS stimulated TNF α production from MDMs (at 4 hours) in the presence of patient plasma at either day 1 or day 5 of the trial +/- the EP2/4 receptor antagonists MF/PF.

Patients were subdivided into those who did not develop infection in the HAS arm (i. n=32) and the standard of care arm (iii. n=41) plus those who did develop infection in the HAS arm (ii. n=20) and those who did develop infection in the standard of care arm (iv. n=14). In all of these subgroups there was a significant increase in TNF α production in the presence of day 1 plasma when cells were pre treated with EP receptor antagonists, this was more pronounced in the patients who went onto develop infection. Median and IQR shown (data not normally distributed). Wilcoxon test used to compare paired sample. *** $p < 0.0001$. ** $p < 0.01$

Exploring changes in %PGE₂ binding capacity pre and post HAS treatment versus the percentage change in plasma PGE₂ there did not appear to be a consistent pattern correlating with patients who did or did not develop infection (figure 5.14). Patients in the bottom right quadrant of figure 5.14 had improved PGE₂ binding capacity with a decrease in total PGE₂ however these were a mixture of patients who did and did not develop infection. Patients in the top right hand quadrant of figure 5.14 had improved

PGE₂ binding capacity but had increased the amount of PGE₂ measured in their plasma at day 5 – again some had developed infection, some had not.

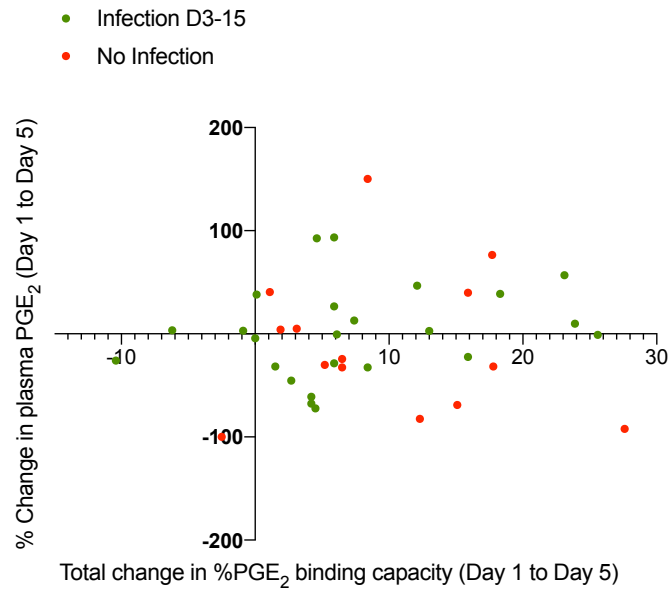


Figure 5.14. Percentage change in plasma PGE₂ between day 1-5 (y-axis) versus total change in %PGE₂ binding capacity between day 1-5 (x-axis) in patients in the HAS treatment arm (n=40).

Therefore it appears the reduction in PGE₂ is not sufficient, when binding capacity changes are similar in both arms, to result in an immune improvement response as evidenced by the MDM assay.

5.3.2.3.5. Other ligands may impact on albumins ability to bind PGE₂

As serum albumin increased there was an improvement in the albumin-PGE₂ binding capacity, however this was not consistent in all samples (figure 5.15) and could be reflective of:

- a) Other ligands competing for PGE₂ binding sites on albumin
- b) The concentration of albumin increasing but the functionality not improving
- c) Other factors impacting the binding assay

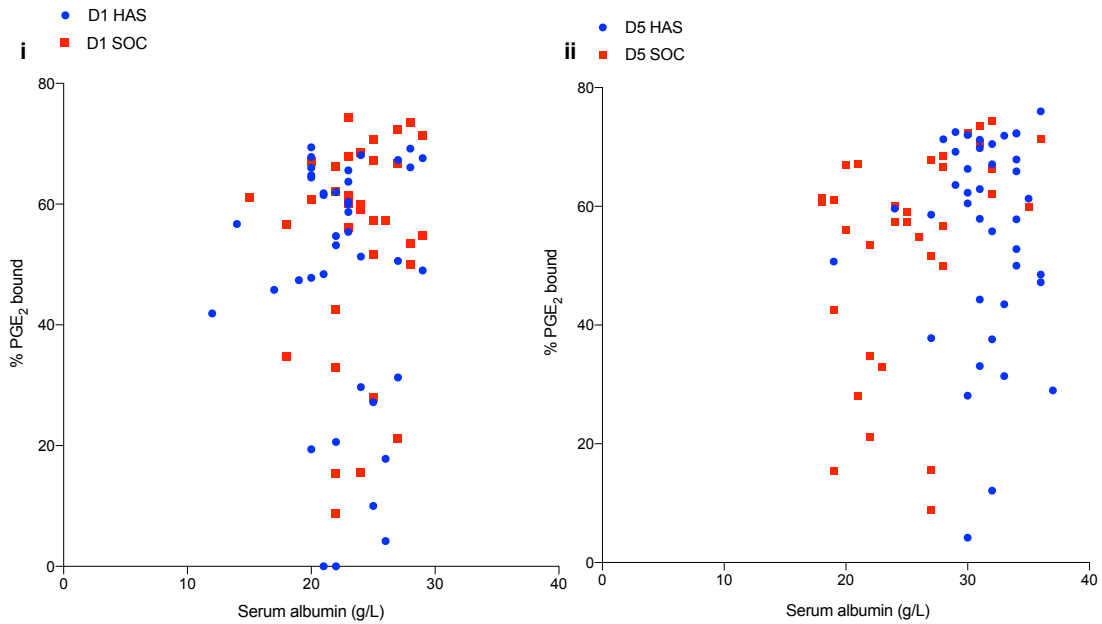


Figure 5.15. Serum albumin versus the percentage of PGE₂ bound to albumin at day 1 (i) and day 5 (ii) in the 20% HAS treatment arm (blue) and standard of care arm (red). n=84 patients

In the single arm feasibility sample analysis (chapter 4) bilirubin impacted on the counting efficacy of the albumin-PGE₂ binding assay due to the yellow discolouration of sample. In this sample analysis a new counting fluid was used with a higher count efficacy, however a decrease in bilirubin still correlated with an increase in the amount of PGE₂ bound to albumin (figure 5.16).

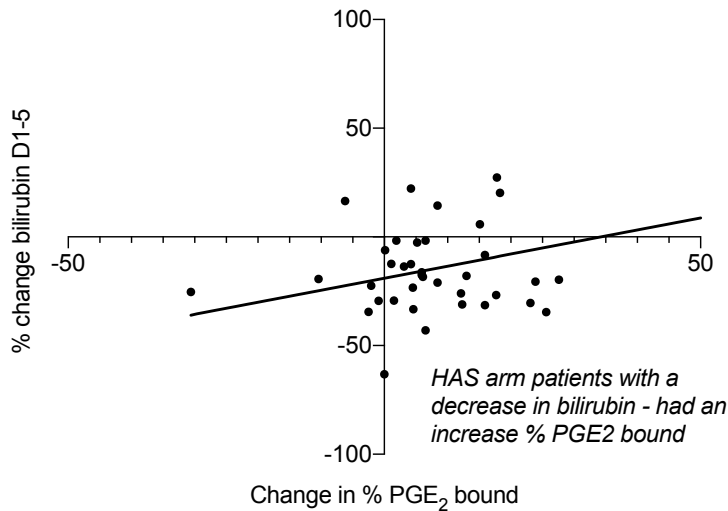


Figure 5.16. Change in the percentage of PGE₂ bound to albumin (day 1 to day 5) as compared to the percentage change in serum bilirubin between day 1 to day 5 in the HAS treatment arm patients n=42 patients. R² =0.1432 95% CI 0.0653 to 0.6219 p=0.0196

5.3.2.3.6. Oxidation of albumin is not significantly different in patients treated with 20% HAS

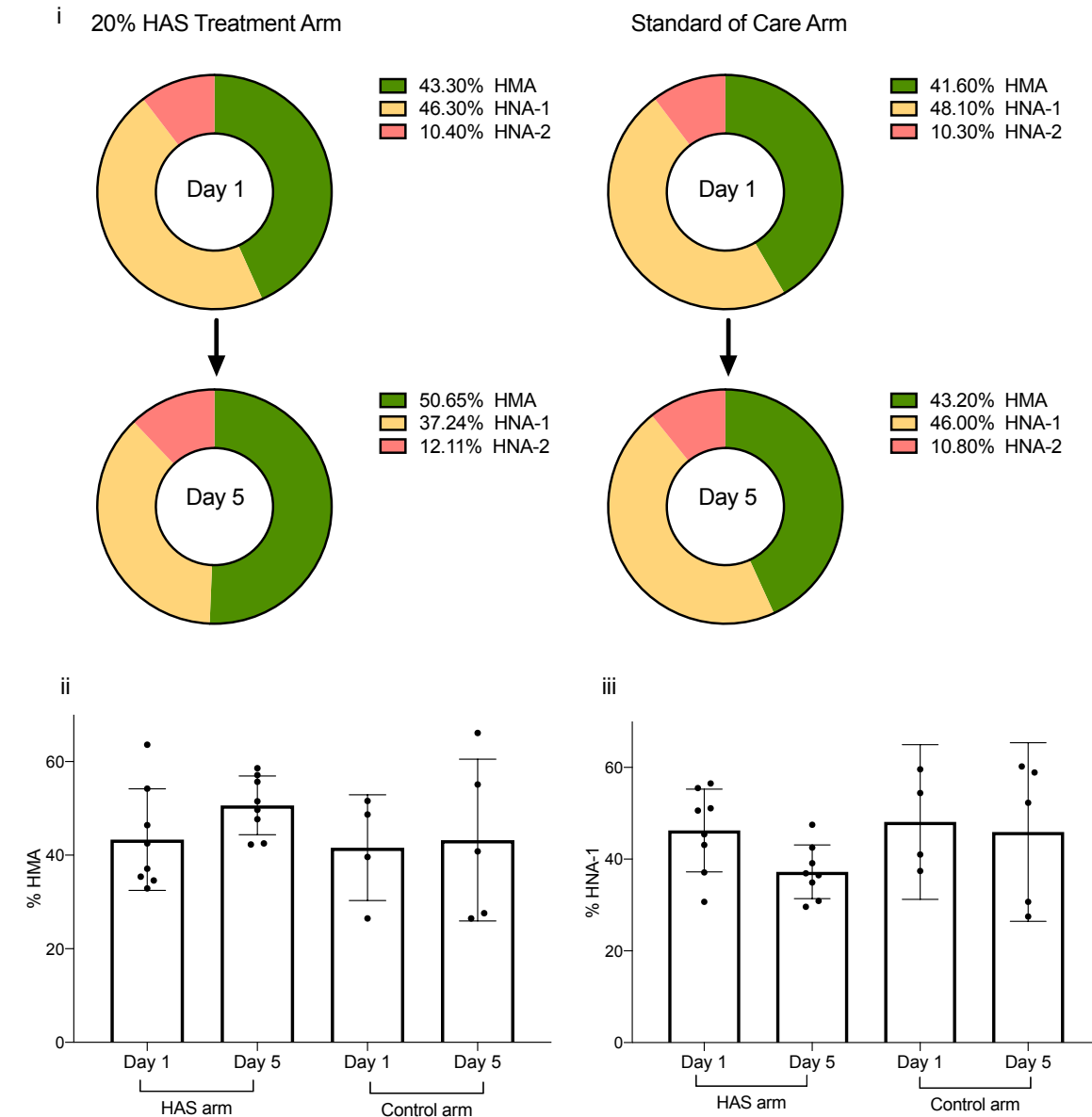


Figure 5.17. Mean proportion of healthy (HMA) versus reversible oxidized (HNA-1) and irreversibly oxidized (HNA-2) albumin present in patient plasma at day 5 of the trial.
 (ii) Mean human mercaptoalbumin (HMA) after IV HAS therapy (n=8) and standard of care (n=4). (iii). HNA-1 after IV HAS therapy (n=8) and standard of care (n=4).

The proportion of oxidised albumin present in a small number patient plasma samples was assessed using HPLC. There was little change in irreversibly oxidised HNA-2 in both arms (figure 5.17i). The proportion of ‘healthy’ non-oxidised HMA albumin present in HAS treated patients plasma trended to increase post treatment (figure 5.17ii) with a

corresponding decrease in reversibly oxidised HNA-1 (figure 5.17iii). Numbers were too low to reach statistical significance. In healthy volunteer sample (n=3) HMA was 67.5% (s.d. 2.3), HNA1 29.2% (s.d. 2.8) and HNA2 3.3% (s.d. 0.93). Therefore, even in post HAS treatment samples, there was nowhere near as much 'healthy' non oxidised HMA as in the healthy volunteer samples. However, numbers analysed were small and these needs to be considered when evaluating these results.

5.3.2.4. Markers of vascular filling and injury: Plasma renin decreases post treatment but serum creatinine measurements remain unchanged

In 20% HAS treated patients there was a significant reduction in plasma renin at day 5, this reduction did not occur in standard of care patients (figure 5.18i). However there was no corresponding reduction in creatinine (figure 5.18ii). This was in line with the larger clinical trial findings (table 5.6). There was no increase in ANP in HAS or standard of care treated patients at day 5 (figure 5.18iii). Syndecan-1, as a measure of glycocalyx breakdown, was unchanged in both the HAS treatment and standard of care groups at day 5 (figure 5.18iv.).

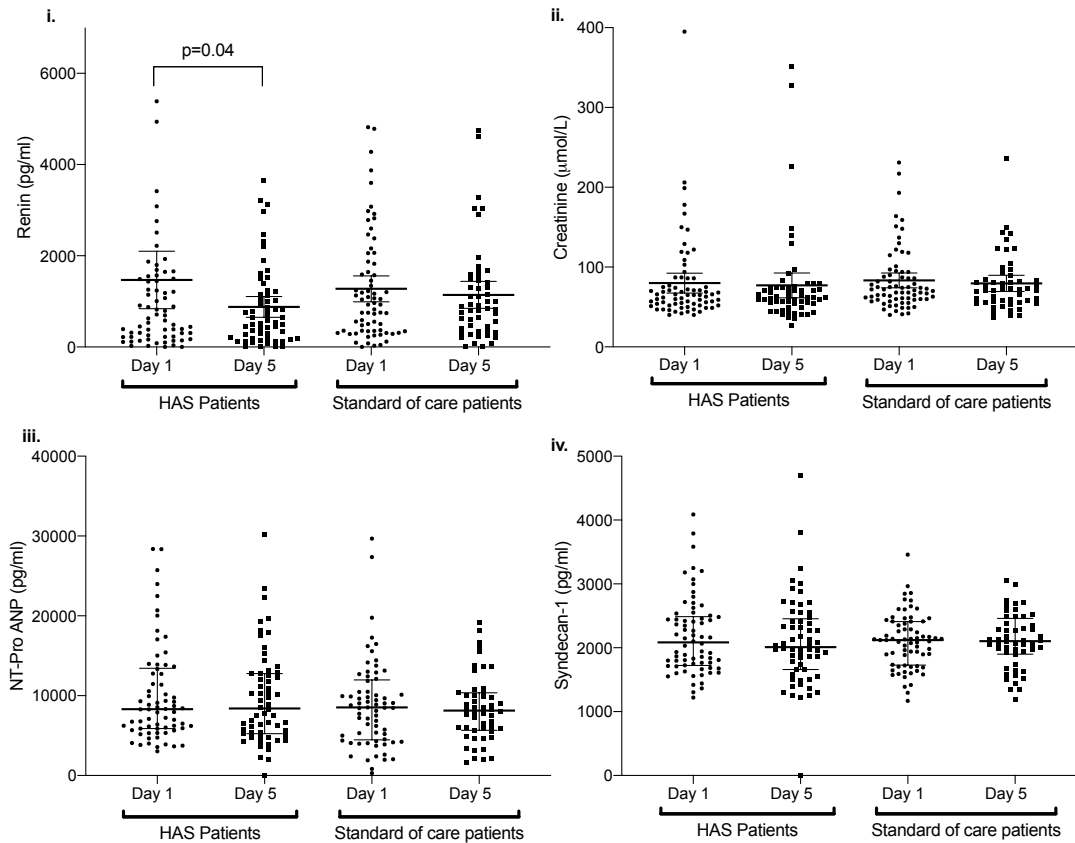


Figure 5.18. Biomarkers of vascular filling and injury at day 1 and 5 in 20% HAS and standard of care n=59 HAS treatment paired patient samples and n=49 standard of care paired patient treatment samples (i) Plasma renin in HAS and standard of care at day 1 and 5. Renin falls by a mean of 635.8pg/mL in HAS treated patients (p=0.432, CI -1251 to 20.18 pg/mL). There are no changes in (ii) creatinine (iii) ANP or (iv) syndecan-1 between days or treatment arms. *Horizontal bars represent mean, error bars 95% CI.*

5.3.2.5. Exploring baseline and day 5 measures which may indicate a 20% HAS treatment response in the plasma analysis sub study

Clinical and plasma analysis was explored to see if there may have been baseline or day 5 measures which could indicate why certain patients had gone on to reach the primary endpoint of the study (infection, renal failure or death in the trial treatment period) or whether it was possible to predict which patients may have never benefited from 20% HAS therapy and should have been excluded from this study population.

Patients prescribed antibiotics at baseline, in both treatment arms, were more likely to go onto hit the primary composite endpoint of the study (table 5.11). Other clinical and plasma analysis measures did not consistently reveal predictive measures of who would hit the primary endpoint. In particular achieving the target serum albumin threshold of > 30g/L in the HAS treatment arm was not associated with whether the patient reached the primary endpoint.

	20% HAS treatment arm		Standard of care arm	
	Primary endpoint n=28	No primary endpoint n=44	Primary endpoint n=26	No primary endpoint n=46
Baseline –no. (%):				
Infection	9 (32%)	10 (23%)	7 (27%)	14 (30%)
Antibiotics	18 (64%)	21 (48%)	14 (54%)	20 (43%)
Baseline – median (IQR)				
Creatinine (umol/L)	66 (42)	63.5 (23.5)	66.5 (21.3)	74.5 (32.5)
Serum albumin (g/L)	22.0 (6.5)	23.0 (4.3)	22.5 (4.8)	24.0 (5.0)
PGE ₂ (pg/mL)	886.1 (905.6)	708 (652.5)	772.7 (540.5)	711.6 (769.1)
%PGE ₂ binding	54.7 (16.2)	59.4 (36.6)	55.9 (9.4)	57.1 (18.2)
MDM TNF α (ng/mL)	10116.3(5090.4)	11861.2 (4324.7)	10139.6(3750.3)	11799.1(5830.5)
sCD14 (ng/mL)	2500 (8037.9)	3785.0 (8105.0)	5020.0(11600.0)	1005.0 (5179.0)
LBP (ng/mL)	2090.0 (4715.0)	1625.0 (3362.5)	1910.0 (3939.0)	2140.0 (4675.2)
Day 5 – median (IQR)				
Serum albumin (g/L)	31.0 (3.5)	32.0 (4.0)	24.0 (8.0)	24.0 (8.0)
PGE ₂ (pg/mL)	670.0 (503.3)	650 (512.5)	753.2 (538.6)	569.4 (536.2)
%PGE ₂ binding	62.9 (15.4)	61.3 (24.9)	60.1 (11.6)	59.5 (22.7)
MDM TNF (ng/mL)	10642.5(4411.7)	12031.0 (3658.7)	10071.8(3825.3)	11212.8(3225.5)

Table 5.11. Baseline and day 5 measures in patients who hit the composite primary endpoint versus those who did not, as measured in each treatment arm.

5.3.3. Development of an approach to validate infection diagnosis in clinical research settings

The diagnosis of infection in clinical practice and in the setting of a clinical trial is a huge challenge as there is currently no single objective measure to confirm or refute the new diagnosis of an infection. For the purposes of this clinical trial, evaluating the impact of targeted IV 20% HAS infusions on the development of infection, a site clinician's diagnosis marked this endpoint. Given the size of the trial, one may assume that any inaccuracies would be equal in both study arms. However, it was possible that an open-labeled trial of a treatment that is widely used might be open to bias. This prompted the following blinded infection review and an attempt to explore new biomarkers which could

aid in making an infection diagnosis more objective in future clinical trials with infection as an endpoint.

5.3.3.1. Independent Infection Case Report Form Review

There were 177 infection CRFs for 177 patient-trial episodes of site clinician’s diagnosis of infection between treatment days 3-15. These CRFs came from 156 patients (13 patients had 2 CRFs, 12 patients had 3 CRFs). 146/156 of these patients contributed to the infection component of the primary endpoint. 83 of these CRFs were in the treatment arm (78/83 contributed to the infection component of the primary endpoint) and 73 in the control arm (68/73 contributed to the infection component of the primary endpoint).

5.3.3.1.1. External reviewers agreement with infection diagnosis (reviewer’s clinical opinion)

The lead reviewer agreed with a clinical diagnosis of infection in 80.2% (142/177) of cases and disagreed in 19.8% of cases (Table 5.12a). There was concordance between the 3 reviewers in 74.6% of cases (132/177 cases). In the 45 cases with disagreement: 35 cases had only 1 reviewer disagreeing with the lead reviewer. 10 cases had 2 reviewers disagreeing. When there was disagreement with the lead reviewer the others usually did not believe there was an infection whereas the lead reviewer thought there was (24/35 cases in which one reviewer disagreed 9/10 cases in which both other reviewers disagreed).

5.12a.		5.12b.	
Infection	Total	Infection	Total
C.Difficile	1	C.Difficile	1
Fungal infection	4	Fungal infection	3
Intra-abdominal infection	5	Intra-abdominal infection	3
Lower respiratory tract infection	55	Lower respiratory tract infection	22
Other infection not covered	31	Other infection not covered	4
SBP	14	SBP	8
Soft tissue/skin infection	13	Soft tissue/skin infection	13
Spontaneous bacteraemia	9	Spontaneous bacteraemia	2
UTI	10	UTI	1
Total	142	Total	57

Table 5.12. Types of Infection when (a.) clinical opinion was that there was enough evidence to support a diagnosis of infection and (b.) when there was enough evidence in the CRF to meet the pre defined criteria for infection.

5.3.3.1.2. External reviewers agreement with infection diagnosis (using pre-defined criteria)

Only 57/142 (40.1%) of cases thought to have infection (in the reviewers opinion) had enough evidence presented in the CRF to meet the pre-defined criteria (see table 5.2) This meant, in total, only 57/177 (32.2%) infection CRFs met the pre-defined criteria for infection (table 5.12b). There was almost 100% concordance between reviewers when the pre-defined criteria was used. The most common reasons for cases not meeting the pre-defined criteria was absence of clinical symptoms or signs being completed on the CRFs or follow up microbiology results not being completed i.e. missing data, rather than negative findings inserted into the CRF. This particularly applied to the LRTI and 'other infection' groups which markedly decreased in number when the pre-defined criteria were applied. Most commonly this was due to clinical symptoms or examination findings of a LRTI not being reported.

Dividing patients into treatment arms, there was similar percentage disagreement with CRF inaccuracy in both arms. There were 12 cases in each arm where the reviewers thought (clinical opinion) there was not a diagnosis of infection but infection had contributed to the primary endpoint. Using the pre-defined infection criteria there were 52 cases in the HAS arm which contributed to the primary endpoint which did not meet criteria and 43 cases in the standard of care arm.

When the clinician's opinion was that there was not enough evidence of infection, data quality on the infection CRF was poor. A total 72% of 'no infection' CRFs graded quality 1 or 2 i.e. very poor or poor (table 5.13).

Data Quality	ALL INFECTION CRFS (n=177)		Clinical Opinion there was enough evidence of infection (n=142)		Clinical Opinion there was NOT enough evidence of infection (n=35)	
	Total (no. CRFs)	Total (%)	Total (no. CRFs)	Total (%)	Total (no. CRFs)	Total (%)
1	39	22%	22	15%	17	49%
2	31	18%	23	16%	8	23%
3	57	32%	52	37%	5	14%
4	35	20%	31	22%	4	11%
5	15	8%	14	10%	1	3%
	177	100%	142	100%	177	100%

Table 5.13. Graded data entry quality on Infection CRFs. 1=Very poor, 5=Excellent.

Therefore blinded scrutiny of the evidence of infection showed only a small amount of disagreement with site clinician’s diagnosis of infection. In addition this was equal in both study arms so would have had no impact on overall clinical trial outcomes. The main challenge is surrounding accurate diagnosis of respiratory tract infection alongside detailed site data collection to enable external review.

5.3.3.2. Infection dataset exploration

I took the opportunity to investigate this unique infection dataset to discern possible additional approaches to guide clinical research & practice in this important area.

5.3.3.2.1. Positive microbiology

19/57 (33.3%) patients meeting the pre-defined criteria had culture positive infection. In total 44 patients were reported to have culture positive infection (24/44 patients did not meet pre defined criteria but were culture positive – i.e. over half of culture positive patients did not fulfil the pre defined criteria for a diagnosis of infection). 43/44 had the organism detailed on the infection CRF. 37/43 organisms were reported as not having antibiotic resistance (table 5.14).

	RESISTANCE			Total
	No	Yes	Not known	
Gram Negative Organisms: <i>Acinetobacter, Coliform, E.coli, E.faecalis, E.faecium, Enterobacter cloacae, Citrobacter farmeri, Veillonella atypical, Klebsiella, Pseudomonas</i>	21	2	1	24
Gram Positive Organisms: <i>C.Difficile, Staphylococcus (uncharacterised), S.aureus, S.epidermidis, S.haemolyticus, Strep Gallolyticus ssp. Pasteurianus, Streptococcus (uncharacterised).</i>	13		3	16
Fungal: <i>Aspergillus, 'yeast'</i>	2			2
Viral: <i>Influenza A</i>	1			1
Grand Total	37	2	4	43

Table 5.14. Reported organisms alongside reports of whether the organism had been reported as having any antibiotic resistance or not

Gram-negative infection was most common (55.8% of culture positive episodes).

Respiratory infection was by far the most commonly diagnosed infection (table 5.15)

however only 6/55 cases reported positive cultures which reflects clinical practice. 8/14 CRFs supporting an SBP diagnosis did not report a cultured organism.

	C.Dif f	Funga l	Intra - abdo	LRT l	Othe r	SB P	Soft tissu e	Spontaneou s bacteraemia	UT l	Tota l
Gram – ve				3	2	6		6	7	24
Gram +ve	1		1	2	2	2	5	3		16
Fungal		1		1						2
Viral					1					1

Table 5.15. Cultured Organisms in different types of infection

5.3.2.2.2. Plasma biomarkers in patients who developed infection

143 individual patients were selected for blinded sample analysis. 39 of these patients had at least 1 infection CRF to support a new diagnosis of infection submitted between D3-15 of trial treatment. Potential plasma biomarkers of infection (as described in section 5.1.3) were explored in this patient group versus 104 patients who did not get diagnosed with a new infection D3-15 of the trial treatment period.

The mean trial treatment day that a trial patient received a new infection diagnosis was day 6 (s.d. 2.5). Plasma samples were available for analysis at baseline (day 1) and at day 5. At day 5, plasma soluble CD14 was significantly higher in patients diagnosed with a new infection as compared to those who were not (5746ng/mL vs 13596ng/mL, $p=0.0094$, CI 1975ng/mL to 13727ng/mL. Figure 5.19) There were non-significant increases in day 5 plasma PCT and calprotectin in the infection group overall with no changes between days before/after infection developed. There were no differences in LBP, CRP, CD163 or CCL8 in the sample groups analysed when day 1 and day 5 samples were compared. WCC at day 5 was higher in patients diagnosed with infection ($11.4 \times 10^9/L$ vs $8.9 \times 10^9/L$, $p=0.0361$, CI 0.1608 to 4.743).

4/39 patients with sample analysis had an infection CRF completed but were not deemed to have enough evidence of infection. Mean day 5 sCD14 in these patients was low (2593.3ng/mL, table 5.16). PCT, calprotectin, LBP and white cell count tended to be higher in patients with culture positive infection. All biomarkers were lower in the 4 patients (with samples) who did not have enough information to support a diagnosis of infection.

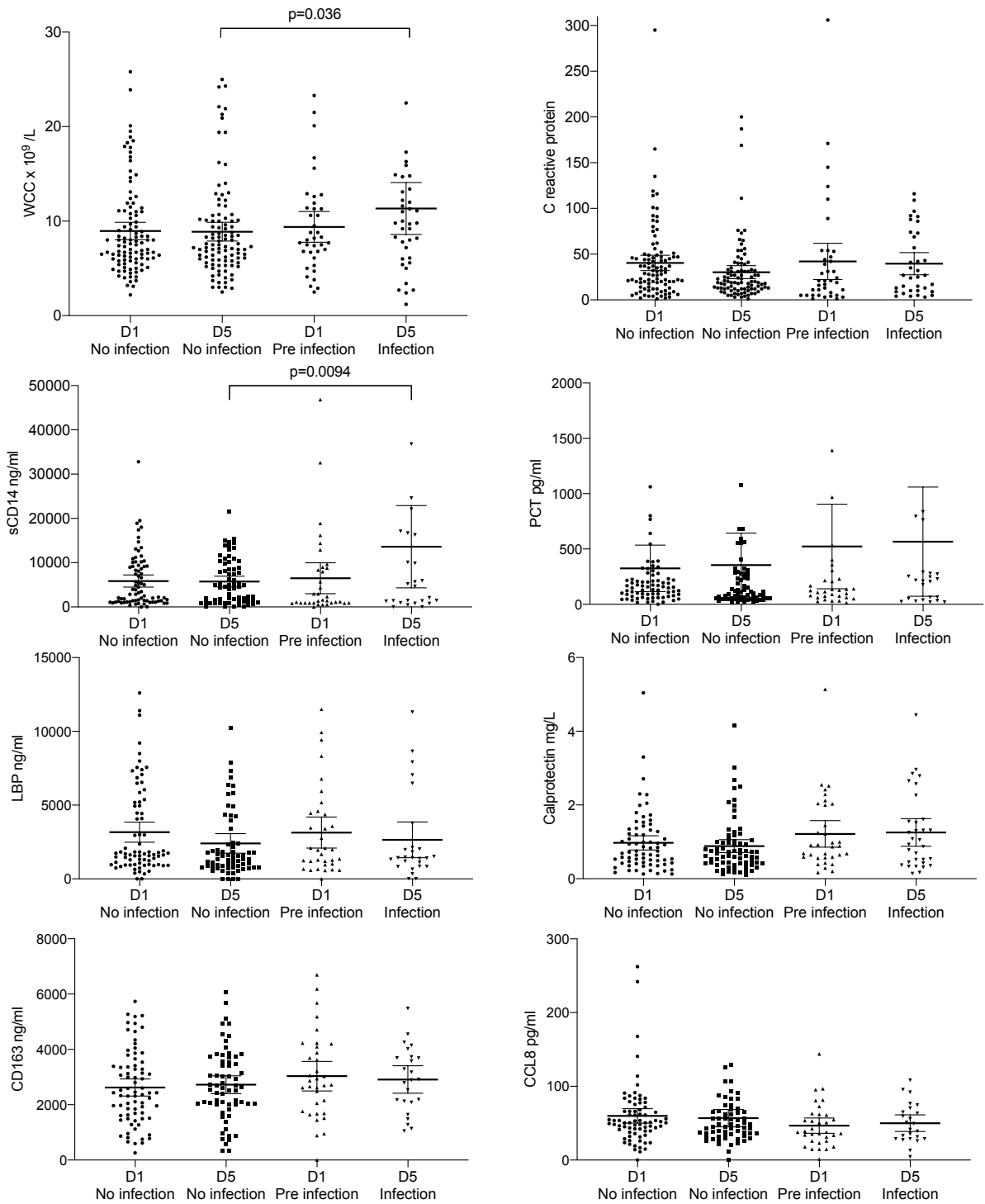


Figure 5.19. Day 1 and Day 5 plasma infection biomarkers

WCC/CRP data from 143 patients (39 patients who developed infection and 104 who did not). Other biomarkers from 111 patients. Comparison of patients who did not develop infection (D1 No infection and D5 No infection, n=76) as compared to patients who went onto develop infection between D3-15 (D1 Pre infection and D5 Infection, n=35).. Horizontal bars represent mean, error bars 95% CI. Students T test used to compare groups.

	No infection*	Infection CRF completed				Clinical Opinion there WAS NOT infection:
		Clinical Opinion there WAS infection:				
		ALL	Fulfilled pre defined criteria	Culture Positive		
<i>n</i>	76	35	12	8	4	
sCD14 (ng/mL)	5,745.6	13,635.5	4,884.3	3,544.8	2,593.3	
PCT (pg/mL)	353.9	609.3	104.5	1,263.1	34.5	
LBP (ng/mL)	2,404.5	2,825.1	2,602.4	3,338.7	810.0	
Calprotectin (mg/L)	0.9	1.3	1.6	1.5	0.9	
CD163 (ng/mL)	2,723.9	2,886.6	3,174.9	3,041.2	2,926.3	
CCL8 (pg/mL)	56.9	54.6	48.6	66.2	26.6	
WCC (x10 ⁹ /L)	8.9	12.2	12.8	14.7	12.3	
CRP (mg/L)	30.3	45.5	35.9	31.1	87.7**	

Table 5.16. Mean day 5 plasma biomarkers of infection in patients with and without infection

*No infection diagnosed by site clinician – therefore no infection CRF completed

Further subdivided into infection CRF outcome. **only 4 patients with one outlier with CRP of 300 thought to have alcoholic hepatitis without infection.

Subdividing the patients with an infection CRF into ‘types of infection’ the numbers of patients in each group become very small apart from LRTI (n=14) and ‘other infection’ (n=11). These were 2 categories where although the CRF reviewers thought there was likely to be an infection there often wasn’t enough evidence to meet the pre-defined criteria (1/14 for LRTI and 2/11 for ‘other infection’). Mean sCD14 in these LRTI patients was 28,013.5ng/mL and 8,807.6ng/mL in the ‘other infection’ patients, higher than the patients without infection.

The only biomarker that correlated well with the traditionally used WCC was LBP (Figure 5.20) (other correlation analysis not shown). sCD14 did not but the correlation graph highlights possible cases where it may be a useful biomarker of infection when WCC is not raised/very low.

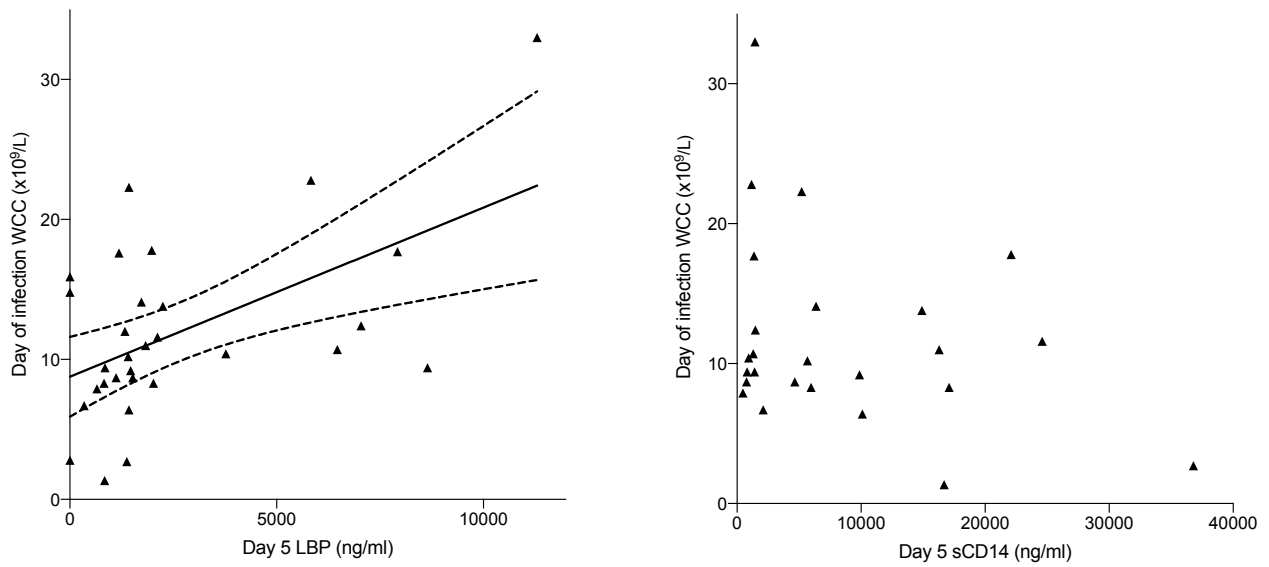


Figure 5.20. Comparison of White Cell Count (WCC) to day 5 LPS-binding protein (LBP) and soluble CD14 (sCD14). WCC on day of infection correlates with plasma LBP ($r^2=0.2865$ $p=0.0023$). sCD14 does not significantly correlate with WCC.

5.3.2.3. Types of infection according to treatment arm

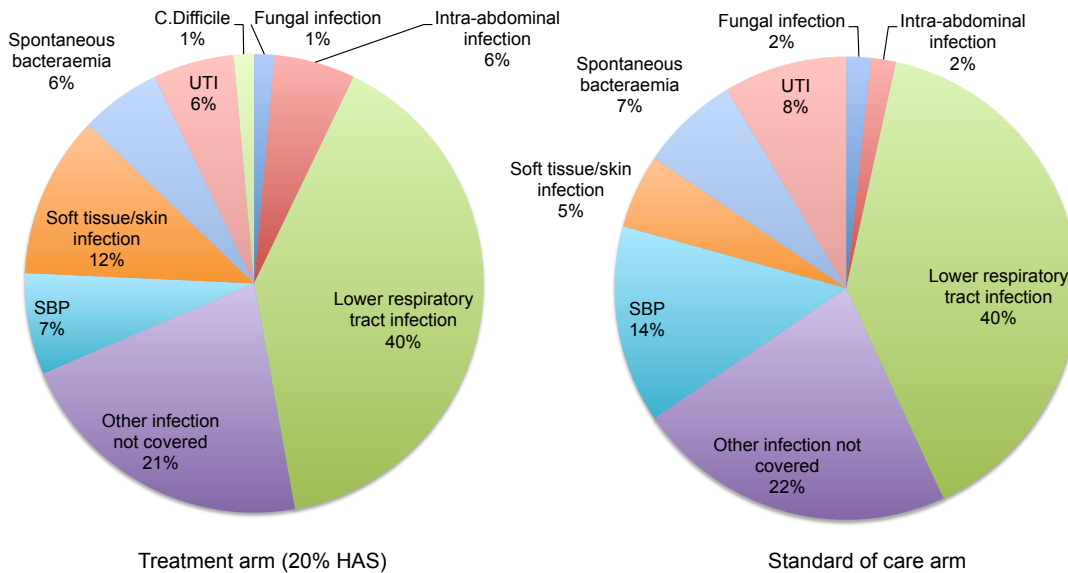


Figure 5.21. Types of infection in each treatment arm. Types of infection when the reviewer deemed there was adequate clinical evidence of infection (clinical judgment rather than pre-defined criteria).

The distribution of infection types was generally not different when patients were treated with 20% HAS (figure 5.21). There was a higher proportion of SBP and lower proportion of soft tissue infection in the standard of care arm as compared to the HAS arm.

5.5. SUMMARY

- Administering IV 20% HAS to hospitalised decompensated cirrhosis patients in order to increase serum albumin >30g/L does not decrease incidence of infection, renal failure or death
 - Targeted albumin therapy achieved a serum albumin >30 g/L whereas there was no significant increase in serum albumin in the standard of care group.
 - The study was appropriately powered
 - There was a 3-fold increase in pulmonary oedema, although numbers remained low
- Infused Albumin has no immunomodulatory effect
 - Plasma mediated MDM dysfunction was not changed in a larger patient setting and not different in HAS treated patients as compared to standard of care patients
 - There were no changes in plasma cytokines
 - There was a small effect on overall plasma PGE₂ concentration
 - There was a small improvement in albumin-binding function and oxidation
- There were no clinically meaningful changes in plasma markers of vascular filling with 20% HAS treatment
- Infection diagnosis is challenging in decompensated cirrhosis
 - Clinician diagnosis followed by blinded validation appears useful for clinical trials, but still lacks consistency
 - Respiratory tract infection is most common, perhaps making culture positivity a poor method of validation and chest radiograph reporting may be of value
 - sCD14 may be a useful biomarker in clinical studies to support the diagnosis of infection alongside WCC but requires validation in prospective studies
- Preliminary work showed no particular clinical or plasma analysis measures that predicted patients that reached the primary endpoint aside from prescription of antibiotics at baseline, but further work is required

5.6. CONCLUSIONS

5.6.1. Administering IV 20% HAS to hospitalised decompensated cirrhosis patients in order to increase serum albumin >30g/L does not decrease incidence of infection, renal failure or death

In a large interventional trial the albumin administration protocol proved effective in raising and maintaining serum albumin levels >30g/L in the treatment arm whereas albumin levels in the standard of care arm remained <30g/L. Despite the protocol efficacy there was no impact of IV 20% HAS therapy on reducing infection, renal failure or death in the trial treatment period or in any of the secondary outcomes. Event rates were as predicted therefore the study was appropriately powered and there was not significant loss to follow up as expected with this inpatient study.

Blinded exploratory plasma analysis, with a control arm, supported the clinical outcomes of the study that there was no immunomodulatory effect and although there were some improvements in the functional quality of circulating albumin this remained much lower than levels seen in healthy volunteers.

These findings are in contrast with the recently published ANSWER study⁷³ which did not aim to increase serum albumin levels but this occurred as a consequence of regular outpatient administration. The difference in study outcomes may have been due to patients in this study having more advanced disease (admitted to hospital, mean MELD 20) therefore it may be too late for 20% HAS to provide a clinical benefit. In this study standard of care arm patients were managed in a similar way to the treatment arm patients (all inpatients being managed by the same clinicians, seen at similar frequencies) where as in the ANSWER study patients in the standard of care arm were seen less frequently and the perceived 20% HAS benefit may have been confounded by a better 'standard of care' in the treatment arm.

As seen in the serious adverse event (SAE) reporting it appears that some 20% HAS patients may be at increased risk of developing pulmonary oedema. This was challenging to interpret as even the best clinicians with invasive monitoring in an ICU setting may not always be able to accurately differentiate between pulmonary oedema driven by fluid overload, acute respiratory distress syndrome secondary to infection and bilateral pneumonia. However, more respiratory events in general were reported in the

HAS treatment arm which raises concerns, particularly when 20% HAS provided no clinical benefit.

The median amount of 20% HAS administered per treatment arm patient was 1000mLs which equates to a median cost increase of £900 (not including staff time, infusion equipment). This must also be taken into account when considering 20% HAS prescription.

The clinical study had a number of limitations. It was unblinded which meant clinicians may have had the tendency to over report SAEs and events, such as infection, in the treatment or standard of care arm. Infection is difficult to accurately diagnose (discussed in 5.6.4). The 20% HAS intervention was allowed to be administered in the standard of care arm as it would have been unethical to stop this therefore event rates may have been decreased in the standard of care arm due to patients receiving small amounts of 20% HAS. However, patients in the standard of care arm received much lower volumes of HAS (median 100mLs vs 1000mLs) and their serum albumin did not increment to the >30g/L target. It is unlikely that such tiny volumes of 20% HAS had a genuine clinical effect. So despite the pragmatic design the protocol was effective and there remained no clinical benefit across all investigated endpoints.

5.6.2. Infused albumin resulted in an improvement in the functional quality of circulating albumin, without immunomodulatory effects clinically and ex vivo

My hypothesis was that giving 20% HAS to increase serum albumin >30g/L would decrease PGE₂ by improving the amount and functional quality of circulating albumin and that subsequently this would decrease the incidence of infection and its complications. Blinded plasma analysis of a sample of patients from the larger RCT somewhat supported the mechanisms underlying hypothesis, despite no difference in clinical outcomes. It is likely that this hypothesis had grossly simplified the clinical reality of these patients and there are a huge number of other factors impacting on the patients' clinical outcomes. Of course 20% HAS may impact on these other mechanistic pathways – but evidently not enough to change outcome when administered in this way.

The PGE₂-albumin binding capacity improved post 20% HAS administration at similar levels seen in the feasibility study, approximately 8% improved binding (chapter 4). This however was still not to the level of healthy individuals. In addition the control arm patients, who did not have a sustained increment in their serum albumin, also had an

improvement in their PGE₂-albumin binding function at day 5. Therefore, the improvement in the function of circulating albumin as a ligand binder is likely to be significantly impacted by the amount of other ligands available e.g. drugs, bilirubin, cytokines. It is likely that this has as much or more of an effect than supplying 'new albumin' in the form of 20% HAS infusion, hence the results seen in the control arm patients. The decrease in partially oxidized albumin (HNA1 – see figure 5.12) was consistent with some functional improvement in PGE₂-albumin binding post 20% HAS infusion, as when albumin is oxidized at the Cys-34 residue there is a conformational change in the binding site of PGE₂¹⁶⁰.

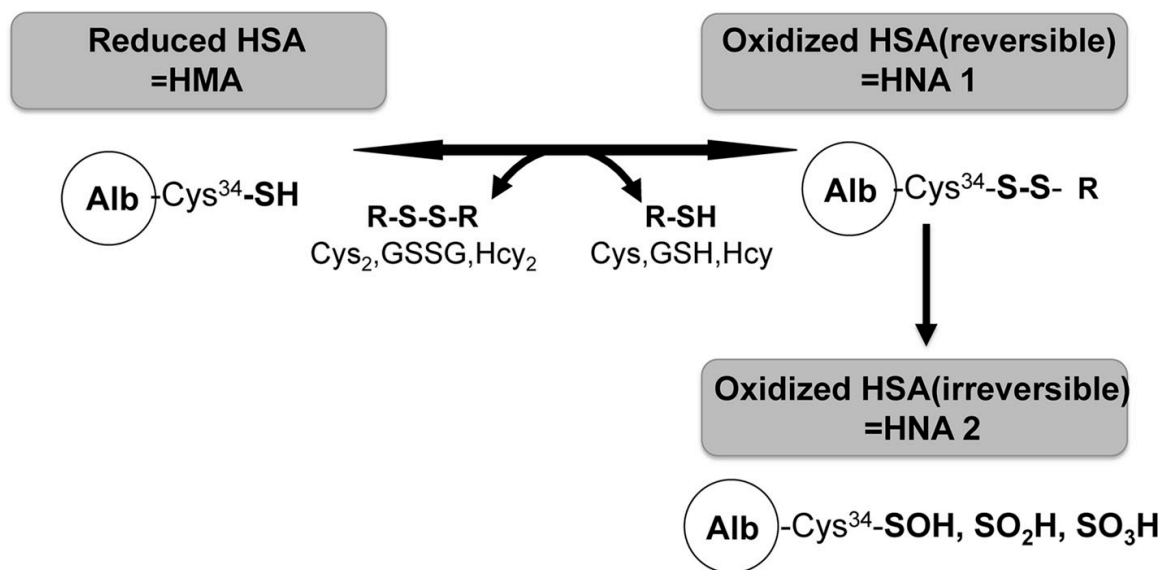


Figure 5.22. Redox states of human serum albumin, taken from Setoyama, et al. ²¹⁶

Redox states of human serum albumin (HSA). Depending on its redox state, plasma HSA can be divided into reduced HSA (human mercaptoalbumin, HMA) and oxidized HSA (human nonmercaptoalbumin, HNA). Oxidized HSA (HNA) is a mixture of the reversibly and irreversibly oxidized forms. Reversibly oxidized HSA (HNA 1) has mixed disulfide bonds with a thiol compound such as cysteine, homocysteine, or glutathione in the blood. In irreversibly oxidized HSA (HNA 2), the free thiol group is more highly oxidized to sulfenic acid ($-\text{SOH}$), sulfinic acid ($-\text{SO}_2\text{H}$), and sulfonic acid ($-\text{SO}_3\text{H}$). *Alb*=albumin, *Cys*=cysteine, *GSH*=glutathione, *GSSG*=oxidized glutathione, *Hcy*=homocysteine

The total measured plasma levels of PGE₂ were decreased after albumin infusion and not in the standard of care arm, however this was nowhere near the level of healthy volunteers²¹⁵. Additionally, there was a substantial improvement in MDM dysfunction when PGE₂ receptors were blocked in the presence of day 1 plasma samples.

However, plasma mediated MDM dysfunction was unchanged in a larger patient setting and not different in HAS treated patients as compared to standard of care patients. This

ex vivo plasma mediated immune dysfunction assay was then consistent with clinical findings. Therefore one must conclude that although infusing 20% HAS resulted in some improvement to the quality of circulating plasma albumin and a decrease in PGE₂ this was not enough to impact on immune dysfunction and risk of infection.

Patient response in the assay was heterogeneous and therefore previous outcomes in the single arm study (chapter 3) may have just captured a small sample of 'responder' patients, with the effect phased out in a larger study. The assay had to be changed with the larger number of samples and monocytes were used from the blood donation service, it is possible that this made the assay less sensitive. However, dividing patients into those who did and did not develop infection still showed no differences – therefore it was not possible to identify a group of patients within this cohort who may benefit from IV 20% HAS used in this way.

5.6.3. Targeted infused albumin had no impact on renal dysfunction when used in this setting

There were no clinically meaningful changes in plasma markers of vascular filling with 20% HAS treatment. 20% HAS reduced plasma renin in the exploratory sub study but this was not supported by a decrease in creatinine, this questions the value of renin as a clinically meaningful biomarker. In the 828 patient RCT there was less renal dysfunction in the 20% HAS arm although this did not reach significance. 20% HAS is an established treatment for prevention of renal dysfunction in SBP, LVP and part of HRS management²¹⁷. Therefore many patients in the standard of care arm at risk of renal dysfunction may have received HAS for these indications which is perhaps why significance was not achieved in the renal benefit seen.

5.6.4. Development of an approach to validate infection diagnosis in clinical research settings

With no gold standard of infection diagnosis in decompensated cirrhosis implementing a regulatory process for site clinicians' diagnosis was a major challenge. Methods have not been previously well described in other studies in the same setting^{55,56,87,218} therefore I sought to instigate and evaluate a structured and transparent approach. The supporting information surrounding a patient's infection diagnosis at site most often led to the agreement that there was infection by the external reviewers. Most importantly there was a similar number of disagreements in each treatment arm, therefore there was no reason

to question the primary endpoint assessment. This process therefore is fit for purpose for RCTs but could be improved for descriptive or epidemiological studies.

The pre-defined criteria for infection (table 5.2) improved reviewer concordance but when strictly applied decreased the rate of infection in both treatment arm by a large proportion. I identified the quality of infection CRF completion as an important confounder here.

Respiratory tract was the most common infection, making culture positivity a poor method of validation. Signs and symptoms of LRTI were required using the selected pre defined infection criteria (table 5.2) however these have been consistently shown to be poor predictors of true infection²¹⁹. A better approach might be using a more objective measure such as blinded chest x-ray review.

Culture negative infection are estimated to be between 30-50% in patients with decompensated cirrhosis⁹¹. 44/156 patients in the RCT (28.2%) had positive cultures, this is therefore consistent with other studies. The majority of infections were gram negative (around 55%). Resistant organisms were recorded less often than expected, this may be due to follow up data entry being poor when sensitivities became available at site.

Exploration of day 5 plasma biomarkers of infection showed only sCD14 was significantly raised and potentially useful as a 'rule out' biomarker of infection in a clinical trial setting. However, differences between infection and non-infection patients were similar to traditionally measured WCC therefore its utility in clinical practice may be limited. A better approach, in blinded clinical trials, may be to utilize a clinician data scoring system alongside objective tests. Halkin, et al.²²⁰ review the performance of diagnostic tests, using likelihood ratios, and compare them to the power of clinical assessment. They show the discriminative power of a test or a clinical assessment is similar but combination of the two greatly increases diagnostic accuracy (see figure 5.23 as an example). Future work could therefore evaluate using clinician pretest probability of infection plus sCD14 and WCC on the diagnostic likelihood of an infection being present. This would have to be evaluated in an observational study where accurate data collection surrounding infection was of primary importance.

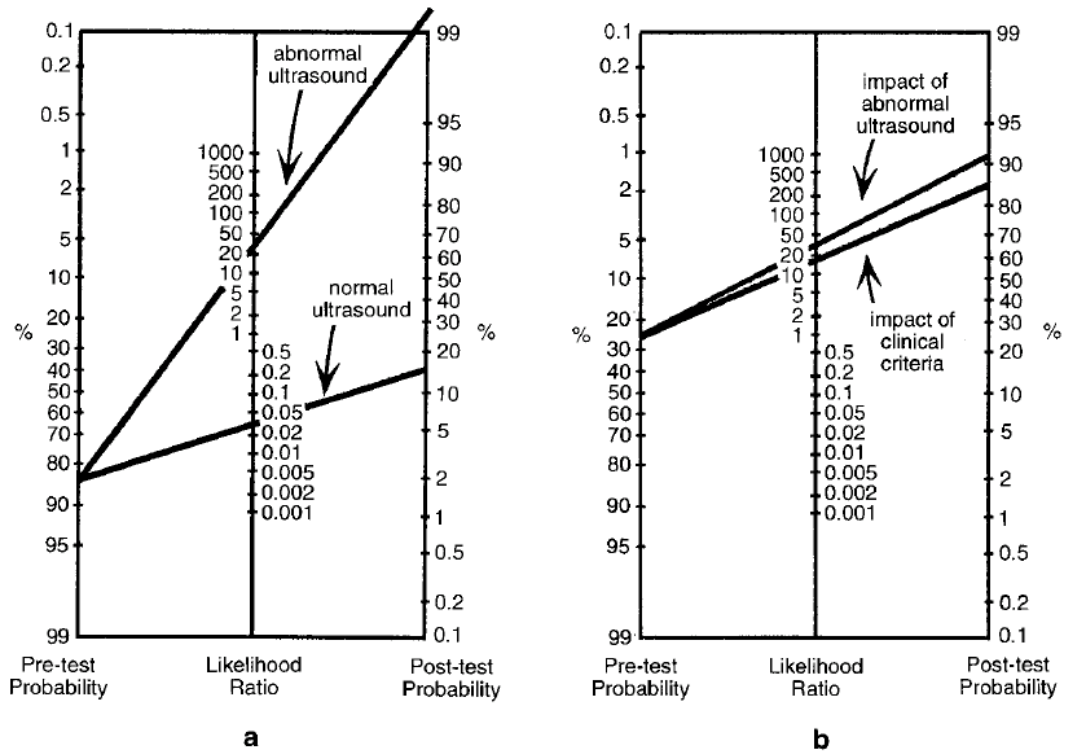


Figure 5.23. Magnitude of impact from a refined clinical assessment vs. that from an ultrasound in the diagnosis of deep venous thrombosis taken from Halkin, et al. ²²⁰.

CHAPTER 6: GENERAL DISCUSSION

My thesis sought to test the hypothesis that administering intravenous albumin solution in order to increase plasma albumin levels to >30g/L (near normal) would decrease incidence of infection in hospitalised decompensated cirrhosis patients. Previous data indicated the potential importance of PGE₂ in innate immune dysfunction in liver cirrhosis patients¹¹ and identified a hypothetical role for albumin to bind and inactivate PGE₂ and improve this dysfunction.

Albumin infusions are extremely popular amongst hepatologists and are widely recommended. However, clinical trials of 20% HAS infusions have shown conflicting results. Benefit has been demonstrated in SBP⁵⁴ but not non-SBP infections⁵⁶, with the latter prematurely terminated because of lethal pulmonary oedema in the albumin arm. Perhaps surprisingly *no* fluid was given as part of standard care in these trials. Recent meta-analyses found no evidence of benefit for all-cause mortality following any interventions in HRS,²²¹ nor differences between albumin versus other plasma expanders for mortality following LVP²²². Few other clinical specialists use albumin outside of plasmapheresis.

During my thesis, I developed and validated clinical trial endpoints, in particular related to infection, and laboratory assays to investigate albumin-PGE₂ binding and immune function. This enabled me to test my hypothesis and perform a unique investigation into the effects of albumin in decompensated cirrhosis using data linked to samples collected from the 35 site ATTIRE randomised clinical trial.

A new IV 20% Human Albumin Solution (HAS) treatment regimen was developed and tested which was found to effectively increase serum albumin to the desired range^{103,212} in a way which was feasible for decompensated cirrhosis patients in busy healthcare settings. Clinical data collected whilst testing the infusion regimen allowed for the improvement and refinement of a trial protocol²²³ and outcomes to test this intervention in a multicenter randomised control trial.

Assays developed in chapters 3 and 4 enabled me to explore the impact of patient IV albumin administration on ex vivo immune dysfunction and albumin binding capacity. However despite the improvements seen in albumin-PGE₂ binding capacity and reduction in plasma PGE₂ concentration in the albumin treated patients, there was no effect on plasma-mediated macrophage dysfunction in our randomized control trial. These findings were mirrored by the trial findings that showed no clinical benefit for

albumin therapy to increase and maintain serum albumin ≥ 30 g/L in hospitalised cirrhosis patients. Most specifically for my thesis there was no effect on infection, with no differences in incidence of new infection, nor outcome in patients admitted with infection or receiving antibiotics at enrolment.

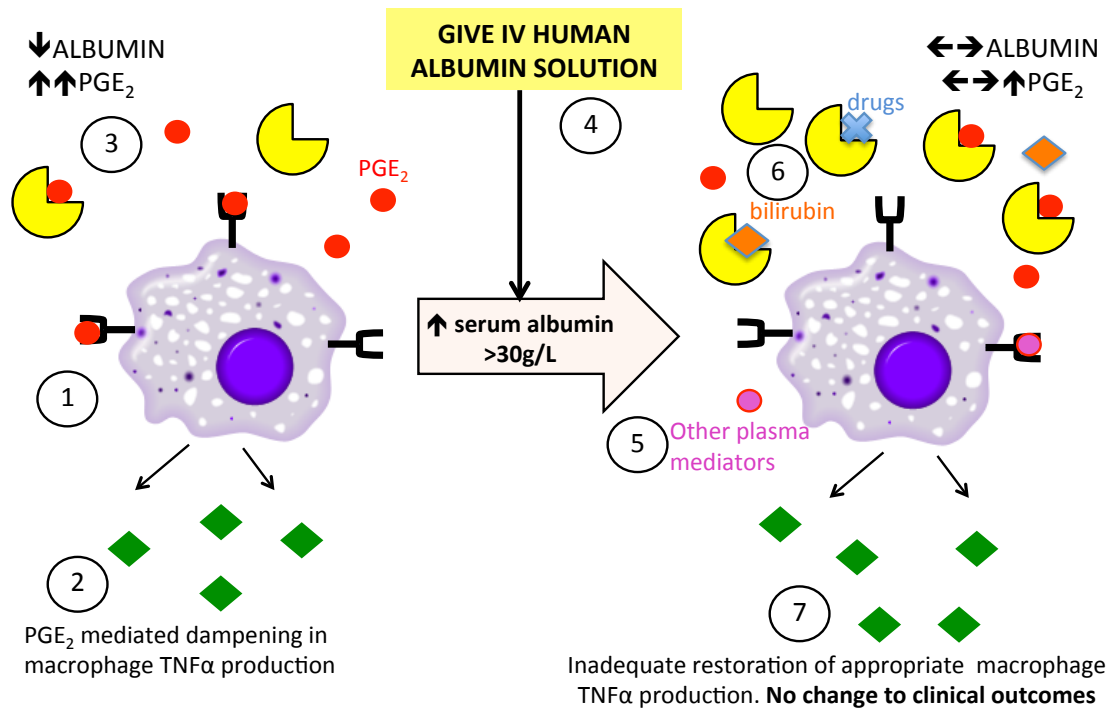


Figure 6.1. Schematic of hypothesis and proposed explanation for studied outcomes

1) A macrophage in presence of increased and more bioavailable PGE₂ which binds EP receptors on the macrophage surface. 2) PGE₂ inhibits Fc receptor mediated phagocytosis and NADPH oxidase mediated bacterial killing producing a down regulated Th1 response leading to decreased pro-inflammatory cytokine production (e.g. TNF). 3) Albumin binds and catalyses PGE₂ however levels are low in decompensated cirrhosis. 4) Giving IV HAS to these patients to increase serum albumin levels >30g/L causes an improvement in albumin's ligand binding ability, including the ability to bind PGE₂. 5) However, other plasma mediators of immune suppression may also be present. 6) In addition, the increased concentrations of competing albumin binding ligands such as bilirubin and drugs in AD patients may displace PGE₂ from albumin. 7) Ultimately albumin infusions have no overall impact on immune function or reduction in incidence of infection in patients with decompensated cirrhosis.

Explanations for the findings in this thesis

To summarise, I reject my original hypothesis that prophylactic intravenous human albumin infusions, to increase serum albumin >30g/L, prevent acute decompensation patients from developing infection. Figure 6.1 provides an explanation for my findings, which are related back to my original research questions as follows:

1. PGE₂ is raised in acute decompensation patients and does dampen immune response ex vivo

Using a large number of samples I confirmed LPS stimulated TNF α production from healthy volunteer MDMs is a reliable assay of cirrhosis plasma mediated MDM dysfunction. LPS stimulated TNF α production from HV-MDMs and MM6 cells significantly improved in the presence of post HAS treated patient plasma (serum albumin >30g/L) versus pre treatment plasma (serum albumin <30g/L) in the single arm study but not in the RCT. Results supported a PGE₂ dependent mechanism for the some of the suppressive effect of patient plasma on these cells. However, despite antagonising the effect of PGE₂, the suppression of TNF α production was not entirely reversed. An explanation for this, and the differing results seen between the single arm and RCT studies in this assay, is the presence of other unmeasured circulating mediators of immune suppression which may simply take over from the role of mediators, such as PGE₂. Figure 6.2. summarises the numerous immune cells and non-cellular components in various compartments of the body that may be effected as cirrhosis progresses to acute decompensation²²⁴. These components have not been addressed in this thesis. The pathophysiological processes are highly complex, subject to ongoing investigation and not yet fully understood. None have yet been translated into a successful therapeutic target in a clinical setting.

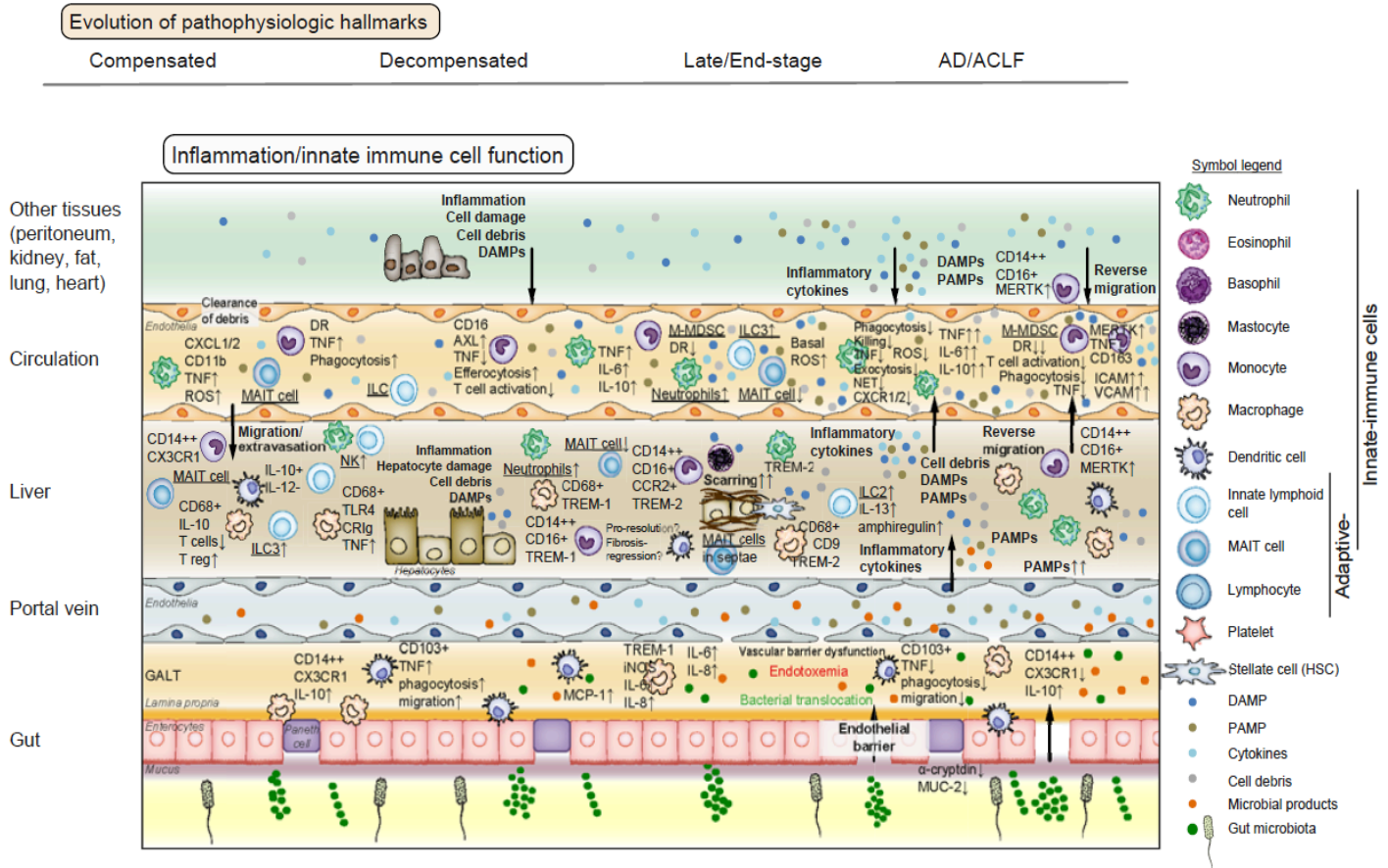


Figure 6.2. Current concept of diverse innate immune cell actions in various tissues and compartments in the context of cirrhosis-associated immune dysfunction. Taken from Bernsmeier, et al. 224

2. IV HAS binds and catalyses circulating PGE₂

This thesis explored the concept of albumin as a drug, rather than a simple volume expander. I established the albumin-PGE₂ hypothesis was plausible by demonstrating the binding affinity of albumin – PGE₂ is low (Kd approximately 270µM) which suggests that physiological decreases in circulating albumin and increases in PGE₂ concentration could result in significant increases in free circulating PGE₂ to pathophysiological levels. This finding was supported by decreased post treatment PGE₂ concentration (EIA measured) in the albumin treatment arm in the RCT. There was a significant improvement of albumin-PGE₂ binding in AD patients after infusion with 20% HAS when serum albumin >30g/L in both the single arm feasibility study and the RCT, to the same effect. It is likely that confounding factors, such a general improvement in patient's clinical condition, contributed to the observed effect as there was also some improvement in albumin-PGE₂ in control arm patients.

3. IV albumin solution increases serum albumin to near normal levels in AD patients, however this does not improve the 'effective albumin concentration' as seen in healthy volunteers which may explain the lack of clinical impact

Bernardi, et al.²²⁵ describe the 'effective albumin concentration' in plasma as the proportion of albumin present that maintains a fully preserved structure and function. I targeted a serum albumin of >30g/L (near normal) however, my results strongly suggest that the functional quality of albumin present is as important as the quantity. Although the infusion protocol increased serum albumin in AD patients and improved binding capacity, this did not reach the levels in healthy volunteers. Commercially available albumin for infusion does not maintain the same properties as healthy circulating albumin¹⁶³, which may have contributed to lack of effect. In addition, AD patients will have multiple other circulating ligands, such as bilirubin and drugs, that will also compete for binding sites on circulating albumin limiting any improvement in binding capacity.

The quantity of oxidised albumin (HNA1 and HNA2) present post treatment remained much lower than that of healthy volunteers. Therefore, despite the improvement in the amount and functional quality of albumin present post IV HAS infusions this does not appear to have been sufficient to have a beneficial clinical effect in these unwell patients. Alcaraz-Quiles, et al.¹⁶⁹ suggested that irreversibly oxidised albumin (HNA-2) itself promoted the release of pro inflammatory cytokines that may contribute to ongoing systemic inflammation.

Given the median (IQR) volume of HAS infused to patients in targeted albumin arm was 1000 (700-1500) mL, which raised albumin >30g/L, compared with 100 (0-600) mL in standard care, my results suggest that infused albumin does not have the capacity to achieve the 'effective albumin concentration' in AD patients.

4. IV HAS had no demonstrable impact on immune dysfunction nor rates of infection in AD patients.

Ex vivo analysis, using samples from HAS treated patients versus standard of care patients, showed no change in plasma mediated MDM dysfunction and no change in measured plasma cytokines. This was entirely consistent with clinical outcomes.

This highlights the importance of translating 'bench side outcomes' to 'bedside outcomes' in adequately powered clinical trials. There have been many studies analysing the impact of HAS treatment in liver disease on markers of immune function

ex vivo^{61,65,75,169,226,227} and in animal models^{11,61}. It is important we understand possible mechanisms of action but more importantly that hypothesis are tested in vivo prior to drawing firm conclusions. IV albumin administration was first given more than 70 years ago²²⁸ and use has become widespread amongst hepatologists. However, there have been a lack of randomised clinical studies to support increasing use.

It is possible that patients with acute decompensation of cirrhosis are too advanced in their disease course to benefit from IV albumin treatment with the therapeutic intentions described in this thesis. The severity of existing albumin damage, amount of circulating ligand and established immune-paresis alongside pending development of extra hepatic multi organ failure may be too great for albumin infusions to overcome.

Future work

Targeting different patient populations

Recently published evidence supporting the use of IV albumin in decreasing mortality in liver cirrhosis has been in outpatients who require regular LVP⁷³. 431 patients with uncomplicated ascites on diuretics were randomised to weekly outpatient HAS infusions or no additional intervention (standard medical therapy). These patients had earlier stage disease (MELD 12-13 as opposed to a mean MELD of 20 for patients studied in my thesis) without recurrent hospital admission. The study had a pragmatic approach and was unblinded. Overall 18-month survival was significantly higher in the standard therapy plus HAS than in the standard medical therapy group (Kaplan-Meier estimates 77% vs. 66%; $p=0.028$), resulting in a 38% reduction in the mortality hazard ratio (0.62 [95% CI 0.40–0.95]). There were additional benefits with lower incidence rate ratio (IRR) for infection (SBP and non-SBP) and renal dysfunction. However unlike the standard therapy group, the HAS group had weekly medical professional contact when IV albumin was administered which could possibly have caused a confounding effect by improving standard of care in this group. Post hoc analysis found that HAS treatment arm patients who incremented their serum albumin levels to near normal¹⁷⁵ had better outcomes.

In contrast the MACHT⁷⁴ study, a double-blind, placebo-controlled trial, patients with advanced cirrhosis (MELD 17-18) awaiting liver transplantation received outpatient fortnightly treatment with midodrine and albumin. This slightly suppressed vasoconstrictor activity but did not prevent complications of cirrhosis or improve survival. However, only 9 patients were treated for the entire year, the median length of treatment was actually only

80 days and the mortality rate in both arms was very low due to patients undergoing timely liver transplantation. Perhaps therefore a greater dose of albumin, perhaps using the infusion protocol described in this thesis, or longer duration of treatment is required to benefit patients with less advanced disease and should be targeted at those not close to receiving a liver transplant.

Targeting PGE₂ receptors

Work has recently been completed within our group characterizing the EP4 receptor as the main driver of PGE₂ related immunosuppression in decompensated cirrhosis. A more precise therapeutic target may be selective EP4 receptor antagonism. Several EP4 antagonists are currently undergoing clinical trials (indications ranging from pain to cancer) and this may represent a future drug to improve immune function in cirrhosis²²⁹.

Improving the function of albumin

Although my thesis demonstrates administering IV HAS improves the functional capacity of circulating albumin in AD patients, this does not reach the same level as healthy individuals. The production of IV albumin from human blood has not changed for many years and the sanitisation process is known to damage the albumin²³⁰. In this thesis I demonstrate batch-to-batch variation in the quality and quantity of albumin available for infusion, even from the same supplier. Recombinant albumin for infusion is sold as a superior product, however its expense limits its use to prolonging half-life of drugs rather than for infusion. In this thesis I show that recombinant albumin for infusion binds PGE₂ in much the same way as albumin obtained from human blood, this could suggest it would be of equivalent efficacy in vivo. One small study has shown similar pharmacokinetic profiles²³¹.

Future work could focus on producing a new protein fragment focussing upon the medicinal properties albumin has. Two possible ideas are:

1. Increasing the number of binding sites on the albumin molecule: producing a protein fragment as a dimer/trimer of the functionally important albumin binding sites would increase functional efficacy.
2. Decreasing the likelihood of albumin oxidation in vivo: The polypeptide 49-307 of human albumin contains the Sudlow domain 2A, which is the specific site of PGE₂ binding, the tight intra-molecular interactions within the hepta-helix make this domain

highly stable and compact. This domain lacks the recognition site for the MHC related Fc cellular receptor, therefore could present completely different pharmacodynamics features in vivo compared with full length albumin²³² potentially making it more stable and less likely to undergo oxidation.

Overall the most important finding from my thesis is a complete lack of effect of large volumes of albumin on clinical outcomes in hospitalised AD patients, which was matched by my laboratory analyses. Hepatologists need to reevaluate the way in which pre clinical albumin studies or studies without a control arm are currently interpreted and put into practice.

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