THE ROLE OF SERUM MARKERS OF ANGIOGENESIS AND FIBROSIS IN PREDICTING THE PRESENCE OF PORTAL HYPERTENSION

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Thesis submitted for the degree of Doctor of Medicine (Research)

University College London

Statement of Originality

‘I, Brian Hogan, 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.’

Brian John Hogan

London

February 2023

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Abstract

Background:

Portal hypertension occurs when the venous pressure in the portal vein increases above 5mmHg. In the UK, 90% of patients with portal hypertension have liver cirrhosis as the underlying aetiology. Currently invasive techniques, either gastrointestinal endoscopy or hepatic venous pressure studies, are required to confirm the diagnosis of portal hypertension. Increased portal pressure is associated with increased clinical complications and clinically significant portal hypertension occurs when the hepatic venous pressure gradient (HVPG) rises above 10mmHg. This is associated with an increased risk of ascites, varices, and variceal bleeding.

Methods:

Ethical approval was obtained from the NHS Research Ethics Service and local approvals from the Royal Free Hospital Research & Innovation office. Potential participants who were having HVPG measured as part of their routine care were recruited, after giving informed consent. 100 Participants had venous samples taken from peripheral blood and from the hepatic vein during the procedure. Angiogenic Tie-2 receptor cytokines, Angiopoietin-1 and -2 were measured, along with a liver fibrosis marker, the ELF test, and these were correlated with the HPVG, with traditional markers of liver fibrosis, and with patient outcomes.

Results:

The Angiopoietin-2/Angiopoietin-1 ratio was elevated in patients with cirrhosis and predicted an HVPG ≥ 12 with an AUROC of 0.804 ($p=0.003$). The ELF test predicted an HVPG ≥ 12 with an AUROC of 0.918 ($p<0.001$) in patients with liver cirrhosis. Both and elevated Angiopoietin-2/Angiopoietin-1 ratio and an elevated ELF test were associated with increased short-term mortality.

Conclusions:

In our cohort of 100 participants, we showed that both the Angiopoietin-2/Angiopoietin-1 ratio and the ELF test correlated with HVPG and deserve further validation for their potential role as diagnostic tests to rule-out clinically significant portal hypertension. The same tests were able to predict short-term mortality and may be useful biomarkers of significant disease endpoints.

Impact Statement

We are facing a globally increasing incidence of liver disease which has translated into an escalation in liver related deaths in the UK by 400% since 1970[1]. Liver disease predominantly affects adults in their 4th – 6th decade and is now the 2nd most common cause of working life years lost in Europe, after only ischaemic heart disease[2]. The most common causes of liver disease in the UK are Metabolic Associated Liver Disease (associated with obesity, Type 2 diabetes and the metabolic syndrome), Alcohol related liver disease and viral hepatitis. These liver diseases can be treated or prevented from progressing if detected at an early stage, reducing the risks to patients of developing the complications of end stage liver disease and improving survival. There is a stigma associated with liver disease which has led to variable practice in the UK and some examples of poor care being delivered to patients[3].

Most early liver disease is not symptomatic and there is an urgent need to develop more reliable biomarkers which can be applied to those who are at risk. This will allow the clinician to detect liver disease at an earlier stage and better advise a patient on appropriate action. The ability to offer treatment, including cure, at an earlier stage will encourage discourse within and outside the medical community and help to reverse the stigma associated with liver disease.

This thesis focuses on the detection of portal hypertension, a complication of advanced chronic liver disease associated with life threatening complications such as variceal bleeding, ascites and acute kidney injury. There are many

therapies for portal hypertension known to reduce the risk of future complications, but currently portal hypertension can be diagnosed late in the disease course and clinicians can be unsure at which stage to apply known therapies without invasive testing. The gold standard test for portal hypertension, hepatic venous pressure gradient measurement, involves a balloon catheter being placed in the hepatic vein, near the liver. We aimed to identify new non-invasive blood tests to help detect and monitor this condition.

We have shown that the detection of Angiopoietins in peripheral blood can help to rule in and rule out clinically significant portal hypertension. Further investigation and validation of these results is required but following this our results may allow health care professionals to more easily guide future treatments. This availability of non-invasive testing and the detection of liver disease at an earlier stage would reduce the personal and financial burden of invasive tests and treatments for the patient and for health care system.

These results may lead to the development of novel therapies. The Angiopoietins govern a receptor on endothelial cells, for which a drug inhibitor exists, and which has been trialled in patients with cancer. This may lead to investigations of this therapy to modulate the neovasculogenesis of chronic liver disease which contributes to portal hypertension.

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List of Abbreviations

A1AT	Alpha-1 Anti-trypsin
ACLD	Advanced Chronic Liver Disease
cACLD	compensated Advanced Chronic Liver Disease
dACLD	decompensated Advanced Chronic Liver Disease
ADH	Antidiuretic hormone
ADMA	Asymmetric dimethylarginine
AFP	Alpha-fetoprotein
AKI	Acute Kidney Injury
ALD	Alcohol related Liver Disease
ALT	Alanine Aminotransferase
ANG1	Angiopoietin-1
ANG2	Angiopoietin-2
ARFI	Acoustic Radiation Force Impulse
BH4	Tetrahydrobiopterin
cAMP	cyclic Adenosine Monophosphate
CB	Cannabinoid
CHC	Chronic hepatitis C
CLL	Chronic Lymphocytic Leukaemia
cGMP	cyclic guanosine monophosphate
CPA	Collagen Proportionate Area
COX	Cyclo-oxygenase
CSPH	Clinically significant portal hypertension
DDAH-1	Dimethylarginine dimethylaminohydrolase 1

DSE	Dobutamine Stress Echo
DILI	Drug Induced Liver Injury
ECM	Extracellular Matrix
EHPVO	Extra hepatic portal vein obstruction
ELF	Enhanced Liver Fibrosis test
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial progenitor cells
ET-1	Endothelin-1
FGF	Fibroblast Growth Factors
FHVP	Free hepatic venous pressure
FXR	Farnesoid X receptor
GFR	Glomerular filtration rate
GI	Gastrointestinal
GOV	Gastro-oesophageal varices
HA	Ayaluronic acid
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HE	Hepatic Encephalopathy
HIF	Hypoxia inducible factors
HIV	Human Immunodeficiency Virus
HO-1	Haem Oxygenase -1
HO-2	Haem Oxygenase -2
HPS	Hepatopulmonary syndrome
HRS	Hepatorenal Syndrome

HSC	Hepatic Stellate Cell
HV	Hepatic Vein
HVPG	Hepatic Venous Pressure Gradient
ICA	International Club of Ascites
IGV	Isolated gastric varices
IL-6	Interlukin-6
iNOS	Inducible Nitric Oxide Synthase
INR	International Normalised Ratio
IVC	Inferior Vena Cava
KC	Kupfer Cell
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cell
LSM	Liver Stiffness Measurement
LT	Liver Transplantation
LVP	Large Volume Paracentesis
MELD	Model for End Stage Liver Disease Score
MRA	Magnetic Resonance Angiography
MRE	Magnetic Resonance Elastography
NAFLD	Non-alcohol related fatty liver disease
nNOS	Neuronal Nitric Oxide Synthetase
NRH	Nodular regenerative hyperplasia
NO	Nitric oxide
NSBB	Non-selective Beta Blocker
OD	Optical Density

P3NP	Amino-terminal propeptide of type III procollagen
PAP	Pulmonary Arterial Pressure
PAOP	Pulmonary Artery Occlusion Pressure
PDGF	Platelet derived growth factor
PH	Portal Hypertension
PHG	Portal hypertensive gastropathy
POPH	Portopulmonary hypertension
PPCD	Post-paracentesis circulatory dysfunction
PVPG	Portal venous pressure gradient
PVR	Pulmonary Vascular Resistance
RAAS	Renin-aldosterone-angiotensin system
SA	Splenic Artery
SBP	Spontaneous Bacterial Peritonitis
SEC	Sinusoidal Endothelial Cell
SEMS	Self-expanding Metal Stent
SMA	Superior Mesenteric artery
SNS	Sympathetic nervous system
SOS	Sinusoidal Obstruction Syndrome
SS	Splenic Stiffness
SST	Serum separator tubes
TE	Transient Elastography
TGF-1β	Transforming Growth Factor - 1β
TIMP-1	Tissue inhibitors of matrix metalloproteinases type-1
TIPS	Transjugular Intrahepatic Portosystemic Shunt

TLR4	Toll Like Receptors - 4
TLR9	Toll Like Receptors - 9
TNFα	Tumour Necrosis Factor - alpha
UCL	University College London
ULN	Upper Limit of Normal
UK	United Kingdom
VBL	Variceal band ligation
VEGF	Vascular endothelial growth factor
VSPC	Vascular stem/progenitor cells
VOD	Veno-occlusive disease
WHVP	Wedged hepatic venous pressure

CHAPTER 1: General Introduction

1.1 Definition and Classification Chronic Liver Disease

Chronic liver disease is defined as liver disease which has been present for greater than 26 weeks[4]. It represents a spectrum of disease ranging from mild inflammation to advanced fibrosis with complications. Whilst traditionally patients with advanced chronic liver disease (ACLD) were given the diagnosis of 'cirrhosis', the latter is a histological diagnosis requiring the presence of 'diffuse fibrosis which converts the normal liver architecture into structurally abnormal nodules' which must be visualised on a liver biopsy[5]. Modern nomenclature aims to reflect the opinion that the diagnosis of advanced liver disease does not require histology in all cases and the terms compensated advanced chronic liver disease (cACLD) and decompensated advanced chronic liver disease (dACLD) better represent this approach to diagnosing liver disease non-invasively[6]. In addition, a staging system for ACLD based on the development of complications, has been proposed. This challenges the traditional concept of cirrhosis being a single stage of chronic liver disease and helps to focus appropriate surveillance and therapies more effectively (see table 1.1) [7-9]. The diagnosis of cACLD is defined by the BAVENO VII criteria and is highly suggested by a Liver Stiffness Measurement (LSM) of >15 kPa[10].

	Stage	Features
cACLD	0	no varices, mild portal hypertension
	1	No varices, clinically significant portal hypertension
	2	Varices
dACLD	3	Variceal bleeding
	4	First non-bleeding decompensation
	5	>1 decompensating event

Table 1.1: Clinical stages of advanced chronic liver disease. cACLD = compensated ACLD, dACLD = decompensated ACLD. Adapted from[11]

Progression from cACLD to dACLD represents a significant progression in the liver disease and an associated reduction in median survival from 12 to two years[11].

1.2 Epidemiology of Chronic Liver Disease

The incidence of liver disease is increasing in the UK and mortality rates have increased by 400% since 1970 (see Figure 1.1)[1]. Liver cirrhosis and primary liver cancer are the 2nd most common cause of working life lost amongst 15-64 year olds in Europe, after ischaemic heart disease[2]. It is estimated that 60,000 people in the UK have cirrhosis and there were 282,299 hospital admissions from liver and liver related disease in 2014-15[1 12]. The rising mortality from liver disease in the UK is predominantly related to alcohol use disorder. However, the incidence of metabolic associated fatty liver disease (also known as non-alcohol related fatty liver disease) is rapidly increasing with an estimated prevalence of 23.7% of the adult population in Europe[13]. In the UK 64.6% of the population are classified as overweight or obese, of whom approximately 10% have evidence of advanced liver fibrosis[1 14].

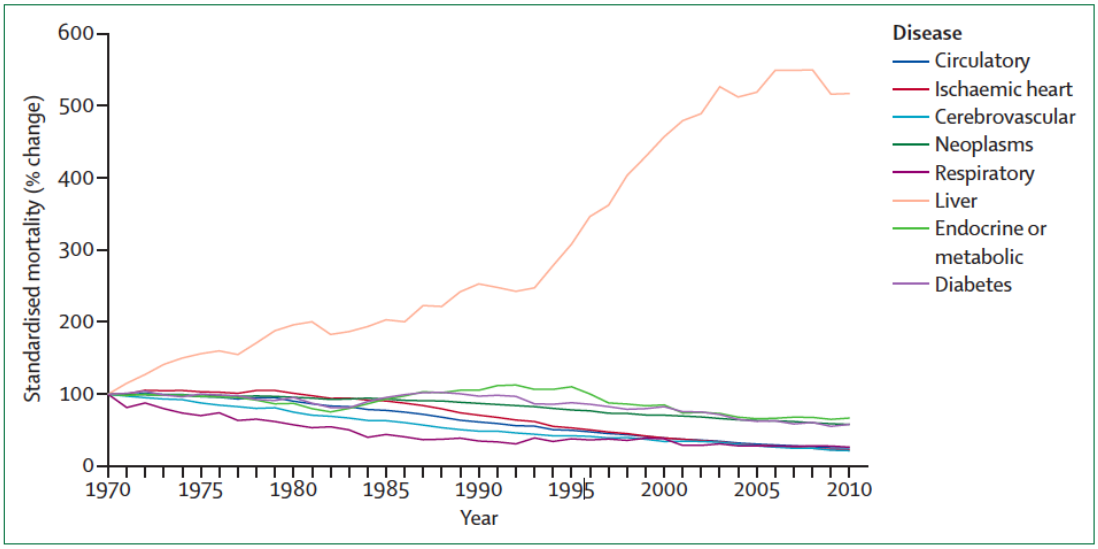

***Figure 1*: Standardised UK mortality rate data**
Data were normalised to 100% in 1970, and subsequent trends plotted using the software Statistical Package for the Social Sciences. Data are from the WHO-HFA database.[4] Analysed by Nick Sheron (September, 2013).

Figure 1.1 A comparison of standardised UK mortality rate data, highlighting the rise in mortality from liver disease, reproduced from Williams et al with permission from Elsevier [License Number: 5486561148816] [1].

1.3 Liver Fibrosis

Liver fibrosis is a dynamic process leading to the deposition of extracellular matrix (ECM) within the liver in response to injury. The deposition of ECM results in a distortion of the liver architecture. It is recognised that the processes of fibrogenesis and angiogenesis occur synchronously and share a common initial pathway in response to tissue injury[15]. The initial injury often results in tissue hypoxia, an increase in free radical oxygen species, hepatocyte apoptosis (activating TLR-9 receptors on hepatic stellate cells) or an increase in bacterial Lipopolysaccharide (LPS) (which activates the TLR-4 receptors on Kuppfer and hepatic stellate cells (HSC)). These processes result in activation of the quiescent HSC, the key regulator of liver fibrosis. The activated HSC

releases pro-fibrotic (TGF-1β, matrix metalloproteinase and tissue inhibitors of metalloproteinases), pro-inflammatory and pro-angiogenic cytokines (PDGF and VEGF) as well as vasoactive substances such as Endothelin[16].

Whilst the deposition of ECM and the development of liver nodules (cirrhosis) is the result of progressive fibrosis, there are three distinct histological phenotypes, which vary depending on the aetiology of liver disease. Biliary diseases tend to result in a portal-portal distribution of fibrosis, chronic viral hepatitis tends to lead to a portal-central distribution (from the portal tracts to the central vein) and alcohol and non-alcohol related fatty liver disease is associated with deposition of ECM around the sinusoids and groups of hepatocytes resulting in a pericellular fibrosis[17]. Fibrosis usually evolves over decades, with a few notable exceptions, such as biliary atresia, Hepatitis C Virus/Human Immunodeficiency Virus co-infection and recurrence of viral hepatitis post liver-transplant [16 18 19].

1.4 Portal Hypertension

1.4.1 Definition of Portal Hypertension

Portal hypertension (PH) is defined as an increased pressure in the portal vein (normally 5-9mmHg)[20]. In modern practice this is assessed by its effect on the gradient between the wedged and free hepatic vein pressures, the hepatic venous pressure gradient (HVPG)[21]. A normal HVPG is < 5mmHg, and a measurement of ≥ 5 mmHg defines sinusoidal portal hypertension.

PH is responsible for many of the complications of ACLD including varices, ascites, encephalopathy and renal impairment and for each 1mmHg increase in the HVPG above normal there is a 3% increase in the annual risk of mortality[22].

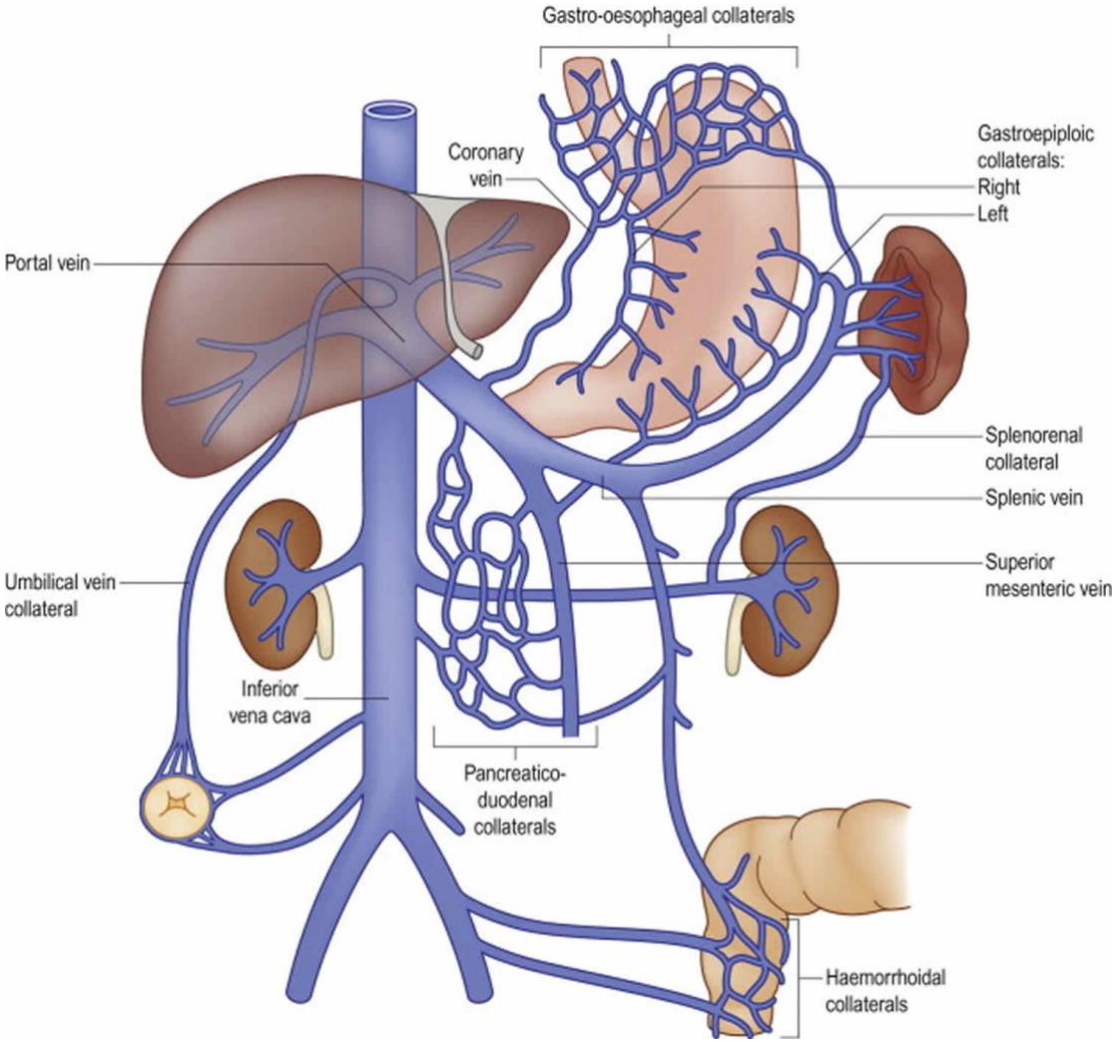

Figure 1.2. The anatomy of the portal venous circulation with common sites of collateral formation in portal hypertension reproduced with permissions.

Various thresholds of HVPG have been described and associated with predictable complications of portal hypertension:

HVPG	Association
<5	Normal
5-9	sub-clinical portal hypertension
≥ 10	development of ascites and varices
≥ 12	increased risk of variceal haemorrhage
≥ 20	failure to control variceal bleeding, high risk of re-bleeding
≥ 22	reduced survival in acute severe alcohol related hepatitis

Table 1.2 Clinical associations of HVPG thresholds. Adapted from [21].

1.4.2 Classification of Portal Hypertension

Portal Hypertension is traditionally classified according to the anatomical level at which the resistance to flow occurs, see figure 1.3 (pre-hepatic, hepatic or post-hepatic). In the United Kingdom 90% of PH is intrahepatic (sinusoidal), related to ACLD. However, in other parts of the world this proportion is smaller with infectious causes of extra-hepatic portal vein thrombosis or obliterative portal venopathy being more frequently encountered[23].

Prehepatic portal hypertension is predominantly related to extra-hepatic portal vein thrombus, which is associated with congenital or acquired pro-thrombotic conditions or local inflammatory processes (acute pancreatitis, abdominal trauma or surgery).

Post-hepatic portal hypertension can be caused by any process obstructing flow from the hepatic veins but is most commonly related to Budd Chiari syndrome (hepatic venous obstruction) and heart failure.

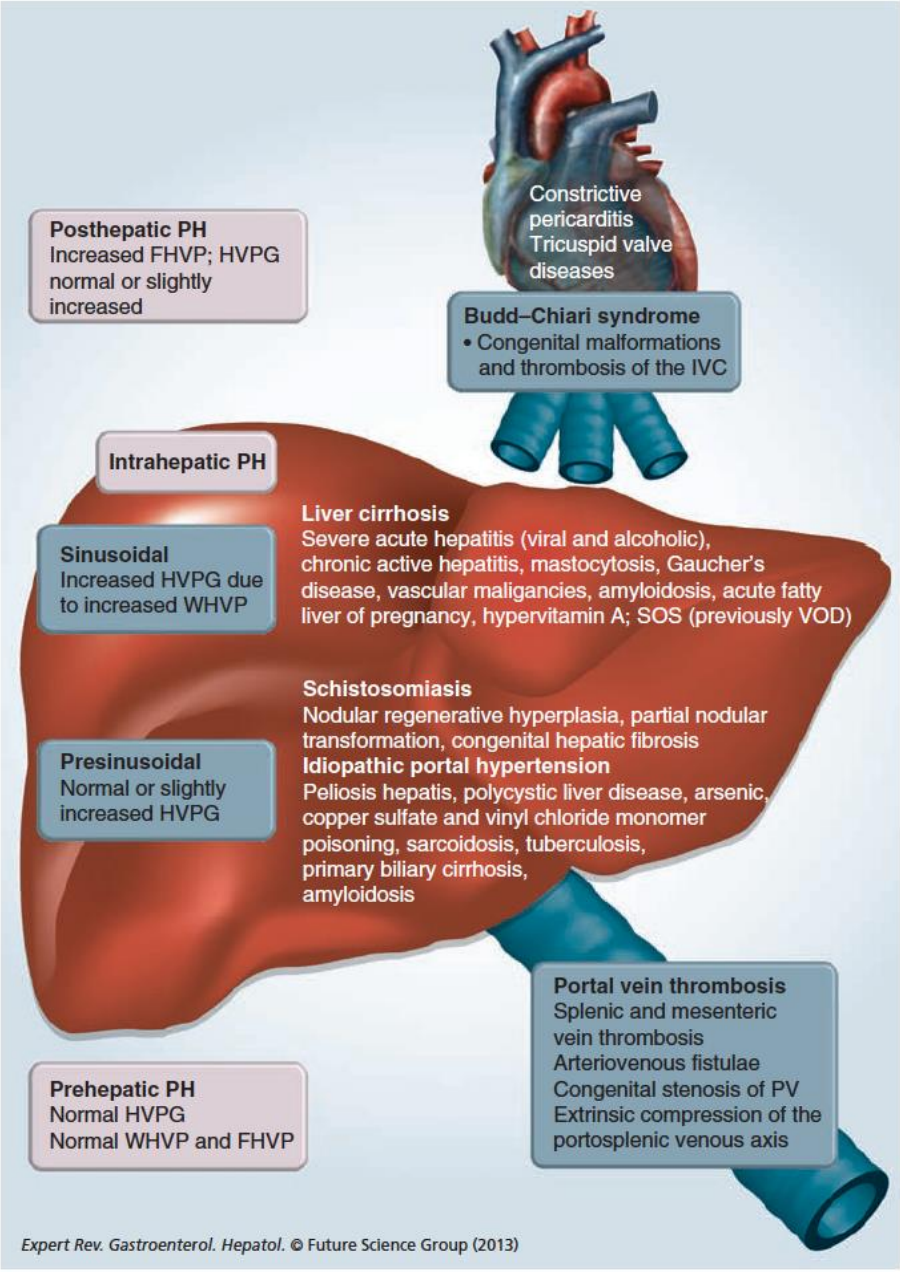

Figure 1.3 The anatomical classification and differential causes of portal hypertension, from Berzigotti et al, reprinted by permission of Informa UK

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1.4.3 Pathophysiology of Portal Hypertension

Application of Ohm's law to the mesenteric portal circulation states that portal pressure (P) is proportional to portal blood flow (Q) and resistance in the portal system (R)[24].

$$(P \propto Q R)$$

The change in liver architecture in advanced fibrosis (fibrosis, nodule formation and sinusoidal capillarisation) increases hepatic resistance and traditionally it was thought that this alone reduced hepatic blood flow. Capillarisation is defined as a loss of the normal liver sinusoidal epithelial cell fenestration and the formation of an organised basement membrane and has been shown to proceed fibrosis development [25-27]. This long-held belief that portal hypertension was a result of increased hepatic resistance and reduced flow alone was first questioned in the 1970's and 1980's when the ability to measure pressure and flow in the portal circulation improved our understanding of hepatic haemodynamics significantly. Groszmann and colleagues published a series of papers showing that there was, in fact, increased hepatic blood flow in patients with chronic liver disease (specifically those with severe alcohol related hepatitis) and demonstrated a significant portosystemic shunt in the same patient group[28 29]. Further work demonstrated that in addition to the mechanical increase in intrahepatic resistance, there is an increase in vascular

tone secondary to endothelial dysfunction and an adaptive increase in splanchnic inflow (see figure 1.4). These adaptive responses are collectively referred to as the dynamic component of portal hypertension, whereas the fixed increase in intrahepatic sinusoidal resistance is known as the mechanical component. It is estimated that approximately 30% of the increase in intrahepatic resistance is related to dynamic factors. Animal models of portal hypertension have confirmed this finding, showing that whilst there is a brief hypodynamic period in the evolution of portal hypertension, by day 4 there is a hyperdynamic circulation with an increased cardiac output [30].

The current synopsis for the development of portal hypertension in cirrhosis is that liver injury, hepatic stellate cell activation, fibrogenesis and sinusoidal epithelial cell capillarisation are associated with intrahepatic liver endothelial cell hyperactivity and vascular remodelling. These processes cause increased intrahepatic vascular resistance, splanchnic arterial vasodilatation, intrahepatic and porto-systemic shunt (collateral) formation. The pooling of blood volume in the splanchnic circulation results in a relative systemic hypovolaemia and a systemic hyperdynamic circulation (reduced systemic vascular resistance and increased cardiac output). This pathological process leads to the familiar complications of varices, variceal haemorrhage, encephalopathy, sodium and fluid retention, ascites and renal impairment. The alterations in the liver microcirculation and molecular mechanisms responsible for these processes are discussed below.

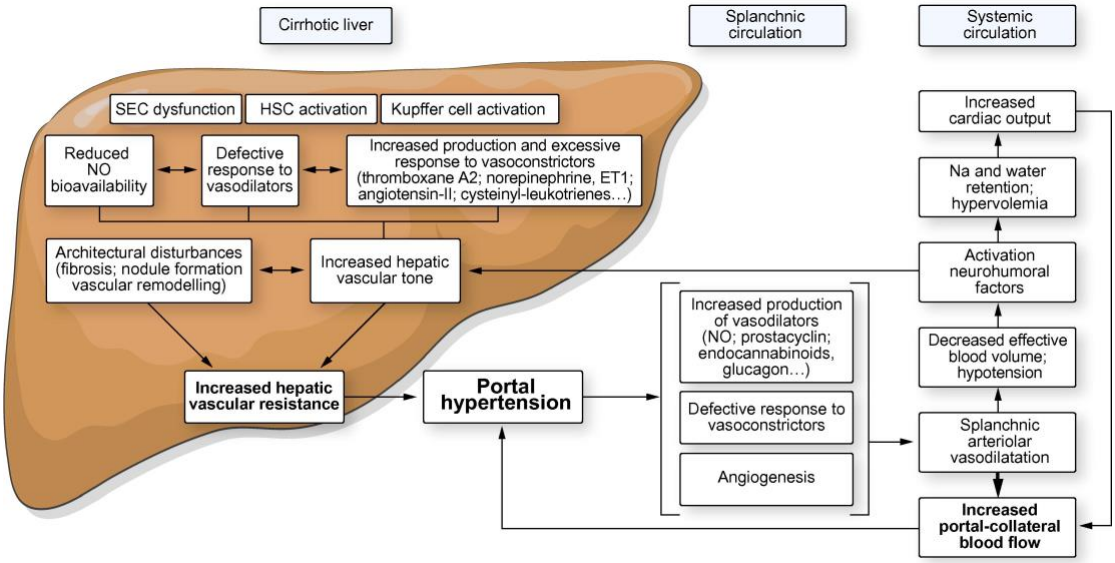

Figure 1.4. Summary of the pathophysiology of portal hypertension, reproduced from Garcia-Pagan et al with permission [CC BY NC ND] [31].

1.4.4 The Liver Microcirculation

The liver has a unique dual blood supply, with 70-80% from the portal venous circulation and 20-30% arising from the hepatic artery[32]. The liver is divided into functional lobules each served by a single central hepatic vein and 6 portal tracts arranged in a hexagonal formation (see figure 1.5). Portal blood flows through the hepatic sinusoids, separated from layers of hepatocytes (each 1 cell thick) by sieve like fenestrations in the liver sinusoidal endothelial cell (LSEC) and then the space of Disse[33]. Flow continues from the sinusoids through the central venules to the hepatic vein and then to the inferior vena cava and the right atrium.

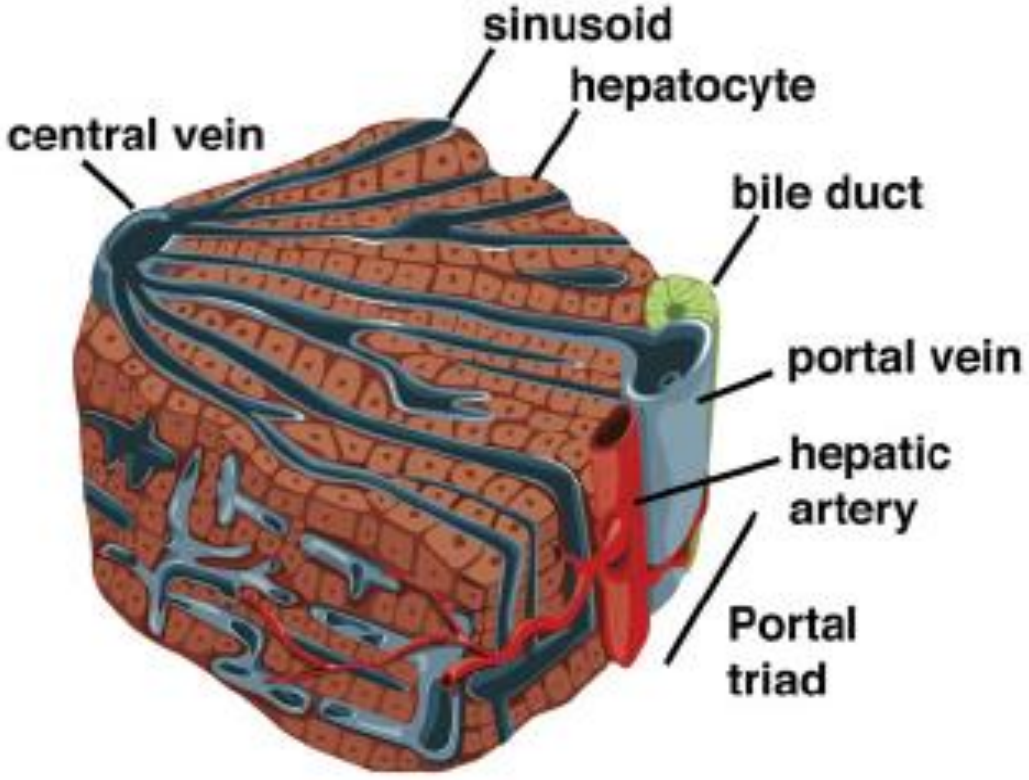

Figure 1.5 ‘Diagram of the structure of a portion of a liver lobule showing the central vein and portal tract with connecting sinusoids’ reproduced from Si-Tayeb et al with permissions from Elsevier [License 5486591468073] [34].

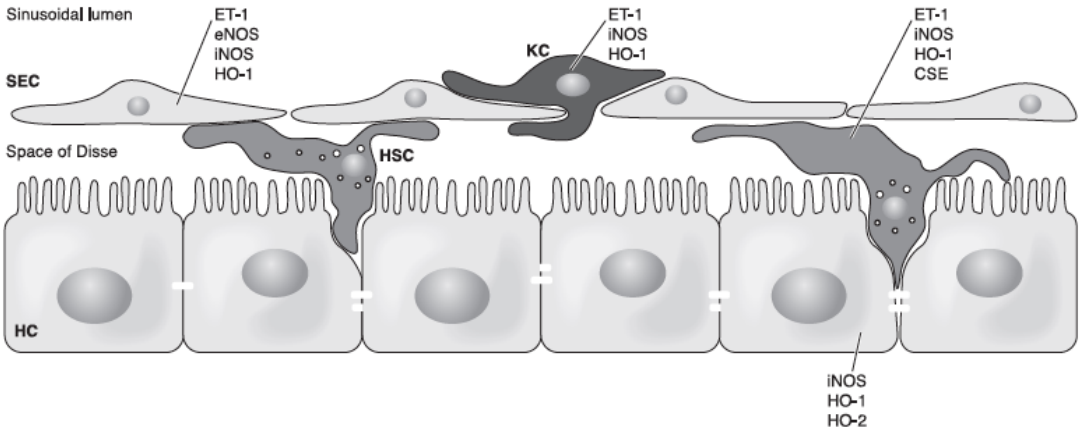

Figure 1.6 ‘Local regulators of the hepatic sinusoidal microcirculation’ reproduced from Vollmar et al, with permissions from the American Physiological Society[33]. ET-1 = endothelin-1; eNOS = endothelial nitric oxide

synthase; HO-1 = haemoxygenase-1; iNOS = inducible nitric oxide synthase; HSC = hepatic stellate cell; SEC = sinusoidal endothelial cell.

Flow through the liver is controlled at the level of the microcirculation by the complex interaction of the LSEC and the hepatic stellate cell (HSC), see figure 1.6. The LSEC is unique from other epithelial cells in it that it possesses fenestrae on the cell surface and lacks a basement membrane. Because of these features the LSEC is often seen as the initial 'line of defense' for the liver from the portal circulation[35]. The hepatocytes and HSCs come into direct contact with circulating cells (especially lymphocytes) and any molecules which pass through these fenestrae into the space of Disse (see Figure 1.6 above). The LSEC plays an important role in maintaining HSC homeostasis, and it has been shown that this process is dependent on the synthesis of nitric oxide and the presence of VEGF [36]. Whilst the contractile nature of endothelial cells is well known, the activated HSC has also been shown to have contractile properties, driven by alpha-smooth muscle actin and myosin, which can further contribute to an increase in vascular tone[16 37].

1.4.5 Endothelial Dysfunction

Endothelial dysfunction encompasses many of the processes central to the development of fibrosis and portal hypertension. It is an alteration in the normal homeostasis governing vascular tone, endothelial cell growth, coagulation and angiogenesis [35]. Inflammation is a trigger for endothelial dysfunction, and in

ACLD inflammation is associated with decompensation and increased mortality[38 39].

1.4.5.1 Reduced Intrahepatic Nitric Oxide

In a physiological state nitric oxide (NO) reduces intrahepatic vascular tone by binding to soluble guanylate cyclase and increasing cyclic guanosine monophosphate (cGMP). This controls calcium efflux from the endothelial cell cytoplasm and leads to a reduction in the contractile state. The generation of NO provides approximately 70% of the balance for the systems which increase vascular tone, the renin-angiotensin-aldosterone system, the sympathetic nervous system and the production of Endothelin [40 41].

NO is a pluripotent, diatomic, colourless, hydrophobic gas which diffuses freely across cell membranes and has a half-life of 3-5 seconds. Due to these properties, it cannot be stored intracellularly and must be synthesised when required. There are 3 isoforms of the enzyme nitric oxide synthase (NOS), which oxidises L-arginine to L-citruilline producing NO. Inducible NOS (iNOS) is associated with the inflammatory response and once activated, for example by endotoxin or TNFα, produces large amounts of NO for an extensive time period. Endothelial NOS (eNOS) is present predominantly in endothelial cells and is responsible for the maintenance of vascular tone, producing smaller quantities of NO. eNOS is regulated by a number of stimuli including stress hormones and mechanical sheer wall stress on vessels, it has been shown to

be essential in maintaining adequate hepatic perfusion[42 43]. The third isomer neuronal NOS (nNOS) is primarily found in the cerebral circulation[44].

In cirrhosis there is a reduction in eNOS activity on sinusoidal endothelial cells leading to reduced NO synthesis, unopposed vasoconstriction and increased intrahepatic vascular tone, with the degree of impaired eNOS activity being directly related to the severity of liver disease[45 46].

There are numerous mechanisms proposed to explain the reduced eNOS activity including decreased receptor phosphorylation, reduced tetrahydrobiopterin (BH4) availability (an essential co-factor for NOS activity), increased levels of asymmetric d-methyl-arginine (which inhibits NO synthesis), increased superoxide NO scavenging and increased caveolin binding with calmodulin, which inhibits eNOS[42 47 48]. Oxidative stress, as demonstrated by the increased circulating malonic dialdehyde levels, and the presence of radicle oxidative species reduce the availability of nitric oxide[49 50].

In addition to the reduction in NO synthesis, there appears to be a reduced responsiveness of the HSC to NO due to a poorly functioning guanylate cyclase signalling pathway, further exacerbating the vasoconstriction [51 52].

1.4.5.2 Increased Extrahepatic (Systemic & Splanchnic) Nitric Oxide

In direct contrast to the intrahepatic circulation, the activity of eNOS in splanchnic and systemic endothelial cells is increased in cirrhosis. There is

excessive NO production induced by local mechanisms, promoting a reduced vascular tone in the splanchnic and systemic systems and increased flow through the portal circulation, aggravating portal hypertension. Many eNOS regulators, including VEGF and inflammatory cytokines have been shown to stimulate eNOS activity. In addition, the increased hepatic portal venous pressure itself elicits a sheer stress on the vascular endothelial cell which is a significant stimulant for NO production, further exacerbating the increased portal pressure [40 44 53].

1.4.5.3 Endothelin-1

Endothelins are potent vasoconstrictors and cause contraction of vascular smooth muscle, predominantly acting on Endothelin-A receptors. Levels of Endothelin-1 have been shown to be increased in patients with cirrhosis in proportion to the severity of liver disease. It is known that Endothelins are released in response to both inflammation and vascular sheer wall stress which are present in portal hypertension. [54-56]. Endothelin-A receptors are present on hepatic stellate cells and contraction of these cells is responsible for a significant contribution to intrahepatic vascular resistance [57 58].

1.4.5.4 Prostanoids

Arachidonic Acid is cleaved by cyclooxygenase-1 (COX-1) into prostaglandin and thromboxane. Thromboxane A2, released from sinusoidal epithelial cells in response to COX-1 up regulation, has been associated with vascular hyper-responsiveness and increased vascular tone in cirrhosis. In support of this

theory, COX-1 inhibitors have been shown to reduce intrahepatic resistance [59-61].

1.4.5.5 Prostacyclins

Prostaglandin-I2 (also called Prostacyclin) is also a product of the COX-1 pathway and released from mesenteric and systemic endothelial cells, it stimulates smooth muscle cell relaxation by increasing intracellular cAMP and levels of Prostaglandin I2 are increased in cirrhosis [62].

1.4.5.6 Endocannabinoids

Endocannabinoids, such as Anandamide, activate cannabinoid receptors (CB-1 and CB2). Endocannabinoids can be released from both platelets and macrophages in response to endotoxin (inflammation) and are mediators of vasodilatation. In cirrhosis there is an up regulation in the number of CB-1 receptors in mesenteric endothelial cells suggesting a role in splanchnic vasodilation[63 64].

1.4.5.7 Glucagon

Glucagon levels are elevated in the portal circulation in models of portal hypertension and results in endothelial smooth muscle relaxation exacerbating vasodilatation [65 66].

1.4.5.8 Impaired RhoA/Rhokinase Signaling

In animal models of cirrhosis, the RhoA/Rhokinase pathway has been shown to be down-regulated resulting in vasodilatation. The RhoA/Rhokinase plays a

role in phosphorylation of myosin light chains, key for endothelial cell contractility[67]. One group has shown that this process can be reversed by the administration of Neuropeptide-Y, a neurotransmitter acting on alpha-adrenoceptors [68].

1.4.6 Development of porto-systemic collaterals (Varices)

The development of porto-systemic collaterals occurs within the liver, as intrahepatic shunts, and outside the liver between the portal and systemic circulation, as extra-hepatic varices. An original hypothesis proposed that these shunts develop from pre-existing small vessels which dilate in response to raised portal pressure. It is now suspected that angiogenesis and neovascularisation play a key role in the development of new vessels, in addition to the enlargement of existing porto-systemic connections. The molecular mechanisms of angiogenesis and neovasculogenesis are described in detail in section 1.9. Briefly, tissue hypoxia and increased sheer wall stress are responsible for the release of pro-angiogenic factors (PDGF, VEGF and Angiopoietin 2) from endothelial cells. These factors promote the development of intra-and extra-hepatic shunts and the enlargement of vessels in the splanchnic circulation.

Varices develop at a rate of 10-14% per year in patients with an HVPG > 10mmHg[21]. Approximately 40% of patients will have varices at the time ACLD is diagnosed [69].

Oesophageal Varices (OV) are graded endoscopically based on their size and the presences of features which increased the bleeding risk. Grade 1 varices completely flatten on the insufflation of air at endoscopy. Grade 2 varices occupy < 50% of the lumen and Grade 3 varices are large enough to occlude the lumen at endoscopy[70].

Gastric Varices are less common than oesophageal varices and are the cause of bleeding in approximately 10-20% of cases in patents with cirrhosis. They are classified according to the Sarin classification (see Figure 1.7). Gastric varices, like ectopic small and large bowel varices, can bleed at lower venous pressures than oesophageal varices[71 72].

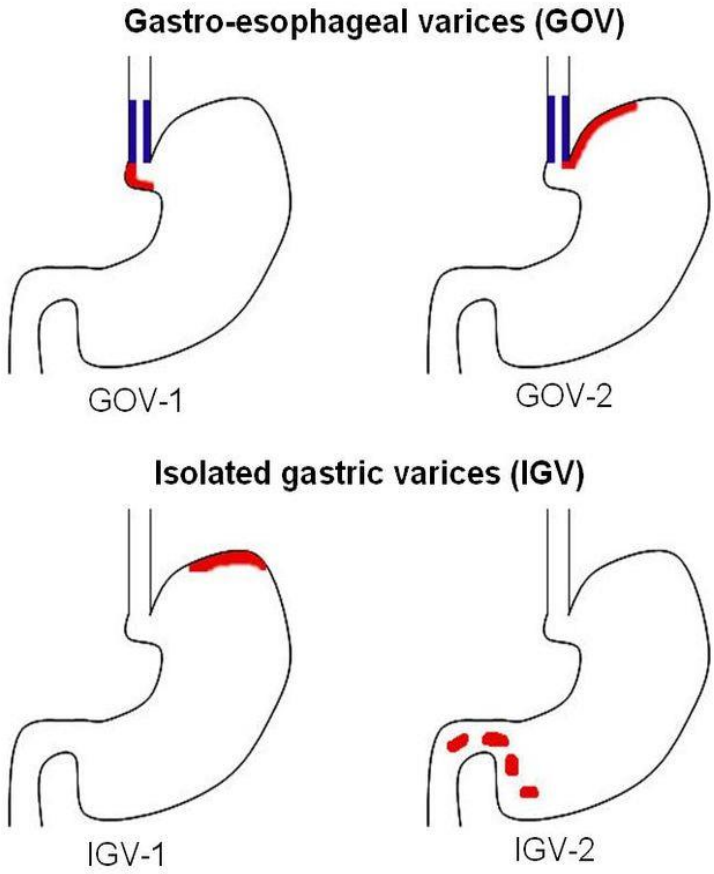

Figure 1.7. Sarin classification of gastric varices[71].

1.4.7 Bacterial Translocation

In ACLD small intestinal bacterial overgrowth, increased gut permeability and impaired immunity facilitate bacterial migration from the intestinal lumen, through the gut mucosa into the portal circulation resulting in systemic infection, particularly with gram negative 'gut' organisms. Gut bacteria can be cultured from the normally sterile mesenteric lymph nodes of approximately 30% of patients with Child-Pugh C cirrhosis[73]. A reduced gut transit time and reduced gastric acid secretion mean than patients with ACLD have bacterial overgrowth[74]. Small intestinal permeability is driven by leakage through 'tight junctions' caused by an inflammatory cascade and increased nitric oxide levels[75] in the extrahepatic portal circulation. ACLD is associated with an increase in pro-inflammatory interleukins and TNF-α further exacerbating intestinal permeability and promoting translocation[76].

The function of T-lymphocytes, neutrophils and monocytes are all impaired in patients with ACLD and, in addition to the bacterial overgrowth and mucosal permeability, a failure in the gut-associated lymphatic system to prevent translocation contributes to the transition of bacteria into the systemic circulation[77].

Importantly, whilst bacterial translocation results from portal hypertension, the presence of bacterial DNA, lipopolysaccharide and peptidoglycans in the portal circulation activate HSCs (via mechanisms including Toll-like receptors) which

further exacerbates intra-hepatic vascular resistance. Therapies directed at reducing the impact of bacterial translocation include selective gut decontamination, prokinetics and probiotics [78] [79].

1.4.8 Portal Hypertensive Gastropathy, Enteropathy and Colopathy

Portal Hypertension results in oedema, congestion and hyperaemia of the gut mucosa. Portal hypertensive gastropathy can affect the whole stomach, is present in 20-28% of patients with portal hypertension and is associated with bleeding, particularly when severe[80] [81]. Similar lesions can develop throughout the gastrointestinal tract and bleeding can occur from vascular ectasia throughout the small bowel and colon [82].

1.4.9 Thrombocytopaenia and Leukopaenia

Portal hypertension and a relative pooling of blood in the splanchnic circulation results in hypersplenism which can influence platelet and leucocyte counts. Sequestration in the spleen results in thrombocytopaenia in approximately 64% and leucopaenia in 5% of patients with cirrhosis. Though splenic sequestration is not the only mechanism of cytopaenias (others being an increased consumption, and a reduced synthesis of thrombopoietin from the liver) it is an important consequence of portal hypertension[83].

1.4.10 Variceal Bleeding

The risk of varices bleeding is approximately 10-20% each year in high-risk patients (characterised by: HVPG > 12; large varices; red wale signs and Child-

Pugh B or C cirrhosis) [84]. The mortality attached to an acute episode of variceal haemorrhage is approximately 5-20% at 6 weeks, increasing to 60% at 1 year[85]. There is a re-bleeding rate of 62% at 2 years [7 86]. Factors associated with increased mortality are impaired renal function, a Child-Pugh score ≥ 9 as well as a MELD score ≥ 18, re-bleeding within 5 days, and Acute on Chronic Liver Failure (which is the development of one or more extra-hepatic organ failure's associated with a decompensating event)[87 88].

1.4.11 Impact of Portal Hypertension on Brain Function

Hepatic encephalopathy (HE) is defined in EASL guidelines as brain dysfunction caused by liver insufficiency and/or portosystemic shunting. HE can lead to a number of neurocognitive symptoms from subclinical changes in behavior or executive function to coma[89]. It is classified as either Type A (associated with acute liver failure), B (in portosystemic shunt or bypass) or C (in cirrhosis)[89].

The development of encephalopathy in ACLD is related to several mechanisms. It is known that bacterial overgrowth and translocation increase ammonia production in the gut and its absorption into the portal circulation. In portal hypertension ammonia can bypass the liver via intra- and extra-hepatic portosystemic shunts. In ACLD there is also an impaired liver urea cycle, increased ammonia production in the kidneys and a reduction in ammonia elimination. Ammonia freely crosses the blood-brain barrier and in the astrocyte is converted to glutamine (by the enzyme glutamine synthetase) which causes cell oedema and neuronal dysfunction. Inflammation, caused by

sepsis and translocation exacerbates encephalopathy and pro-inflammatory cytokines contribute to the astrocyte and microglial dysfunction associated with the syndrome [90-92].

1.4.12 Impact of Portal Hypertension on Cardiac Function

ACLD and portal hypertension are associated with 'cirrhotic cardiomyopathy'. This is an impaired response of the heart to stress and a failure of relaxation (demonstrated by diastolic dysfunction). It is present in approximately 50% of patients with cirrhosis, and is thought to be caused by the endothelial dysfunction associated with portal hypertension. The 3 major features are systolic dysfunction, diastolic dysfunction and a prolonged QT interval[93]. Cirrhotic cardiomyopathy is reversed by transplantation with cardiac function returning to normal in the 6 to 12 months after liver transplant [94].

1.4.13 Systemic Effects of Portal Hypertension

Splanchnic vasodilatation, mediated by increased nitric oxide, prostacyclin, glucagon, endocannabinoids and down regulation of the RhoA/Rho-kinase pathway results in a relative 'pooling' of blood in the splanchnic circulation[31]. In addition, angiogenesis and neovasculogenesis result in hypertrophy of existing vessels and the development of new portosystemic collaterals[95]. Magnetic resonance angiography shows that patients with ACLD have a 43% increase in flow through the descending aorta (a hyperdynamic circulation), a 30% increase in liver blood flow (mostly from an increase in supply from the hepatic artery) and a threefold increase in flow through the superior mesenteric artery

contributing to splanchnic pooling. The degree of 'splanchnic steel' is related to the severity of liver disease, and is increased with a MELD > 15[96].

This splanchnic pooling of blood results in a reduction in peripheral circulating blood volume. Patients with ACLD, awaiting transplantation, have been shown to have a hyperdynamic circulation as demonstrated by a high cardiac index (4.9 L/min/m^2), reduced peripheral vascular resistance (721 dyn/cm^5) and increased plasma renin levels[97 98].

In addition to the 'splanchnic steel' the hyperdynamic peripheral circulation is exacerbated by a number of other mechanisms. The vasodilators which cause splanchnic endothelial dysfunction are also detected in the systemic circulation and have similar effects on the peripheral circulation. In addition, products of bacterial translocation (endotoxins and TNFα) result in a peripheral inflammatory response, whose cytokines cause further vasodilatation.

Reduced peripheral volume and mean arterial pressure stimulate baroreceptor reflexes, which activate the sympathetic nervous system (SNS) and the renin-aldosterone-angiotensin system (RAAS) [99-102].

The RAAS results in increased sodium and water reabsorption, via activation of the sodium/hydrogen pump in the proximal tubule and an increased ADH secretion acting on the distal convoluted tubule. This, along with leakage of fluid into the peritoneum from the splanchnic capillaries, leads to the formation

of ascites, and eventually a dilutional intravascular hyponatraemia (independently associated with a poor prognosis) [103].

1.4.14 Development of Ascites

The formation of ascites is one of the most common and visible complications of cirrhosis and it develops in approximately 60% of patients with cirrhosis within 10 years of diagnosis[104]. The formation of ascites is due to sodium and water retention (discussed in section 1.4.13) and the leakage of fluid from the mesenteric capillaries, where portal hypertension increases the capillary hydrostatic pressure, into the peritoneum [105]. The presence of ascites is associated with a poor prognosis, approximately 40% one year and 50% two year mortality[106].

1.4.15 Impact of Portal Hypertension on Renal Function

Hepatorenal syndrome (HRS) is defined as the occurrence of renal failure in patients with ACLD in the absence of an identifiable cause. AKI is common in hospitalised patients with ACLD, occurring in over 25% of inpatients[107]. HRS is thought to be the aetiology of AKI in less than one quarter of ACLD inpatients with renal dysfunction, with approximately two thirds being related to sepsis, hypovolaemia and vasodilators[108]. The diagnostic criteria for the hepatorenal type acute kidney injury (HRS-AKI) are [109]:

- Diagnosis of cirrhosis and ascites
- Diagnosis of AKI according to ICA-AKI criteria
- No response after 2 consecutive days of diuretic withdrawal and plasma volume expansion with albumin 1 g/kg bodyweight

- Absence of shock
- No current or recent use of nephrotoxic drugs (NSAIDs, aminoglycosides, iodinated contrast media, etc)
- No macroscopic signs of structural kidney injury*, defined as:
    - absence of proteinuria (>500 mg/day)
    - absence of microhaematuria (>50 RBCs per high power field)
    - normal findings on renal ultrasonography

Hepatorenal syndrome is a functional (potentially reversible) renal impairment due to vasoconstriction of the renal arteries in response to the progressive circulatory dysfunction of portal hypertension[110], and MRA studies have shown that there is a 40% reduction in renal blood flow in ACLD[96]. The AKI can be exacerbated by an acute event, such as sepsis or bleeding, which 'stress' an already compromised circulation and in this situation the diagnostic criteria of the International Club of Ascites should be used (ICA-AKI criteria – see Figure 1.8)[109].

Table 2 International Club of Ascites (ICA-AKI) new definitions for the diagnosis and management of AKI in patients with cirrhosis

Subject	Definition		
Baseline sCr	A value of sCr obtained in the previous 3 months, when available, can be used as baseline sCr. In patients with more than one value within the previous 3 months, the value closest to the admission time to the hospital should be used In patients without a previous sCr value, the sCr on admission should be used as baseline		
Definition of AKI	Increase in sCr ≥0.3 mg/dL (≥26.5 μmol/L) within 48 h; ora percentage increase sCr ≥50% from baseline which is known, or presumed, to have occurred within the prior 7 days		
Staging of AKI	Stage 1: increase in sCr ≥0.3 mg/dL (26.5 μmol/L) or an increase in sCr ≥1.5-fold to twofold from baseline Stage 2: increase in sCr >two to threefold from baseline Stage 3: increase of sCr >threefold from baseline or sCr ≥4.0 mg/dL (353.6 μmol/L) with an acute increase ≥0.3 mg/dL (26.5 μmol/L) or initiation of renal replacement therapy		
Progression of AKI	Progression Progression of AKI to a higher stage and/or need for RRT		Regression Regression of AKI to a lower stage
Response to treatment	No response No regression of AKI	Partial response Regression of AKI stage with a reduction of sCr to ≥0.3 mg/dL (26.5 μmol/L) above the baseline value	Full response Return of sCr to a value within 0.3 mg/dL (26.5 μmol/L) of the baseline value

AKI, acute kidney injury; RRT, renal replacement therapy; sCr, serum creatinine.

Figure 1.8. International Club of Ascites definitions for the diagnosis of AKI in patients with cirrhosis. Reproduced from Angeli et al, with permission from Elsevier [CC BY NC ND][109].

Using the AKIN definitions patients admitted with dACLD had 25.6% mortality at 90 days without AKI, this climbed to 40% with AKI stage 1, and further increased to 67.3% with AKI stages 2 or 3. The AUROC to predict transplant-free mortality was 0.68 at 28 days and 0.62 at 90 days [111].

1.4.16 Impact of Portal Hypertension on Lung Function

Patients with portal hypertension can develop two specific liver related pulmonary complications, hepatopulmonary syndrome (HPS) and portopulmonary hypertension (POPH). POPH is present in 5-6% of patients with ACLD being assessed for LT and is diagnosed with a mean PAP > 20 mmHg (along with a PAOP of ≤ 15 mmHg and PVR > 2 Woods units) using Right Heart Catheter studies[112 113]. POPH is caused by an increase in endothelial derived mediations of vasoconstriction from the portal circulation, though interestingly the severity of POPH is not associated with the severity of liver disease or the severity of portal hypertension[114].

HPS consists of a triad of hypoxaemia (alveolar-arterial oxygen gradient), dilated pulmonary vasculature and ACLD. Imaging shows dilated pulmonary capillaries and less frequently arterio-venous shunts. The intrapulmonary shunting is usually confirmed with 'bubble-contrast' echocardiography, where a Right > Left shunt is observed between 3-6 cardiac cycles (a shunt demonstrated within 1-3 cardiac cycles is more suggestive of an intra-cardiac shunt). The pathophysiology is again related to the endothelial dysfunction of portal hypertension, with vasodilatation being mediated by pro-inflammatory

cytokines nitric oxide and the associated angiogenesis and neovasculogenesis playing a role in the development of the intra-pulmonary shunting[115 116].

In addition to these two vascular complications, fluid may accumulate in the pleural cavity, most commonly in the right pleural space. This is termed a hepatic hydrothorax, the aetiology is identical to ascites and it is managed in a similar way[117].

1.4.17 Impact of Portal Hypertension on Immune Function

The unique microcirculation of the liver with its fenestrated epithelial cells delivering blood from the portal circulation and the dual supply from the hepatic artery means the liver receives a large proportion of the cardiac output (>25%) [118]. This constant contact with circulating antigens and lymphocytes allows the liver to be an influential immune organ in health.

It is well established that infections are a leading cause of morbidity in ACLD and primary infections are most commonly from ascites and the urinary tract[119]. The immune paresis of patients with ACLD has been shown to impair both monocyte and neutrophil function and increases the risk of primary and secondary infections, putting them at a greater risk of multi-organ failure and death[77 120 121].

1.5 Assessment of Portal Hypertension

1.5.1 Hepatic Venous Pressure Gradient

Hepatic Venous Pressure Gradient (HVPG) is now established as the 'gold standard' investigation in the assessment of portal hypertension, is an accurate estimate of portal vein pressure[122], and has been shown to predict decompensation[123]. The HVPG is defined as the difference between the wedged hepatic vein pressure (WHVP) (which is a measurement of liver sinusoidal pressure and reflects portal pressure in cirrhosis) and the free hepatic vein pressure (FHVP). The HVPG must be assessed according to internationally recognised techniques to ensure valid results[10 124].

The HVPG is measured by catheterising the hepatic vein, usually via the internal jugular but occasionally via the femoral vein using an ultrasound guided seldinger technique and local anaesthesia before venepuncture. A balloon tip catheter is used to measure the wedged hepatic vein pressure (WHVP) whilst the balloon is inflated, and then the free pressure when the balloon is deflated (FHVP). Occlusion of the HV with the balloon is confirmed with the presence of a wedged trace on the monitor and using radiopaque contrast to demonstrate a 'wedge' without any passage of contrast into the IVC. An accurate assessment of HVPG required these criteria to be followed:

- The patient should be fasted for at least 6 hours.
- The patient should have no, or low dose sedation.
- A transducer should be 'zeroed' at the level of the axilla.
- The Free pressure should be measure close to the Inferior Vena Cava (2-3cm from the junction between the HV and the IVC).
- The wedged traced should be measured for at least 60s and the measurements checked in triplicate for consistency.

HVPG measurement is an invasive investigation[125], however, reported complications are rare and related either to catheter insertion, such as local haematoma or pain at the venepuncture site, vaso-vagal collapse, transient cardiac arrhythmia or Horner's syndrome[126 127]. In one large centre, there have been no reported incidences of mortality related to HVPG measurement in over 12000 procedures performed over 30 years[21].

In patients with cACLD HVPG has been associated with certain milestones in liver disease and can be used to predict prognosis (see table 1.3)[127-135].

HVPG	Clinical Outcome
5-9	**Sub-Clinical Portal Hypertension**
10	**Clinically Significant Portal Hypertension** Presence and development of varices
	Development of Ascites
	Increased risk of HCC development
	Decompensation within 3 months of resection for HCC
12	Variceal rupture/bleeding
16	Increased risk of mortality in ACLD
16	Increased short-term risk after non-hepatic abdominal surgery
20	Failure to control bleeding following variceal haemorrhage
22	Mortality in patient with alcoholic hepatitis

Table 1.3. Threshold levels of HVPG associated with clinical endpoints. Adapted from [10 21 136].

Whilst HVPG is established as the gold standard test for portal hypertension, and its role in prognostication is demonstrated, it is still not universally available or applied to patients with ALCD. This is predominantly related to the cost and availability of the test[137].

1.5.2 Endoscopy

Endoscopy is currently considered the gold standard test to detect oesophageal or gastric varices, and hence by implication the presence of portal hypertension. UK guidelines currently recommended that all patients with ACLD undergo surveillance endoscopy at diagnosis to assess for varices[70]. There is a suggestion by the international Baveno consensus workshop that patients with a liver stiffness (measured by transient elastography) of < 20 kPa and a platelet count of > 150 x10^9/L do not require endoscopy due to the low risk of varices[138]. Screening with endoscopy offers added benefits, as it can stage the size of varices, and assess for PHG and GAVE. Endoscopy is generally well tolerated, but there are a small number of significant associated complications (most importantly GI haemorrhage and perforation) which must be considered[139].

1.5.3 Collage Proportionate Area

Collage Proportionate Area is an estimate of the amount of collagen in the liver using digital image analysis of a Sirius red stained section of liver from a biopsy sample. It has been shown to correlate with clinical decompensation and with

HVPG. A CPA cut-off of between 18 - 25.5% has been shown to predict a clinical decompensating event[140-142].

1.5.4 Direct Portal Pressure Measurement

Direct Portal Pressure Measurement by percutaneous cannulation of the umbilical vein, percutaneous or EUS guided transhepatic approach to the portal vein have been described and are still occasionally used to access the portal vein (for example to treat ectopic varices by embolisation)[143 144]. The normal portal pressure should be between 7 and 12 mmHg, but due to the invasive nature of this test and the risk of intra-abdominal bleeding and sepsis it is not recommended as a routine investigation.

1.5.5 Splenic Pulp Pressure Measurement

Splenic Pulp Pressure Measurement involves placing a needle in the splenic pulp and measuring the pressure via a transducer. It has been shown to correlate with portal pressure and again can be used as an estimate of portal vein pressure[145]. The potential risks of splenic puncture, bleeding and infection, again limit the everyday use of this investigation.

1.5.6 Transient Elastography

Transient Elastography (TE) uses a 1-dimentional ultrasound technology to record the velocity of propagation of a sheer wave through the liver and estimate the stiffness, measured in kilopascals (kPa)[146]. TE it is now used routinely in clinical practice to estimate the stage of liver fibrosis, and is well validated for the use in viral hepatitis. It has more recently been evaluated for

its ability to estimate portal pressure and it performs well in cACLD (again being best validated in viral hepatitis) with an AUROC of 0.93, using the cut-off of 13.6kPa it has a 90% sensitivity to rule-out CSPH[147]. The cut-offs for non-viral liver diseases are higher in reported cases and further evaluation in these aetiologies is still required[148]. It must be remembered that the normal caveats to using TE apply (in that the patient must have fasted for 2 hours, the IQR must be < 30%, the serum ALT < 5x ULN, the correct probe for body mass index used, extra-hepatic cholestasis excluded, right heart failure must be absent and there must be no recent alcohol use disorder) [149]. In a mixed population of patients with mainly cryptogenic and alcohol related chronic liver disease a TE cut-off of 21.6 kPa was able to detect CSPH with a sensitivity of 79% and specificity of 67% (AUROC 0.740 [95% CI: 0.662 – 0.818])[150]. At higher levels of HVPG (> 12 mmHg) the correlation between TE and HVPG is lost and it cannot be used to accurately estimate the HVPG, it is presumed that this is related to the influence of the 'dynamic' component of endothelial dysfunction on portal pressure in advanced portal hypertension[151] [152]. This finding is confirmed by a group who identified that LS and LSPS were only able to accurately predict CSPH in compensated patients with alcohol related ACLD, and in dACLD the MELD score was a better prognostic predictor[153].

There is an ongoing international debate on whether TE can be safely used to rule out oesophageal varices. One recent systematic review demonstrates a specificity ranging from 43-78%, with the liver stiffness cut-offs being higher in non-viral related aetiologies[148]. Most recent BAVENO guidelines suggest that a LSM < 20 kPa combined with a platelet count > 150 x10^9/L can be used to

avoid endoscopy in patients with cACLD. They also suggest that patients with a LSM of > 25 kPa or the combination of a LSM > 20 kPa with a platelet count <150 x10^9/L are likely to have CSPH, may need endoscopy, and should have prophylaxis to prevent variceal bleeding[10].

TE cannot produce a valid measurement of stiffness every patient, for technical reasons most commonly related to body habitus, operator experience or the presence of ascites[154]. The failure rate in one study of 992 patients, using two operators, was 3.2% and the proportion of unreliable scans was 3.9%[155].

The combination of elastography, spleen size and platelet count have been combined in one study as the portal hypertension risk score:

> PH risk score = -5.953 + (0.188 x LS) + (1.583 x sex (1:male; 0: female)) + (26.705 X spleen diameter/platelet count ratio)
>
> The spleen diameter / platelet count ratio was calculated as the ratio of the bipolar diameter of the spleen in millimetres and platelet number/mm.

This score was able to detect clinically significant portal hypertension with an AUROC of 0.935; 95% CI, 0.893–0.977; P <.0001[156]. This model is supported by further work which shows that most patients can be reassured by a low liver stiffness to spleen/platelet score[157 158].

Current guidelines from the European Association for the Study of the Liver suggest the following LSM measurements can be used in assessing chronic liver disease and portal hypertension (see Table 1.4) [159]:

LSM < 8- 10 kPa	**Can be used to rule out cACLD**
LSM > 12-15 kPa	Can be used to rule in cACLD
LSM > 20-25 kPa	Can be used to rule in CSPH

Table 1.4. EASL Liver Stiffness Measurement recommendations for the assessment chronic liver disease and portal hypertension.

1.5.7 Spleen Stiffness

Splenic stiffness (SS) assessed by transient elastography has also been reported as correlating with HVPG and the presence of varices[160 161]. In one study a specificity of 90% was reported to detect Oesophageal varices if a liver and spleen stiffness measurement was combined (using cut-offs of 27.3 kPa and 40.8 kPa)[162].

A cutoff of <21 kPa or >50 kPa can be used in patients with viral hepatitis to rule out, or rule in CSPH respectively[10].

1.5.8 Acoustic Radiation Force Impulse (ARFI) elastography

ARFI measures elasticity of liver tissue, in areas selected by the sonographer, using short duration acoustic pulses. It has been shown to be a reliable predictor of cirrhosis, performing at least as well as transient elastograghy using Fibroscan, mainly in patients with viral hepatitis[163-165]. ARFI has also been shown to be useful in detecting CSPH and both liver and splenic stiffness can

detect an HVPG > 10mmHg with an AUROC of 0.93 and 0.97 respectively[166]. In the same study of 78 patients with chronic liver disease of mixed aetiologies, the investigators reported a sensitivity of 97% and specificity of 89% for liver stiffness to predict CSPH using an ARFI cut-off of 2.17 m/s. Using a cut-off of 2.32 m/s the sensitivity and specificity of splenic stiffness to detect CSPH were 96% and 89%.

1.5.9 Ultrasound and Doppler

Both portal vein flow velocity (reduced in cirrhosis) and spleen size (increased in cirrhosis) have been evaluated as potential tools for the assessment of portal pressure.[167-169] However, due to significant variability in portal vein velocity one study of 39 participants showed no significant correlation between that and HVPG[170].

1.5.10 Magnetic Resonance Elastography (MRE)

MRE uses similar technology to TE, detecting shear wave velocity through the liver. Proponents suggest that it has less inter-observer variability and a greater proportion of the liver can be examined at the same investigation.

Pooled results suggest that MRE can accurately detect advanced fibrosis with an AUROC of 0.93 using a cut-off of 4.11 kPa[171]. It has been assessed as a potential method of detecting portal hypertension in animal models and in small series in humans, but is not yet validated for routine clinical use[172-175]. One of the largest series in humans included 30 patients, of which 14 had CSPH. This

showed that liver T_1 longitudinal relaxation time, SMA and SA arterial flow correlated well with HVPG[176].

1.5.11 Serum Markers predicting Portal Hypertension

The search for a non-invasive test for raised HVPG has led to the assessment of many serum markers. Whilst several potentially useful serum tests have been identified, none of these have been validated. A model including albumin, INR and ALT developed by Berzigotti et al. was able to predict the presence of CSPH with an AUROC of 0.952[177].

Von Willebrand Factor (vWF), a marker of endothelial function, and soluble CD163, a marker of Kuppfer cell activation, have been associated with clinically significant portal hypertension with AUROCs of 0.884 and 0.834 respectively and an sCD163 level of > 3.95 mg/L can predict CSPH with a positive predictive value of 0.99[178 179]. In addition, a vWF level greater than 216 u/dL predicts liver related events or the need for liver transplantation[180]. Although vWF has been shown to be able to predict complications of CSPH, it does not correlate well enough with HVPG to be used as a linear non-invasive biomarker, Spearman's $r = 0.687$; $p<0.001$)[181].

Other markers of the inflammatory cascade were measured in patients with portal hypertension, recruited to a trial of pre-primary prophylaxis with non-selective beta-blockers. Transforming growth factor beta (TGF-β) and heat shock protein-70 (HSP-70) were included in a clinical algorithm where an HVPG > 12mmHg could be excluded with a sensitivity of 87%[182].

Asymmetric dimethylarginine (ADMA) is an enzyme which can inhibit eNOS, thereby reducing NO synthesis and increasing sympathetic tone. ADMA is metabolised by the liver and an increase in ADMA levels has been described in cirrhosis[183]. ADMA levels have also been showed to correlate with HVPG (correlation coefficient 0.77, P< 0.001)[184].

The indocyanine green retention test uses a fluorescent dye which is excreted, unaltered, into bile and clearance from plasma is dependent on hepatic blood flow, hepatocyte function and bile formation. It has been shown to be a potential marker of CSPH in patients with cirrhosis with AUROCs of 0.7932 – 0.832, dependent on the severity of liver disease[185].

Serum markers of fibrosis have also been suggested as potential predictors of portal hypertension and the combination of hyaluronic acid (HA), amino-terminal propeptide of type III procollagen (P3NP) and tissue inhibitors of matrix metalloproteinases type-1 (TIMP-1) has been shown to predict the presence of clinically significant portal hypertension 12 months following liver transplant in patients with Hepatitis C with an AUROC of 0.93[186].

The ELF test (the combination of hyaluronic acid (HA), amino-terminal propeptide of type III procollagen (P3NP) and tissue inhibitors of matrix metalloproteinases type-1 (TIMP-1)) has been combined with sCD163 in one study of two cohorts each of 80 participants. It was able to identify CSPH with an AUROC of 0.9 and using a score cut-off of 1.4 to rule out CSPH there was

an NPV of 0.94 and a PPV of 0.89[187]. Other investigators have shown that it may be possible to use ELF, using a threshold of < 10.1, to rule out high risk portal hypertension (HVPG > 20mmHg)[188].

1.6 Therapies for Portal Hypertension

The treatment of portal hypertension can have significant benefits, if the HVPG is reduced by more than 20% of baseline (or to values below 12mm Hg), the risk of portal hypertensive complications fall significantly and survival is improved[189]. Since these criteria require at least 2 separate invasive HVPG measurements, a surrogate has been developed, by assessing the acute response to intravenous Propranolol, where a reduction in HVPG of > 10% is associated with a longer term haemodynamic response[190 191]. Longer term follow up data has shown that patients who had a ≥ 10% reduction in HVPG in response to propranolol had a lower risk of variceal bleeding (3.6% v 15%) and a lower risk of non-bleeding decompensating events (23% v 33%) after 12 months of surveillance[192].

Therapies specifically for portal hypertension, as opposed to those directed at the underlying aetiology of liver diseases have traditionally been limited to treatments for the common complications such as variceal bleeding, encephalopathy and ascites. The development of anti-fibrotics and anti-angiogenesis agents may allow intervention at an earlier stage to prevent the progression of portal hypertension[193]. In addition, newer agents targeting endothelial dysfunction have shown promising early results.

Standard Medical Therapies for Portal Hypertension

1.6.1 Beta-blockers

The mainstay of non-disease specific treatment for portal hypertension has been non-selective beta-blockers (see figure 1.9).[194] The haemodynamic effects of beta-blockade are more pronounced in patients with clinically significant portal hypertension and the reduction in HVPG is influenced by an increase in free hepatic venous pressure (indicating reduced venous compliance) and a more modest (but still significant) reduction in wedged hepatic venous pressure.[98] In experimental studies a >10% reduction in HVPG can be achieved in 69% of participants and a >20% reduction in HVPG in 40% of participants with CSPH when exposed to Propranolol.[98] In primary prophylaxis of variceal haemorrhage they reduce the risk of bleeding and mortality by 11% and 9% respectively[195 196]. This effect may be improved with Carvedilol (a non-selective beta-blocker with some alpha-blocker effects), which is associated with a higher rate of haemodynamic response (19 v 11%, $p<0.001$) [197 198]. A large international study did not find any benefit for beta-blockade in preventing the formation of varices in early portal hypertension[69], but they may reduce the progression from small to large varices[199].

In the primary prevention of variceal bleeding non-selective beta-blockers reduced bleeding by 11% overall and by 16% (from 30% to 14%) in patients with medium to large varices over a 2 year period[200]. In patients who have already had a variceal haemorrhage the same study showed that the efficacy

of beta-blockers in secondary prophylaxis (in combination with endoscopic variceal ligation) is increased with a reduction in re-bleeding of 21%.

The HVPG guided use of propranolol or carvedilol in patients with CSPH as primary prophylaxis has been shown to reduce the incidence of a combined endpoint which included decompensation (ascites, variceal bleeding, encephalopathy) or death[201]. This trial (the PREDESCI study) suggests that beat blockers may be beneficial as primary prophylaxis of all causes of decompensation, not just variceal haemorrhage.

Beta-blockers have also been shown to reduce the rate of bacterial translocation, potentially reducing the risks of infection in patients with ACLD[202], and prevent episodes of spontaneous bacterial peritonitis[203 204].

Some studies have suggested that the addition of vasodilating agents such as Isosorbide mononitrate or prazosin to therapy with non-selective beta-blockers may add additional benefit to the reduction in portal pressure[205-208]. However, the evidence is conflicting, and their routine addition is not recommended in national guidelines[70].

Though there has been some controversy about the safety of beta-blockers in recent years, they remain the most effective pharmacological treatment currently available to reduce portal pressure. One important study has confirmed that long-term beta-blocker use did not alter short-term mortality following a variceal bleed, and this adds weight to the observation that they do

not increase mortality in dACLD[209 210]. Further data suggesting that beta-blockers can be stopped for short periods without risking acute bleeding episodes (for example for during an acute admission, or for a DSE) are reassuring[211]. Some expert opinion advises that in dACLD consideration should be made for switching back to low doses of Propranolol from Carvedilol, as Carvedilol has a more pronounced effect of lowering systemic arterial pressure which may be detrimental at this stage of the disease[212]. At earlier stages of portal hypertension Carvedilol may be a superior choice of beta-blocker due to the additional benefits of reduced intra-hepatic vascular resistance (due to alpha-1 adrenoceptor antagonist activity within the liver), and potential anti-fibrotic and anti-oxidant effects[213].

Unfortunately, beta-blockers are contraindicated in 15-20% of patients and poorly tolerated in up to 18% of patients[69], which limits their use in a significant number of patients.

HVPG can be used acutely to assess response to treatment for portal hypertension with propranolol and a reduction in HVPG to < 12mmHg or a 20% reduction from the baseline values have been associated with a significant reduction in bleeding, both in primary and secondary prophylaxis[189 214-217]. In a more recent study 58.2% of patients tested showed an acute haemodynamic response to propranolol. Patients were then follow-ed up for 24 months using either propranolol or carvedilol as primary prophylaxis and being an acute responder was associated with a 3.6% variceal bleeding risk during follow-up, in comparison to a 14.9% risk in the group who were initial non-haemodynamic

responders[192]. The obvious clinical advantage in knowing whether a patient is a responder or a non-responder to first line beta-blockers is that non-responders can be offered alternative therapies, such as further titration of beta-blockers, switching to carvedilol, band ligation or portosystemic shunt or entered into clinical trials.

It has been suggested that HVPG should be used routinely to assess response to beta-blockers in the secondary prophylaxis of variceal bleeding. In this scenario 22-67% (median 47%) of non-responders to secondary prophylaxis will re-bleed, which can be compared to a re-bleeding rate without secondary prophylaxis of 63% at 2 years[218 219]. A publication of updated outcomes has shown that an 'à la carte' approach using HVPG guided therapy and the addition of nitrates and/or alpha-blockers could achieve a greater overall reduction in HVPG and a reduced mortality in comparison to a control group who did not have HVPG guided therapy and were treated with nadolol, nitrates and band ligation of varices (29% vs. 43%; hazard ratio = 0.59; 95% confidence interval = 0.35-0.99)[220]. In the same study the overall rebleeding rate was 19% in the HVPG guided treatment group compared to 31% in the control group; HR 0.53; 95% CI 0.29 – 0.98; p=0.04).

One group has evaluated metabolomics data and identified 2 lipid metabolites which can predict the HVPG response to beta-blocker with an AUROC of 0.872, CI 0.754 – 0.989[221].

As described above in section 1.5.1 HVPG requires specialist equipment, training and a standardised approach to measurement. Novel less invasive biomarkers may allow us to predict the individual patient response more easily to therapies without invasive testing.

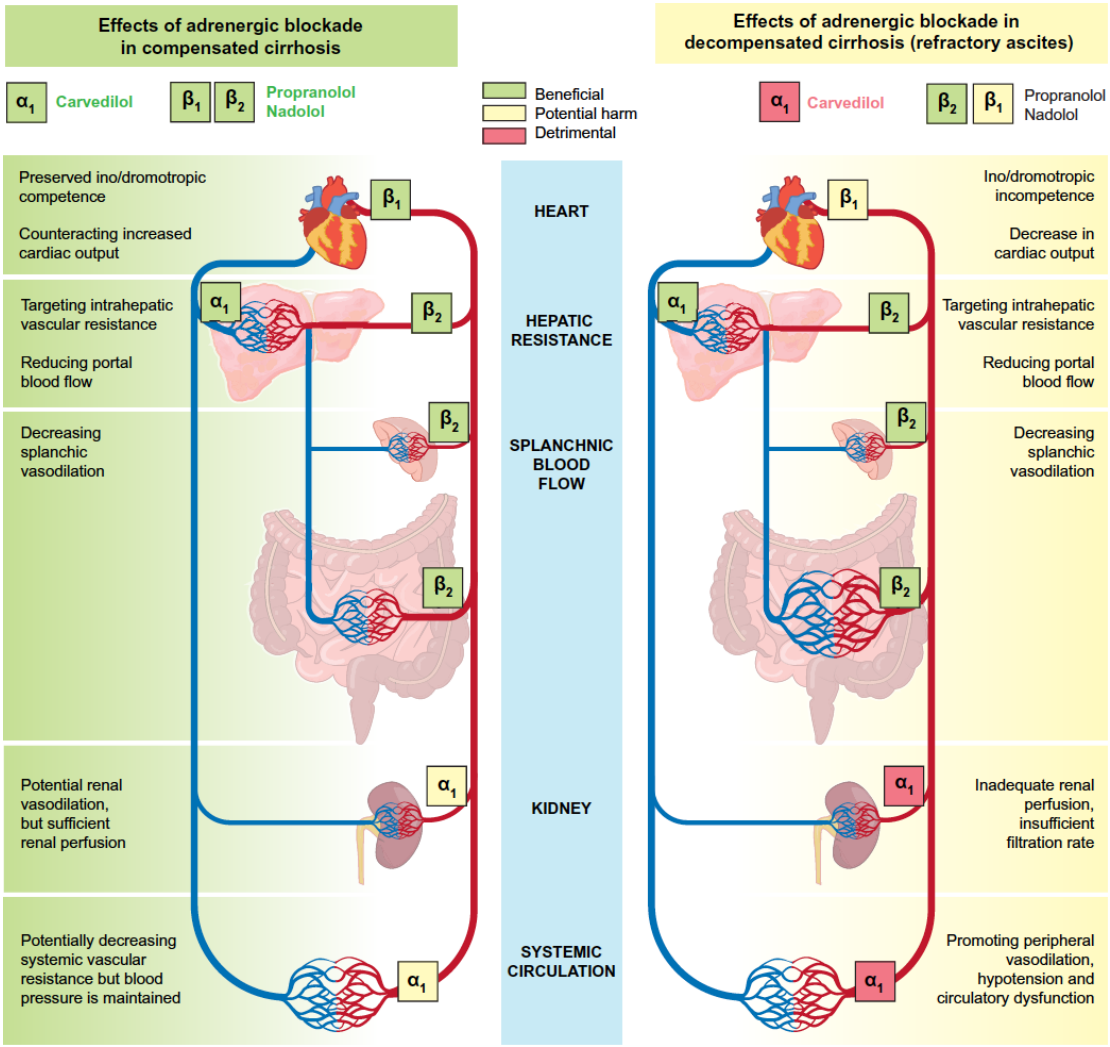

Figure 1.9. The effects of adrenergic blockade in compensated and decompensated cirrhosis taken from Reiberger et al, with the kind permission of Elsevier [License No. 5487131028082][212].

1.6.2 Vasopressin Analogues

Terlipressin (triglycyllysine vasopressin) is a long-acting analogue of vasopressin acting on both Vasopressin-1 (V1), vascular smooth muscle, and Vasopresssin-2 (V2) receptors in the distal convoluted tubule. It has a greater affinity for V1 receptors. V1 receptors are located on systemic, coronary, renal and splanchnic circulations, in addition they are also found on platelets and myometrium. Activation of V1 receptors with Vasopressin or an analogue causes vasoconstriction. V2 receptors are found in the distal collecting tubule and agonism leads to increased water reabsorption[222].

Terlipressin is used in the management of variceal haemorrhage and hepatorenal syndrome where the splanchnic vasoconstriction reduces flow in the varices and improves both systemic mean arterial pressure and renal perfusion pressure[223-225]. Small studies have shown that Terlipressin is equivalent to Noradrenaline in the treatment of HRS[226 227]. In addition, it has recently been shown that an intravenous infusion of Terlipressin provides effective therapy at a lower does, with reduced adverse events when compared to bolus doses[228]. Significant adverse events such as peripheral and cardiac ischaemia can occur in up to 3% of patients when Terlipressin is used at high doses for variceal haemorrhage[229].

1.6.3 Somatostatin and Somatostatin Analogues

Somatostatin and Octreotide (its long-acting synthetic analogue) may be used as an alternative splanchnic vasoconstrictor. Somatostatin is a peptide hormone which reduces blood flow in the mesenteric circulation[230]. A recent

comparison of Terlipressin, Octreotide and Somatostatin suggests that their efficacy in the initial management of variceal bleeding was equivalent, and this could be used where Terlipressin is not available or contra-indicated[231].

1.6.4 Midodrine

Midodrine is an agonist of α_1-adrenoceptors resulting in systemic arteriolar vasoconstriction which is used to treat both dialysis induced hypotension and orthostatic hypotension[232]. Midodrine has a beneficial effect on the systemic haemodynamics of patients with cirrhosis and may be useful in the treatment of post-paracentesis circulatory dysfunction[233-235]. It has also demonstrated effectiveness in the reversal of HRS when used in combination with octreotide, leading to improved GFR and reduction in serum creatinine[236 237]. Midodrine may be effective in preventing post-paracentesis circulatory dysfunction related AKI[238].

1.6.5 Management of Ascites

The pathogenesis of ascites is described in section 1.4.14. It should be managed according to international guidelines with a no-added salt diet, loop diuretics and aldosterone antagonists[106]. Ascites becomes 'diuretic refractory' when it cannot be adequately controlled, either secondary to complications of diuretic therapy 'diuretic-intractable' or poor response to maximal tolerated doses of diuretics 'diuretic resistant'[239].

The initial therapy for refractory ascites is Large Volume Paracentesis (LVP) which involves percutaneous insertion of an intra-peritoneal drain and removal

of ascites. The principal immediate risks associated with this procedure are infection, trauma and visceral perforation, but fortunately risks of serious complications are low[240].

Paracentesis is associated with haemodynamic changes, a condition termed post-paracentesis circulatory dysfunction (PPCD). This is associated with a more rapid re-accumulation of ascites, AKI and an acute rise in portal pressure[241-243]. The risks of PPCD can be reduced by infusing albumin, as a plasma volume expander, to patients having greater than 5L paracentesis and by removing the fluid in a single procedure[244-246]. In patients at a very high risk of PPCD, such as those with established kidney injury, or active sepsis paracentesis should be ideally avoided or if unavoidable limited to less than 5L[247].

As LVP is solely a symptom control strategy patients should also be considered for alternative therapies such as TIPS or Liver Transplantation[106 248 249].

1.6.6 Endoscopic Therapy

Endoscopy currently has a role in both the diagnosis and assessment of portal hypertension and in the prevention and management of bleeding related complications.

1.6.6.1 Endoscopy for diagnosis of portal hypertension

Current national guidelines propose that all patients with ACLD should have an endoscopy at the time of diagnosis, and then further surveillance endoscopies at 1-3 years intervals to assess for the presence of oesophageal varices[70]. The international consensus conference on the management of portal hypertension (the BAVENO conference) has suggested that patients with a platelet count of > 150 plateletsx10^9/L and a liver elastography result of < 20kPa could avoid a screening endoscopy[138 250].

The risk of variceal bleeding is governed by the size of the varix (increased with medium and large varices), increased tension in the variceal wall (indicated by the presence of red spots or red whale markings), the portal pressure (risk increased with HVPG $\geq$ 12) and the severity of liver disease[70].

1.6.6.2 Endoscopic therapy for varices

Variceal band ligation (VBL) is the main endoscopic therapy for oesophageal varices. In comparison with beta-blocker therapy for primary prophylaxis there is a reduction in bleeding events, but no significant difference in mortality at 1 year[251]. The risks of fatal post-banding ulcer bleeding are reported in a meta-analysis as 4/146 (2.7%), however only 3/19 RCTs reported this complication and these studies included 'aggressive' banding programmes with repeat sessions every 1-2 weeks[252].

Balloon Tamponade can be offered for refractory variceal bleeding as a bridge to further endoscopic therapy or portosystemic shunting. This device is

effective at controlling haemorrhage in up to 90% of patients[253]. SEMS is an alternative to balloon tamponade, which can be left in situ for up to 14 days, whilst the patient recovers from the acute episode[254 255].

Injection of gastric varices with tissue adhesive glue, usually N-butyl-cyanoacrylate) has been shown to be superior to VBL in achieving haemostasis (87% v 45%) and has been shown to reduce re-bleeding rates (31% v 54%)[256]. These results are support by further studies[70]. TIPS was found to be superior to glue injection in one study for the secondary prevention of bleeding gastric varices[257].

Balloon-occluded retrograde transvenous obliteration of varices involved catheterising an outflow shunt (gastro-renal or gastric-inferior vena caval) and embolising feeding varices[258]. This technique may be a potential option in patients unsuitable for shunts.

1.6.7 Portosystemic Shunts

Transjugular intrahepatic portosystemic shunts (TIPS) or surgical portosystemic shunts (see figure 1.10) are used to decompress the portal circulation, bypassing the liver and diverting venous flow to the inferior vena cava. Their primary indications are in refractory ascites and variceal bleeding, with surgical shunts being comparable to TIPS in patients with relatively well compensated liver disease (MELD < 14)[259]. Although TIPS have been used for many years, their efficacy has increased significantly since the use of PTFE-covered stents became routine[260].

TIPS should be considered in high risk patients (Child-Pugh B or C) who have active bleeding at endoscopy as there is evidence of reduced mortality[261]. However, in patients with poor synthetic function the benefits must be weighed against the risks, and in those with a MELD > 24 the risks must be considered to be high[262]. An individualised decision is required about the use of TIPS in secondary prophylaxis. The mortality rates for 'salvage' TIPS in variceal haemorrhage are high at around 50%, and data suggest that outcomes with TIPS may be better than secondary prophylaxis with EVL and NSBB in patients with a high HVPG[263 264]. A more recent metanalysis of preventative TIPS in patients at high risk of rebleeding showed that even those who were thought to be traditionally high risk, with a bilirubin >171 umol/L, had an improved 1 year survival when compared to standard secondary prophylaxis of bleeding with beta-blockers and variceal band ligation[265].

When a TIPS is formed for variceal bleeding, traditionally a 10mm covered stent was used, but recent data suggest that the use of an 8mm stent may be as effective in controlling bleeding, but reduce the occurrence of post-shunt encephalopathy[266].

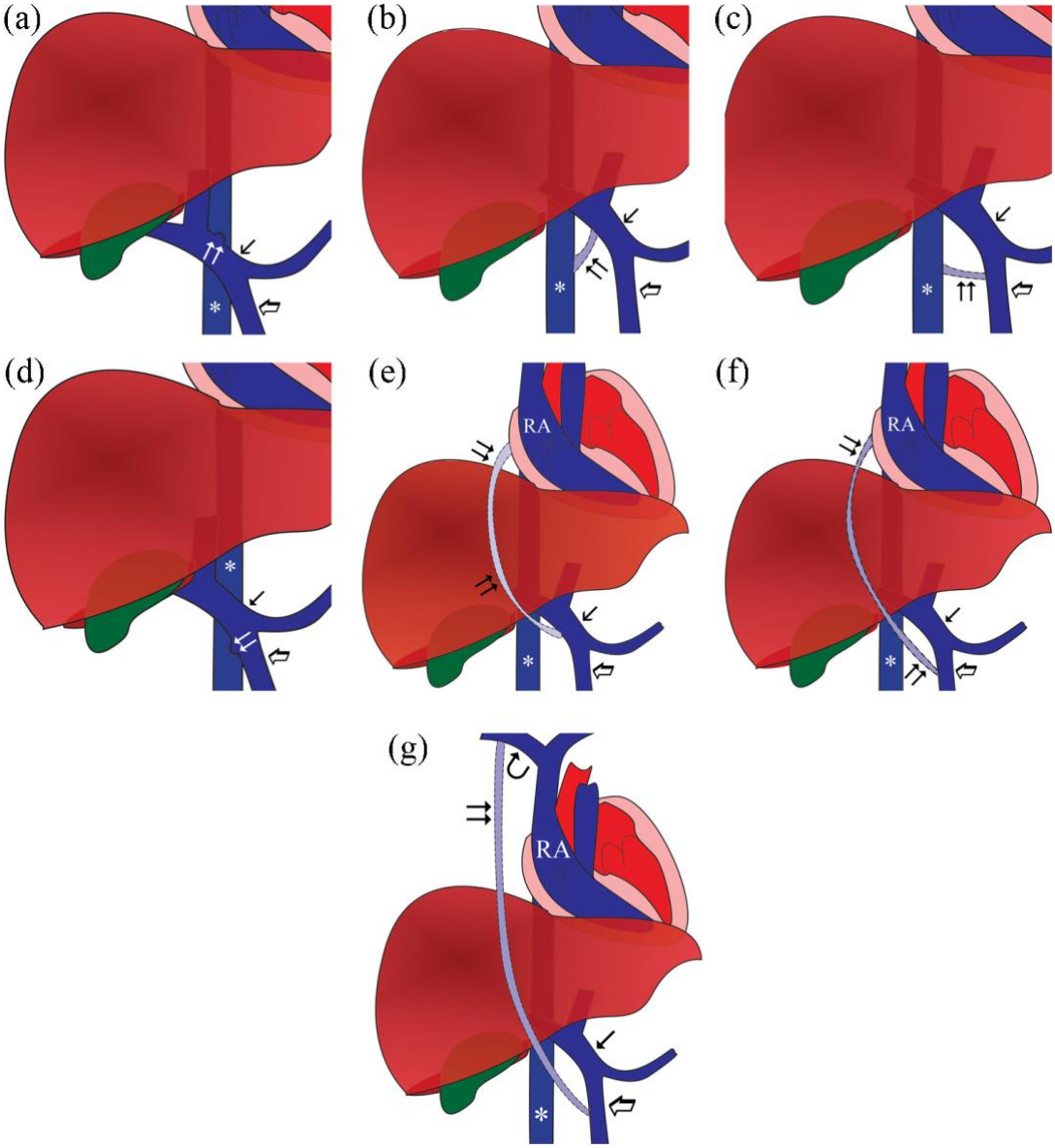

Figure 1.10. Schematics showing types of non-selective portosystemic shunts: [a] Side-to-side portocaval shunt. [b] H-type interposition portocaval graft. [c] H-type interposition mesocaval graft. [d] Side-to-side mesocaval shunt. [e] Portoatrial shunt. [f] Mesoatrial shunt. [g] Mesoinnominate shunt [*IVC=asterisk, superior mesenteric vein=open arrow, portal vein=arrow, right atrium=RA, right innominate vein=curved arrow, graft/anastomosis=double arrow*]. Reproduced from Taslikian et al, with kind permission from Elsevier [License Number 5487221482954][267].

Experimental Medical Therapies

1.6.8 Intensive Lifestyle Programmes

Obesity may increase portal pressure and obese patients with ACLD are 3 times more likely to develop decompensation[268]. A multicentre Spanish study investigated the effects of a 16 week programme of caloric restriction (a reduction of 500-1000 kCal/day) alongside supervised physical activity in a cohort of patient with cirrhosis from mixed aetiologies (NAFLD 24%). After 16 weeks there was an average 5.2% reduction in body weight and a 10.7% reduction in HVPG[269].

1.6.9 Biguanides & Glitazones

Tripathi and colleagues showed that in a rat model of portal hypertension (bile duct ligation) treatment with Metformin reduced liver fibrosis, stellate cell activation and inflammation which all contributed to a reduced hepatic vascular resistance. Animals treated with Metformin showed a significantly reduced portal pressure (15% reduction)[270].

Recently Pioglitazone has been shown to reduce portosystemic shunting by 22-30% and reduce endothelial cell migration in a bile duct ligation model of portal hypertension, suggesting that it may play a role in reducing angiogenesis. Interestingly there was no reduction in portal pressure or splanchnic blood flow in this study[271].

1.6.10 HMG-CoA reductase inhibitors

HMG-CoA reductase inhibitors have been shown to enhance eNOS activity in ECs and hence stimulate production of NO, in one trial of 59 patients Simvastatin reduced HVPG by 8% (and by 11% when used in combination with β-blockers)[272]. A recent clinical trial of a combination of Simvastatin and standard medical therapy reduced mortality rates after variceal bleeding, predominantly due to a reduction in deaths from infection[273]. Further investigation into the clinical role of statins in portal hypertension is required.

1.6.11 FXR-antagonists

The farnesoid X receptor (FXR) is involved in a number of liver and gut pathways including bile acid metabolism, fibrogenesis and gut immunity and has been implicated in a number of gut an liver pathologies, most notably non-alcohol related fatty liver disease and cholangiopathies[274]. It has been shown that FXR agonists can reduce the severity of steatohepatitis in NAFLD, fibrosis progression and may be able to reduce the incidence of SBP[275 276].

FXR-antagonists, such as obetocholic acid, have also been shown to increase dimethylarginine dimethylaminohydrolase 1 **(**DDAH-1), a gene which plays a role in the generation of nitric oxide, and improve portal haemodynamics in animal models[277 278]. More recently the mechanism of FXR agonists actions on endothelial function have been described, where an experimental FXR agonist PX20606 reduced portal pressure via up regulation of eNOS, cystathionase and DDAH-1 and down regulation of endothelin-1 and p-moesin. In the same study

there was a reduction in IL-6, TNF-a, LPS-binding protein and bacterial growth in FXR agonist treated animals and a reduction in VEGF, ANG1 and ANG2 expression[279].

1.6.12 Anti-coagulant therapy

There has been evidence for some time that anticoagulants may be effective agents to modulate the progression of liver fibrosis. Early results of an RCT suggested a beneficial effect of Warfarin at reducing liver fibrosis in patients who had received a liver transplant for hepatitis C[280]. It is known that thrombin activates PAR receptors which are found on both HSCs and ECs and are implicated in the fibrotic pathway[281]. The direct thrombin inhibitor Rivaroxaban has been shown to reduce portal pressure in animal models of portal hypertension[282].

1.6.13 Selective Vasopressin (V_{1a}) Receptor Agonists

A selective partial agonist of the V_{1a} receptor (FE 204038) causes vasoconstriction of the splanchnic circulation, reducing portal pressure and improving renal perfusion. It is not active on the V_2 receptors which are responsible for water retention in the distal collecting tubule. In animal models this experimental drug has been able to reduce portal pressure and ascites without compromising serum sodium or causing significant increases in mean arterial pressure or systemic vascular resistance[283].

1.6.14 Selective Vasopressin (V_2) Receptor Antagonists

This class of drug, also known as 'Vaptans' are orally administered V_2 receptor antagonists, and act at the distal collecting tubule. They prevent reabsorption of free water, with the clinical benefits in cirrhosis of reducing ascites formation and treating hyponatraemia. Though physiologically they offer potential benefit, they are not in clinical use due to concerns over their association both with abnormal liver function tests and increased mortality in one study, and no demonstrable benefit in mortality[284-286].

1.6.15 Anti-microbial Therapy

It has been proposed that anti-biotic therapy (Rifaximin / Norfloxacin) may reduce the bacterial translocation associated TLR-4 activation of HSCs, and therefore modulate intra-hepatic vascular tone[287]. To support this theory there is evidence that Rifaximin use is associated with better survival in patient with portal hypertension and encephalopathy[288]. A study of short-term use of Rifaximin (over 4 weeks) did not have any effect on portal pressure in patients with cirrhosis and ascites, predominantly because of continued alcohol misuse by the participants[289]. Later studies, however, did show a reduction in HVPG with microbiome targeted therapies[79]. In addition, these investigators reported only minor effects on bacterial translocation and the activity of pro-inflammatory cytokines[290]. The use of Rifaximin has been associated with a reduction in the number of admissions, and re-admissions, to hospital[291].

1.6.16 Anti-oxidant Therapy

Anti-oxidant therapy with vitamin C, chocolate and superoxide dismutase's may improve portal haemodynamics by improving the availability of intrahepatic NO[49 292 293].

1.6.17 COX inhibitors

Inhibition of COX-1 reduces the formation of thromboxane and can lower portal pressure in animal models of cirrhosis[60].

1.7 Nodular Regenerative Hyperplasia

Nodular regenerative hyperplasia (NRH) is characterised by the formation of small nodules in the liver <3mm), in the absence of significant fibrosis. The nodules are composed of two cell layers of hypertrophied hepatocytes with the majority of cases being initially asymptomatic[294]. Clinically, NRH can be associated with portal hypertension leading to the complications of varices, bleeding, ascites and encephalopathy.

The cause of NRH is still incompletely understood but may be related to vascular anomalies, causing localised tissue hypoperfusion and impaired endothelial cell function at the level of the small hepatic veins, resulting in an obliterative venopathy[295]. It is associated with the use of immunosuppressant and anti-cancer drugs, auto-immune and inflammatory conditions, HIV and prothrombotic phenotypes (especially myeloproliferative disease)[296].

HVPG is not an accurate reflection of portal pressure in NRH. The absence of sinusoidal capillarisation (as in cirrhosis) means that the wedged HVP is not reliably equivalent to the portal vein pressure. Haemodynamic studies have shown that the HVPG was a mean of 8.7mmHg lower than the portal venous pressure gradient (PVPG)[297].

TIPS is a useful therapy for NRH and is often well tolerated due to the preserved synthetic liver function[298].

1.8 Vasculogenesis, Angiogenesis and Neovasculogenesis

The term 'Vasculogenesis' describes the embryonic development of new vessels from the vascular plexus (see Figure 1.11). Angiogenesis (both sprouting and non-sprouting) describe the 'budding' or development of new vessels from existing systems. In neovasculogenesis, there is development of new vessels from endothelial precursors cells that migrate to sites of tissue injury and hypoxia. Both neovasculogenesis and angiogenesis can occur in adults in response to hypoxia and inflammation[299 300].

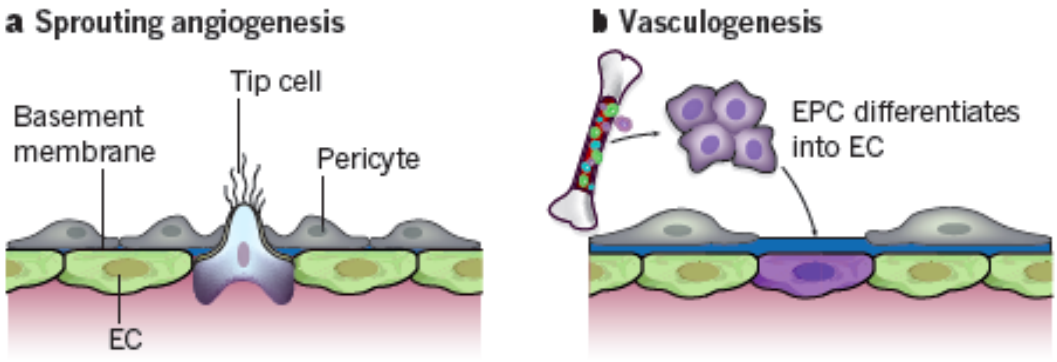

Figure 1.11. Vessel formation can occur by sprouting angiogenesis (a), or by (b) the recruitment of bone-marrowderived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs; b) reproduced from Carmeliet et al, with kind permissions from Springer Nature [License Number 5487230837793][301].

In health the endothelial cell is protected by signals which promote vessel stabilisation, VEGF, ANG1 and FGF. Cells are connected by junctional molecules (VE-cadherin and claudins) and surrounded by pericytes, which release the stabilising factors VEGF and ANG1. In disease, either hypoxia, inflammation or tumour promote the release of ANG2, which causes the pericyte to detach from the EC, in turn loosening the junction with its neighbours. Further mediators, such as NOTCH, neuropilins, placental growth factor and fibroblast growth factor promote one cell to develop into a tip, followed by a stalk, elongating into a new vessel. A reversal of the initial process then promotes vessel maturity with PDGF, ANG1 and TGF-β promoting pericyte attachment[301].

1.9 Neovasculogenesis and Angiogenesis in Liver Disease

The triad of pathological processes contributing to the development of chronic liver disease are Inflammation, Fibrosis and Angiogenesis/Neovasculogenesis. Neovasculogenesis is the process where new blood vessels are formed from endothelial progenitor and is known to be an essential part of both wound healing and fibrosis development. In chronic liver disease the capillarisation of sinusoids, development of intra and extra-hepatic portosystemic shunts and the development of hepatocellular carcinomas may mean Neovasculogenesis plays an important role in the pathogenesis of these complications [302].

Morphological analysis of intrahepatic shunting in liver disease shows the development of new vessels from portal venous branches to hepatic venules [303]. The development of LSECs from endothelial progenitor cells (EPCs) in the bone marrow in response to hepatic necrosis, and the ability of transplanted 'quiescent' EPCs to control fibrogenesis suggests that neovasculogenesis also plays a role in the development of new vessels in ACLD [304 305].

We know that as part of the inflammatory cascade pro-angiogenic cytokines (PDGF, TGF-B1 and VEGF) are released, in addition the fibrotic process and deposition of ECM induces areas of tissue hypoxia, stimulating the release of tissue Hypoxia Inducible Factors (HIF-1α)[306 307]. Several studies have confirmed an increase in VEGF in models of cirrhosis[308].

The role of angiogenesis in portal hypertension is further implied from studies that show an increase in VEGF expression in the mesenteric circulation of

animal models of portal hypertension and that VEGFR-2 inhibition in animal models reduces the development of collateral vessels[309 310].

Recently, it has been shown that in healthy adults stem/progenitor cells are resident in the mesentery and in models of portal hypertension they can differentiate into vascular stem/progenitor cells (VSPC) that are responsible for the neovasculogenesis seen in portal hypertension. They also showed that cytoplasmic polyadenylation element binding protein-4 regulates the differentiation to VSPCs [311].

1.9.1 Hypoxia Inducible Factors

The hypoxia inducible factors (HIFs) are a family of heterodimeric transcription factors that act as master regulators of a homeostatic transcriptional response to hypoxia in virtually all cells and tissues. In hypoxic conditions they are able to translocate to the cell nucleus where they can up-regulate target genes [312]. Whilst there are numerous genetic targets which the HIFs have been shown to up regulate the two most important targets involved in angiogenesis/neovasculogenesis are VEGF and Angiopoietins [313-315].

1.9.2 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) was the first angiogenic growth factor to be identified, is the most well characterised, and the most pivotal factor involved in the formation of new vessels either by neovasculogenesis or angiogenic sprouting [316]. It is actually a family of closely related factors the

most important of which is VEGF-A, which activates VEGFR-2 (Figure 1.12). Soluble isoforms of VEGF promote vessel enlargement, whilst matrix-bound isoforms promote sprouting[301]. It is thought that VEGF mediates much of it's effect on vascular permeability through a nitric oxide dependent pathway [317].

VEFG is over expressed in the hepatic and mesenteric circulation in portal hypertension and appears to play a role in to the development of portal hypertension and it's complications such as ascites [318].

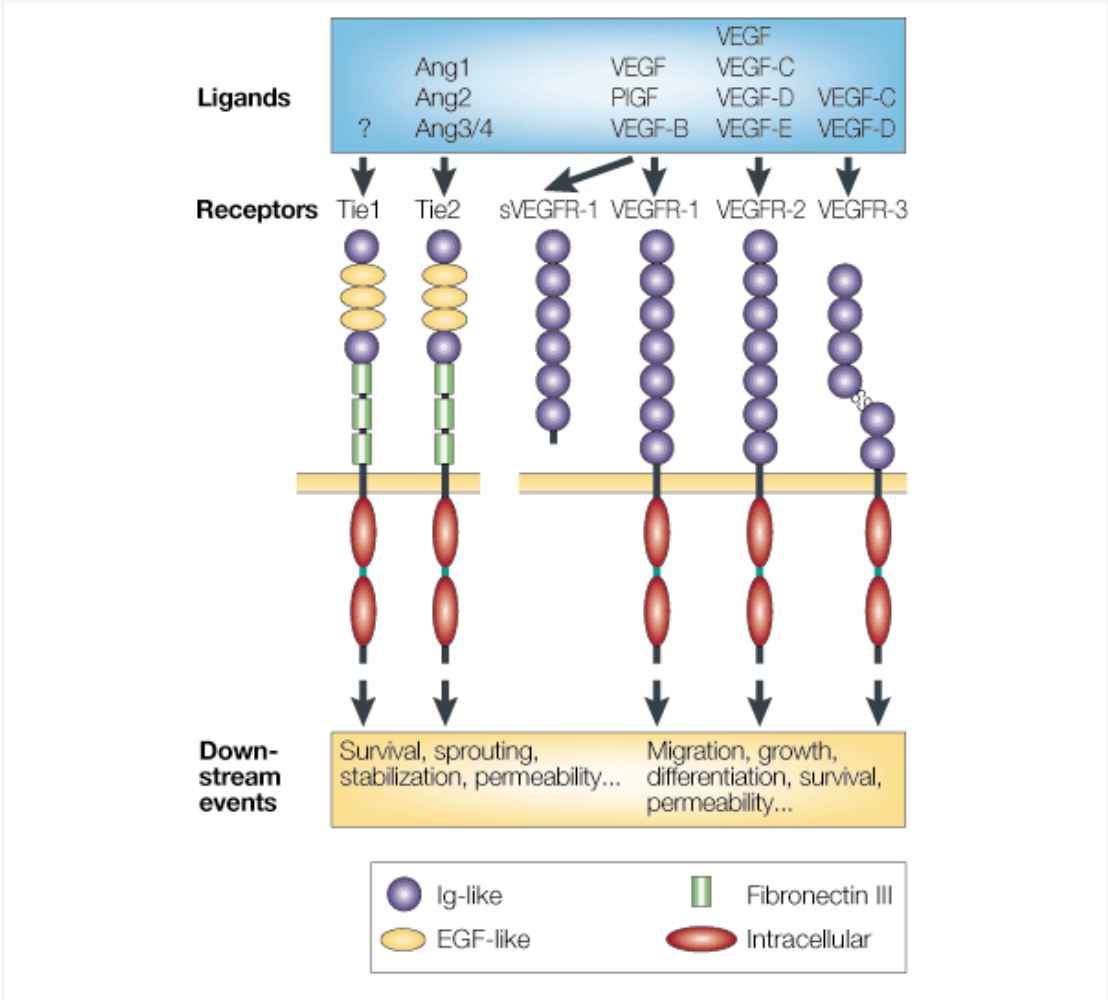

Figure 1.12 Structure of endothelial-cell receptor tyrosine kinases and growth factors involved in vasculogenesis, angiogenesis and lymphangiogenesis.

Reproduced from Jones et al, with kind permissions from Springer Nature [License Number 5487231319703][319].

1.9.3 Platelet Derived Growth Factor (PDGF)

In addition to VEGF, PDGF is also secreted by endothelial cells in portal hypertension. PDGF-β is an important factor promoting maturity and stabilising the newly enveloping vessels (acting as a chemoattractant for pericytes), and at some stage in their development vessels become independent of VEGF and reliant on PDGF for the development of smooth muscle cells and a pericytes covering to provide stability [299].

1.9.4 Fibroblast Growth Factors

The FGF family have a number of roles in controlling vessel development and they have been shown to stimulate the release of ANG2 and VEGF in disease. At low levels FGF is required in health to maintain vascular integrity[301].

1.10 Angiopoietins and TIE receptors

Angiopoietins are 70-kDa glycoproteins which contain an amino-terminal angiopoietin-specific domain, a coiled-coil domain, a linker peptide and a carboxyl-terminal fibrinogen homology domain[320]. There are 3 known Angiopoietins, but only types 1 and 2 have been well characterised. ANG1 is secreted by pericytes and smooth muscle cells, whereas ANG2 is produced by the endothelial cells (particularly tip cells). Both Angiopoietin-1 (ANG1) and Angiopoietin-2 (ANG2) are ligands of the tyrosine kinase receptor Tie-2 that is expressed mainly on vascular endothelial cells (Figure 1.13). The Tie-1

receptor does not exert any intracellular effect but can regulate the binding of molecules to Tie-2 [320-322]. In embryogenesis the Tie-2 pathways are vital for effective angiogenesis and vessel development, however in adult life they change to have more of a protective role preventing unfavourable responses to tissue injury.

ANG1 has been shown to be an agonist of Tie2 both in vivo and in vitro. When Tie2 is activated by ANG1 it exerts its cellular effects via several complementary but separate mechanisms. Firstly, activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway promotes cell survival and inhibits cell migration. Secondly, inhibition of NF-kB and reduced activity of leucocyte adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin) have an anti-inflammatory effect. Thirdly, downregulation of cadherin and platelet endothelial cell adhesion molecule-1 stabilise cell-cell junctions and prevent leakage[323-325].

ANG2 expression is increased in cells undergoing active remodelling, or in tumour neovasculogenesis. For tumour angiogenesis or neovasculogenesis to occur the maintenance effects of ANG1 must be antagonised by ANG2 (which displaces ANG1 on the Tie2 receptor binding site). Although ANG2 does not activate the Tie2 receptor it prevents the ongoing 'background' protective mechanisms that are promoted by ANG1. This allows de-stabilisation of the endothelial cell and loosening of the gap junctions providing an environment that is favourable for angiogenesis/neovasculogenesis[326-328].

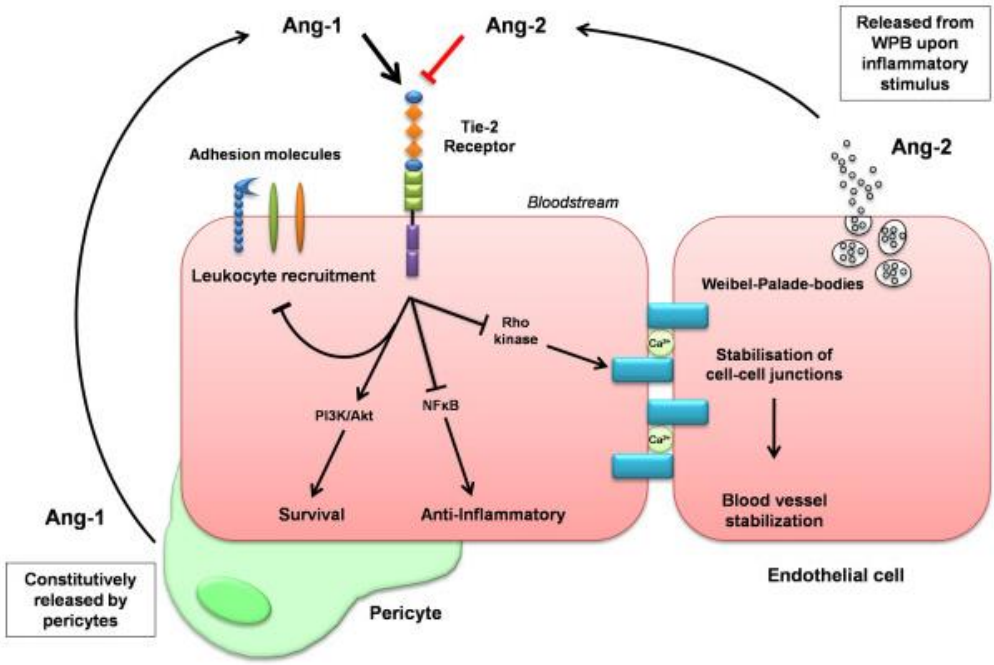

Figure 1.13 A schematic model of the angiopoietin-Tie2 ligand-receptor system. Reproduced from van Meurs et al, with kind permission of Springer Nature [Licence Number 5487240167640][320].

ANG2 is stored with endothelial cell Weibel-Palade bodies and is known to be released in response to a number of stimulants including thrombin, HIF-1α, TNF-α and VEGF[329]. Crucially, in the absence of VEGF, ANG2 cannot destabilise EC walls and therefore angiogenesis/neovasculogenesis cannot occur[300 316 330 331].

A constant flux between ANG1 and ANG2 (controlled by local mediators) is required to promote the destabilisation and growth of new vessels and then their maturation and stabilisation as part of the fibrotic/tissue remodelling process (Figure 1.14).

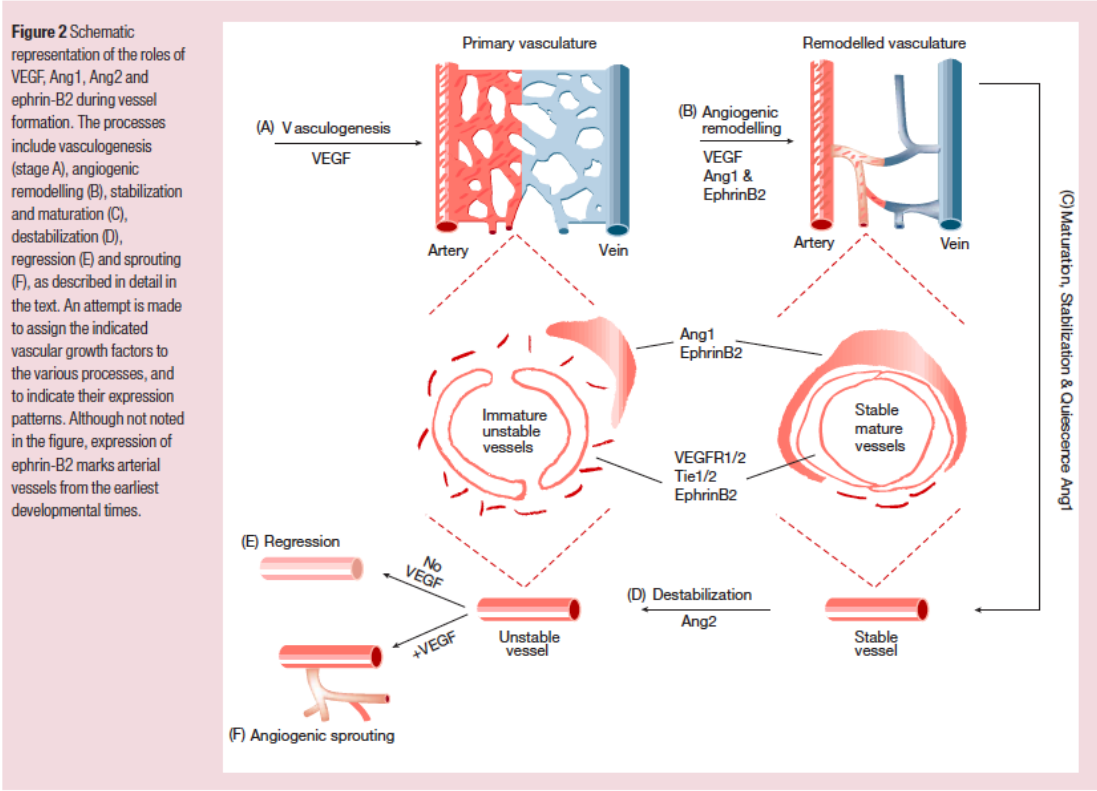

Figure 2 Schematic representation of the roles of VEGF, Ang1, Ang2 and ephrin-B2 during vessel formation. The processes include vasculogenesis (stage A), angiogenic remodelling (B), stabilization and maturation (C), destabilization (D), regression (E) and sprouting (F), as described in detail in the text. An attempt is made to assign the indicated vascular growth factors to the various processes, and to indicate their expression patterns. Although not noted in the figure, expression of ephrin-B2 marks arterial vessels from the earliest developmental times.

Figure 1.14 Schematic representation of the roles of VEGF, ANG1, ANG2 and ephrin-B2 during vessel formation. Reproduced from Yancopoulous et al, with kind permission from Springer Nature [License Number 5487240432408][316].

1.11 NOTCH and WNT pathway

The NOTCH/WNT pathway is essential for stalk development, VEGF induces the tip cells to express delta like ligand 4 (DLL4), which activates NOTCH. This process restricts further branching in the stalk and promotes elongation and stabilisation of the vessel [301].

1.12 Angiopoietins in Liver Disease

The initial investigation of Angiopoietins in liver disease focused on their potential role in tumour neovasculogenesis in hepatocellular carcinoma,

however, it has since been shown that VEGF levels are elevated in chronic liver disease and in portal hypertension[332].

It has been shown that the HSC can secrete ANG1 [333], and that ANG2 levels are elevated post partial-hepatectomy[334].

The largest study of ANG2 in patients with ACLD was by Scholz and colleagues. They reported elevated ANG2 levels in patients with cirrhosis, when compared to healthy controls. They also reported an increase in ANG2 levels in cirrhotic patients with HCC, which was significantly higher than that in patients with cirrhosis but no HCC. There was a difference in the severity of liver disease in the two groups, with the non-HCC group having predominantly Child-Pugh grade B and C patients (HCC group 44/93 CP A, 26/93 CP B, 11/93 CP C; non-HCC group 60/180 CP A, 65 / 180 CP B, 55/180 CP C)[335].

Another study of 147 patients with alcohol related liver disease showed that the cases had significantly elevated ANG2 and VEGF levels compared to controls and that the levels increased with increasing severity of liver disease assessed by Child-Pugh score and MELD[336]. The ANG2 levels were 3.90 ng/mL in patients with MELD < 20 and 11.37 in patient with MELD $\geq$ 20 ($P<0.001$).

It has been shown that ANG2 was corelates with liver fibrosis markers and is reduced in patients with Hepatitis C who had received anti-viral therapy[337].

Separate authors have also reported a reduction in serum ANG2 levels in combination with a reduction in HVPG in 66 patients who had been cured of Hepatitis C with anti-viral therapy[338]. The same results suggested that the reduction in ANG 2 was correlated to a reduction in HVPG with a Spearman's correlation of 0.267 (p=0.030).

A relationship between increased ANG2 levels and poor survival has been demonstrated in a study of 191 patients with ACLD, a high MELD score and an AKI[339].

It has been shown that ANG2 mRNA expression is increased in HCC when compared to normal liver tissue, and that a higher ANG2/ANG1 mRNA ratio was associated with tumour portal vein invasion, tumour diameter, micro vessel density and a reduced survival[340 341]. More recently it has been confirmed that increased ANG2 expression is associated with poorer survival and along with 4 other genes has been able to predict the rate of tumour growth[342].

Hsieh and colleagues measured serum levels of VEGF-A and ANG2 before and after TACE in patients with HCC. They showed that prior to TACE patients with HCC staging > 2 had significantly higher ANG2 and VEGF-A levels and that Child-Pugh class B patients had higher ANG2 levels than Child-Pugh A patients. Following TACE there was a significant increase in both ANG2 and VEGF[343].

Salcedo *et al* measured the serum levels of vascular endothelial growth factor (VEGF), angiopoietin-2 (ANG2) and soluble Tie-2 (the tyrosine kinase receptor for angiopoietin) in 36 patients who were undergoing treatment for chronic hepatitis C (CHC) virus. Prior to treatment all patients had a liver biopsy to assess the grade of inflammation and stage of fibrosis. Fibrosis was assessed by the METAVIR score and 10 patients had stage F1 disease, 20 patients F2 and 6 F3. The grade of activity on the biopsies was predominantly A2. Baseline levels of VEGF in the CHC group showed no significant difference when compared to 15 healthy controls, who had normal liver function tests and had tested negative for hepatitis B and C viruses. The serum VEGF level did correlate with grade, but not stage of fibrosis prior to treatment. Serum levels of ANG2 were significantly different in CHC and control groups ($p<0.05$) but were unable to differentiate between varying stages of fibrosis. After standard treatment with Interferon and Ribavirin for 24 or 48 weeks for genotypes 2/3 and 1/4 respectively there was a significant reduction in both serum VEGF and ANG2 levels with respect to pre-treatment values and this decrease correlated with a reduction in the serum ALT. The response was greatest in the responder v. non-responder groups[344].

It has been shown that ANG1 is upregulated and ANG2 downregulated in Focal Nodular Hyperplasia (FNH) and that this predominance of ANG1 promotes the thickened and disorganised smooth muscle cell distribution around the vessels seen in FNH[345].

1.13 Angiopoietins in Non-Liver Diseases

The close relationship between angiogenesis and inflammation has been shown in many diseases and the Angiopoietin/Tie2 axis appear to play a crucial role in these processes [346] [347].

ANG2 has been shown to be over expressed in many pro-inflammatory conditions such as acute myocardial infarction, acute respiratory distress syndrome, septic shock, critical illness and higher levels of ANG2 have been associated with more severe disease in sepsis and critical illness[320] [348]. It has long been established that the breakdown of the endothelial barrier is a major contributor to the progression of multi-organ failure and the ANG1/ANG2 ratio and Tie-2 interaction may be an important component of this pathological process[349] [350]. Fiedler and colleagues showed that ANG2 sensitised the endothelial cell to TNF-α and was a crucial factor in the inflammatory process in a model of sepsis[351] [352].

Higher ANG2 levels have been associated with more severe presentations of CLL and a shorter overall survival after treatment[353].

1.14 Anti-Angiogenic Therapy in Portal Hypertension

Fernandez and colleagues showed that a monoclonal antibody (Sorafenib a multipotent tyrosine kinase inhibitor targeting Raf kinase, VEGFR-2 and -3, PDGF receptor β[354] and inhibitors of VEGFR-2 phosphorylation) resulted in a 52% reduction in portal-systemic collateral formation in mice and rats if used for 5-7 days after portal vein stenosis [355] [356]. Interestingly in the same study,

investigators found that if the VEGFR-2 inhibitor was given after this initial time period it was unable to ameliorate the development of portal hypertension. In a second study the same group showed that a different VEGF inhibitor, Rapamycin, did reduce portal hypertension when administered > 2weeks after the initial liver injury, and that when used in combination with Imatinib, a PDGF inhibitor, there was a reduction in portal pressure of 40% and in SMA blood flow by 30% [357].

In an animal model of portal hypertension Sunitinib, another tyrosine kinase inhibitor, can reduce fibrosis (reducing collagen accumulation by 30%) and portal pressure by 40% [358]. In a similar experiment Rapamycin, an inhibitor of VEGF synthesis, reduced portal collateral formation by 67% and attenuated the splanchnic hyperdynamics[357].

It is known that Sorafenib reduces collagen formation in animal models of liver disease by inducing HSC apoptosis and inhibiting HSC proliferation [359].

Pinter and colleagues studied the effect of Sorafenib (400mg twice daily) in patients being treated for hepatocellular carcinoma. After 2 weeks of treatment there was a reduction in HVPG of > 20% from baseline in 4/12 patients, and a downregulation of VEGF, PDGF, PlGF and RhoA kinase mRNA activity in the same 4 patients[360].

1.15 Anti-Angiopoietin Targeted Therapy

Trebananib is a peptibody (peptide-Fc fusion protein) which selectively binds ANG1 and ANG2 preventing Tie2 receptor activation and is currently being assessed as an anti-angiogenic adjuvant treatment in oncology[361]. It has been evaluated in combination with other anti-cancer agents and appears to be efficacious and have an acceptable safety profile [362-364]. Its use has been assessed in hepatocellular carcinoma but results were similar to Sorafenib alone[365].

Nesvacumab is a human monoclonal antibody which binds Angiopoietin-2, preventing its interaction with the Tie-2 receptor. This drug has successfully completed Phase-1 trials in patients with advanced solid organ tumours with an acceptable safety profile[366].

It has been suggested that a dual inhibitory strategy of ANG1 and ANG2 would be superior to ANG2 inhibition alone in preventing tumour angiogenesis, as unopposed ANG1 action would still lead to vessel maturation in newly formed tumour vessels[367].

1.16 GENERAL AIMS

1. **To measure ELF, ANG1 and ANG2 in patients undergoing measurement of HVPG.**
2. **To assess the efficacy of ELF, ANG1 and ANG2 as biomarkers of HVPG.**
3. **To determine the performance of ELF, ANG1 and ANG2 as biomarkers of the extent of liver fibrosis assessed by measurement of collagen proportionate area in patients undergoing liver biopsy.**
4. **To assess the efficacy of ELF, ANG1 and ANG2 in predicting prognosis in liver disease.**

Chapter 2: Serum levels of Angiopoietins, ELF and Collagen Proportionate Area in patients presenting for Transjugular Liver Biopsy and Hepatic Venous Pressure Measurement

2.1 BACKGROUND

Angiopoietins are proteins that interact with the Tie-2 receptor to promote or prevent angiogenesis. Angiopoietin-1 (ANG1) binds to the Tie-2 receptor and promotes the stabilisation of endothelial cells. In the presence of tissue injury increased levels of Angiopoietin-2 (ANG2) displaces ANG1 and allows the destabilisation of endothelial cells which allows angiogenesis and neovasculogenesis[328].

ELF is an established diagnostic test for estimating the degree of fibrosis in patients with chronic liver disease and thresholds have been defined for the diagnosis of advanced fibrosis and cirrhosis.[368 369] The ELF test generates a continuous unitless numerical variable value that exhibits a near linear correlation with the amount of fibrosis in the liver[370 371].

Collagen proportionate area is a quantitative measure of the amount of liver collagen that binds Sirius red expressed as a proportion of liver visible in a histological field of a liver biopsy. The proportion of tissue taking up the Sirius red dye can be measured using automated image analysis in which a grid is applied to the histological field and the ratio of red staining squares is measured as a proportion of all squares in the grid. This is an alternative means of

quantifying liver fibrosis that differs from histological staging in a number of important ways. First, it generates a continuous quantitative variable. Secondly it does not incorporate any architectural assessment of the liver and so does not differentiate between cirrhosis and other stages of fibrosis and so removes the categorical distinction between “advanced fibrosis” and “cirrhosis”. Thirdly by measuring the extent of fibrosis in cirrhotic biopsies, unlike histological staging it can be used to measure differing degrees of fibrosis in cirrhotic biopsies in which the degree of collagen deposition can be correlated with the severity of decompensation[372]. We aimed to assess these variables in a prospective cohort of patients with liver disease.

2.2 OBJECTIVE

To measure the serum levels of Angiopoietins and ELF in patients attending for HVPG and transjugular liver biopsy and to assess collagen proportionate area.

2.3 METHODS

2.3.1 Study Design

The study protocol was approved by the sponsor and the chief investigator.

This study was carried out at the Royal Free Hospital, London, a tertiary liver transplant centre in the United Kingdom. As part of this study, samples were also collected from 3 participants at the Scottish Liver Transplant Unit, Edinburgh, UK. We recruited patients who attended for transjugular liver biopsy

or hepatic venous pressure measurement for evaluation of chronic liver disease.

2.3.2 Research Ethics Committee Approval

Ethical approval was obtained from the NHS Research Ethics Service on the 10th March 2011 (Ref: 11/H0718/8), who approved the study protocol, team and information leaflets.

NHS Research & Development approval was obtained on the 15th April 2011 and the Sponsor of the study was University College London.

2.3.3 Study Population

Inclusion criteria

- Patients attending for trans-jugular liver biopsy or hepatic venous pressure measurement as part of the investigation of chronic liver disease or isolated portal hypertension.
- Ability to give informed consent for the study and agreement to participate.
- Age ≥ 18 years of age.

Exclusion criteria

- Unwilling or unable to provide informed consent.
- Aged < 18 years of age.

2.3.4 Study Procedure

Potential participants were informed of the study and given a patient information leaflet when they we informed about their appointment in the interventional radiology unit. On the day of the procedure, they were introduced to the research co-ordinator and given further verbal information to complement the written information on the study. They were then invited to provide written informed consent in person.

2.3.5 Data Collection

After obtaining the participants written informed consent the following data were recorded:

- Sex
- Age
- Ethnicity
- Aetiology of liver disease
- Year of primary liver diagnosis

2.3.6 Collection of Serum

Prior to the procedure peripheral blood was collected from participants into plain yellow topped serum separator tubes (SST). At the time of measurement of the HVPG, samples were obtained from the hepatic vein and, again collected into SST. The samples were then processed in accordance with the following procedure.

1. Samples were allowed to clot in the SST for a minimum of 30 minutes.
2. Samples were then spun in a centrifuge for 15 mins at 1000g.

3. The separated serum was then removed from the SST and stored in 1ml cryogenic tubes.
4. Each sample was labelled only with a participant's unique trial number.
5. Cryogenic tubes were stored at -80^{o}C prior to analysis.

2.3.7 Angiopoietin-1 Measurement

Angiopoietin-1 levels were measured using human angiopoietin immunoassays (Quantinkine® ELISA, R&D Systems, Minneapolis, MN, USA). These are quantitative sandwich enzyme-linked immunoassays (ELISA) developed to measure human Angiopoietin levels. The ELISA was carried out according to the manufacturer's procedure as below. The assays were performed in the UCL-RFH Biobank Laboratory.

2.3.8 Preparation of reagents for Angiopoietin-1 Assay

96-well plate: A monoclonal antibody specific for human Angiopoietin-1 is pre-coated onto the microplate.

Human Angiopoietin-1 Standard: Recombinant human Angiopoietin-1 in a buffered protein base with preservatives. A 40,000 pg/mL solution was diluted as in the assay procedure below.

Human Angiopoietin-1 Conjugate: Monoclonal antibody specific for human Angiopoietin-1 conjugated to horseradish peroxidase with preservatives.

Assay Diluent: Buffered protein base with preservatives.

Calibrator diluent: Buffered protein base with preservatives, made with 20mls of concentrate and 80mls of distilled water to a total volume of 100mls.

Wash buffer: Buffered surfactant with preservative made from concentrate with 20mls of wash buffer concentrate in 480mls of distilled water.

Colour Reagent A: Stabilised hydrogen peroxidase

Colour Reagent B: Stabilised chromogen (tetramethylbenzidine)

Substrate Solution: A mixture of colour reagent A and colour reagent B in equal volumes (24ml in total), which was protected from the light prior to use.

Stop Solution: 2N Sulphuric Acid

2.3.9 Angiopoietin-1 Assay Procedure

1. A 96-well plate map was designed with capacity for each sample and including standards to be tested, in duplicate.
2. Serum samples were removed from storage and allowed to thaw to room temperature.
3. Serum samples were diluted according to a 50-fold dilution (10uL of serum with 490 uL of calibrator diluent).
4. Standard solutions were re-constituted.
    a. 1mL of distilled water was added to the stock solution (40000 pg/mL).
    b. This was further diluted to provide standard samples as in Figure 2.1
5. 100uL of assay diluent was added to each well.
6. 50uL of either standard or sample was added to the diluent according to the plate map. This was covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital plate shaker at 500 rpm.

7. Each well was aspirated and washed with a squirt bottle a total of 4 times with a wash buffer solution. At the end of the washes remaining buffer was removed by inverting the plate on clean paper towels.
8. 200uL of Angiopoietin-1 conjugate was added to each well. The plate was covered with an adhesive strip and incubated for a further 2 hours at room temperature on the plate shaker as above.
9. Each well was washed as in step 7 above.
10. 200uL of substrate solution was added to each well. The plate was protected from light with foil and incubated on the bench at room temperature for 30 minutes
11. 50uL of stop solution was added to each well.
12. Optical density (OD) was measured using a FLUOstar Galaxy microplate reader (BMG Labtechnologies, BMG LABTECH Ltd, Aylesbury, UK). Absorbance was measured at 460nm and at 544nm. The OD at 460nm was subtracted from the reading at 544nm to correct for any optical imperfections on the plate.
13. The average of the two duplicate readings for each standard was calculated.
14. A standard curve was derived from the measured OD for each of the standard concentrations of Angiopoietin-1 (and the result was multiplied by the dilution factor from step 3, to provide the final concentration).

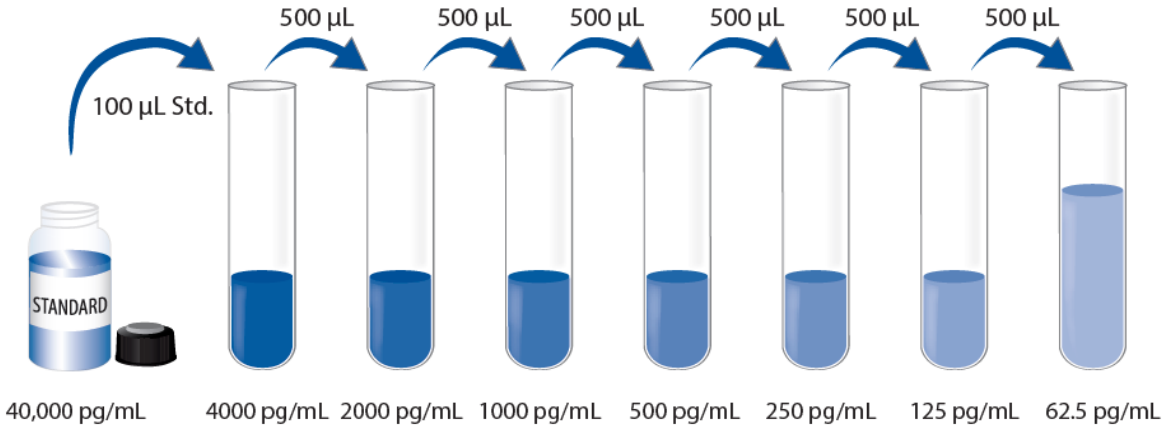

Figure 2.1. The dilution of standard, reference samples, for the Angiopoietin-1 ELISA.

Appropriate PPE was worn (lab coat, gloves and a mask to prevent contamination (as both Angiopoietin-1 and -2 is detectable in human saliva)).

2.3.10 Angiopoietin-2 Measurement

Angiopoietin-2 levels were measured using human angiopoietin immunoassays (Quantinkine® ELISA, R&D Systems, Minneapolis, MN, USA). These are quantitative sandwich enzyme immunoassay designed to measure human Angiopoietin levels. The ELISA was carried out according to the manufacturer's procedure as below. The assays were carried out in the UCL-RFH Biobank Laboratory.

2.3.11 Preparation of reagents for Angiopoietin-2 Assay

96-well plate: A monoclonal antibody specific for human Angiopoietin-2 is pre-coated onto the microplate.

Human Angiopoietin-2 Standard: Recombinant human Angiopoietin-2 in a buffered protein base with preservatives. A 30,000 pg/mL solution was diluted as in the assay procedure below.

Human Angiopoietin-2 Conjugate: Monoclonal antibody specific for human Angiopoietin-2 conjugated to horseradish peroxidase with preservatives.

Assay Diluent: Buffered protein base with blue dye and preservatives.

Calibrator diluent: Buffered protein base with preservatives, made with 20mls of concentrate and 80mls of distilled water to a total volume of 100mls.

Wash buffer: Buffered surfactant with preservative made from concentrate with 20mls of wash buffer concentrate and 480mls of distilled water.

Colour Reagent A: Stabilised hydrogen peroxidase

Colour Reagent B: Stabilised chromogen (tetramethylbenzidine)

Substrate Solution: A mixture of colour reagent A and colour reagent B in equal volumes (24ml in total), which was protected from the light prior to use.

Stop Solution: 2N Sulphuric Acid

2.3.12 Angiopoietin-2 Assay Procedure

1. A 96-well plate map was designed with capacity for each sample or standard to be tested in duplicate.
2. Serum samples were removed from storage and allowed to thaw to room temperature.
3. Serum samples were diluted according to a 5-fold dilution (50uL of serum with 200 uL of calibrator diluent).
4. Standard solutions were re-constituted.

a. 1mL of distilled water was added to the stock solution (30,000 pg/mL).
b. This was further diluted to provide standard samples as in Figure 2.2

5. 100uL of assay diluent was added to each well.
6. 50uL of either standard or sample was added to the diluent according to the plate map. This was covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital plate shaker at 500 rpm.
7. Each well was aspirated and washed with a squirt bottle a total of 4 times with a wash buffer (buffered surfactant with preservative). At the end of the washes remaining buffer was removed by blotting (inverting) the plate on clean paper towels.
8. 200uL of Angiopoietin-2 conjugate was added to each well. The plate was covered with an adhesive strip and incubated for a further 2 hours at room temperature on the plate shaker as above.
9. Each well was washed as in step 7 above.
10. 200uL of substrate solution was added to each well. The plate was protected from light and incubated on the bench at room temperature for 30 minutes.
11. 50uL of stop solution was added to each well.
12. Optical density was measured using a FLUOstar Galaxy microplate reader (BMG LABTECH Ltd, Aylesbury, UK). Absorbance was measured at 460nm and at 544nm. The OD at 460nm was

subtracted from the reading at 544nm to correct for any optical imperfections on the plate.

13. The average of the two duplicate readings for each standard was calculated.
14. A standard curve was derived from the measured OD for each of the standard concentrations of Angiopoietin-1 (and the result was multiplied by 5 to allow for the dilution in step 3)

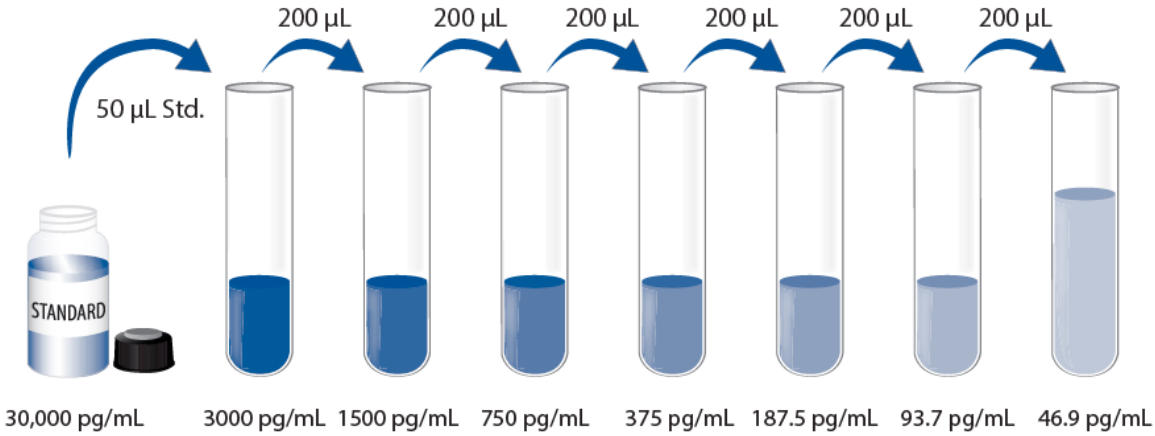

Figure 2.2. The dilution of standard, reference samples, for the Angiopoietin-2 ELISA.

2.3.13 ELF measurement

Serum samples were thawed to room temperature and the levels of hyaluronic acid (HA), amino terminal propeptide of type III procollagen (P3NP) and tissue inhibitor of metalloproteinase 1 (TIMP-1) were assayed on the Advia Centaur Classic Immunoassay System (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). These assays were carried out in the Siemens laboratory at the Royal Free Hospital by a single operator. The ELF score was then calculated according to the published algorithm:[368]

$$\text{ELF score} = 2.494 + 0.846 \ln(C_{HA}) + 0.735 \ln(C_{P3NP}) + 0.391 \ln(C_{TIMP1})$$

2.3.14 Histological Processing

Liver biopsy samples were formalin fixed, paraffin embedded, and stained with hematoxylin and eosin, and the Gordon and Sweet method for reticulin. All samples were reviewed by the same pathologist and the Ishak stage score was recorded. The Ishak stage score was selected for all samples, regardless of aetiology to maintain uniformity of reporting. Cirrhosis was defined as an Ishak score of 5 or 6.

ISHAK SCORE[373]

Stage	Description
0	No fibrosis
1	Fibrous expansion of some portal areas, with or without short fibrous septa
2	Fibrous expansion of most portal areas, with or without short fibrous septa
3	Fibrous expansion of most portal areas, with occasional portal to portal bridging
4	Fibrous expansion of portal areas with marked bridging (portal to portal) as well as portal to central
5	Marked bridging (portal to portal and/or portal to central) with occasional nodules (incomplete cirrhosis)
6	Cirrhosis, probable or definite

Table 2.1 – Ishak Fibrosis Score

Participants with known hepatocellular carcinoma did not have a transjugular biopsy performed to reduce the risk of tumour seeding. In these participants a histological sample obtained at the time of liver resection, if undertaken, was used to assess fibrosis stage and CPA.

2.3.15 Collagen Proportionate Area (CPA)

Sections of each histological sample were stained with picro-Sirius Red for digital image analysis, which was performed by a single pathologist. After whole-section digital image capture, CPA was measured using a visual basic script for Zeiss Axiovision (version 4.8.2.) in which binary segmentation of RGB colour channels was used to distinguish liver tissue from collagen. The CPA measurement process included a manual editing step to eliminate image artifacts, and operator-dependent thresholding to determine the stained area of the section. The CPA was calculated as the area occupied by stained collagen as a proportion of the area of the whole parenchyma and expressed as a percentage.

2.3.16 Statistical Analysis

Medians were compared using the appropriate non-parametric test (Mann-Whitney U). SPSS v22, IBM, USA was used for all the statistical analysis.

2.4 RESULTS

2.4.1 Demographics: 100 participants were recruited to the study and the demographics are summarised in table 1. The median age was 55 years (IQR 46-62 years).

The aetiologies seen were hepatitis C (27%), alcohol related liver disease (25%), autoimmune hepatitis (13%), non-alcohol related fatty liver disease (9%), cryptogenic (6%), hepatitis B (6%), nodular regenerative hyperplasia (4%), drug induced liver injury (3%) and other (7%). The 7 patients classified as having 'other' liver diseases consisted of 1 participant with each of alpha-1 antitrypsin related liver disease, hepatic amyloidosis, Budd-Chiari syndrome, extra-hepatic portal vein thrombosis and chronic lymphocytic leukaemia and two patients with both non-alcohol and alcohol related fatty liver disease.

	All Participants (n=100)	Participants without Cirrhosis (n=48)	Participants with Cirrhosis (n=52)	
Median (IQR) Age (years) (n=100)	55 (46 – 62)	55 (46 – 62)	55 (45 – 63)	p=0.981
Male : Female (%)	65 : 35	58 : 42	71 : 29	
Aetiology				
Hepatitis C	27	18	9	
Alcohol	25	3	22	
Autoimmune	13	9	4	
NAFLD	9	2	7	
Cryptogenic	6	2	4	
Hepatitis B	6	3	3	
NRH	4	4	0	
DILI	3	3	0	
ALD/NAFLD	2	-	2	
EHPVO	1	1	0	
A1AT	1	-	1	
Amyloid	1	1	0	
Budd-Chiari	1	1	0	
CLL	1	1	0	
Cirrhosis (%)	52	0	100	
HCC (%)	17	6	26.9	p=0.013
Post-Transplant (%)	25	38	14	
Median (IQR) HVPG (mmHG)	7 (4 – 14)	4 (3 – 5)	13 (8 – 17)	p<0.001
ISHAK Stage (%)				
0	19	19	-	
1	12	12	-	
2	5	5	-	
3	6	6	-	
4	1	1	-	
5	10	-	10	
6	37	-	37	
Unknown	10	5	5	
CPA (IQR) (%) n=85	11 (5 – 26)	6 (4 - 10)	26 (17 – 31)	p<0.001
Median (IQR) ELF (peripheral vein) n=100	10.49 (8.86 – 12.22)	9.00 (8.23 – 10.73)	11.59 (10.08 – 13.27)	p<0.001
Median (IQR) ELF (Hepatic vein) N=87	10.04 (8.51 – 11.91)	8.53 (7.86 – 10.04)	11.58 (9.94 – 14.04)	p<0.001
Angiopoietin 1 (ANG1) pg/mL n=83	23990 (15730 – 37480)	30090 (20240 – 50990)	19690 (10252 – 31115)	p<0.001
Angiopoietin 2 (ANG2) pg/mL n=83	4017 (2386 – 6235)	3300 (2325 – 4262)	5577 (2721 – 9230)	p<0.001
ANG2 / ANG1 Ratio n=83	0.1688 (0.0805 – 0.3590)	0.0982 (0.0593 – 0.1724)	0.2853 (0.1683 – 0.6355)	p<0.001

Bilirubin (IQR) (umol/L)	14 (9 – 63)	10 (8 – 16)	31 (12 – 214)	p<0.001
INR (IQR)	1.1 (1.0 – 1.6)	1.0 (1.0 – 1.2)	1.3 (1.1 – 2.0)	p<0.001
Sodium (IQR) (mmol/L)	140 (137 – 142)	141 (139 – 143)	137 (134 – 141)	p<0.001
Creatinine (IQR) (umol/L)	80 (64 – 102)	86 (65 – 102)	76 (59 – 104)	p=0.161
MELD Score (IQR)	9 (7 – 16)	7 (6 – 9)	13 (9 – 24)	p<0.001
UKELD Score (IQR)	48 (45 – 54)	46 (44 – 48)	53 (47 – 62)	p<0.001
ALT (IQR) (iU/L)	48 (27 – 88)	51 (26 – 87)	42 (29 – 89)	p=0.548
AST (IQR) (iU/L)	58 (35 – 114)	52 (30 – 89)	42 (28 – 185)	p=0.069
ALP (IQR) (iU/L)	110 (82 – 171)	99 74 – 150)	122 (90 – 191)	p=0.548
Albumin (IQR) (g/L)	40 (34 – 44)	42 (40 – 45)	37 (29 – 42)	p=0.003
Alpha-fetoprotein (IQR) (kunits/L)	4.5 (2.68 – 7.90)	4.45 (2.5 – 7.9)	4.55 (2.88 – 8.88)	p=0.778
APRI Score (IQR)	1.10 (0.60 – 2.98)	0.93 (0.37 – 1.73)	1.54 (0.75 – 4.30)	p=0.069
FIB-4 Score (IQR)	3.79 (1.85 – 7.12)	2.07 (1.39 – 5.05)	5.31 (3.22 – 9.91)	p=0.001

Table 2.2 – Demographics of the study population and summary of liver blood tests, angiopoietin levels, ELF score, CPA and liver severity scores.

Twenty-five percent of patients were recruited post-liver transplantation. The aetiologies in the participants recruited post-liver transplant were Hepatitis C (19/25), Autoimmune (3/25), Cryptogenic (2/25) and Budd Chiari (1/25). The degree of fibrosis varied in this group of post-transplant patients (see figure 2.3)

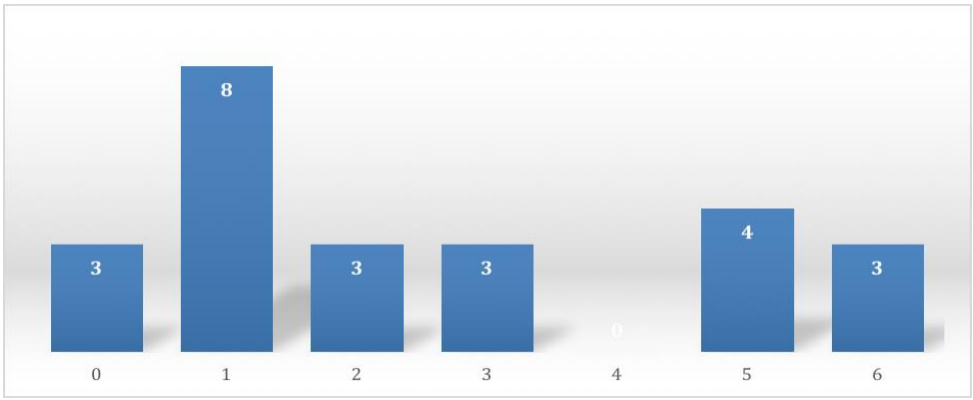

Figure 2.3 – Frequency of Ishak fibrosis score in participants who had previously received a liver transplant (one post-transplant patient did not have an Ishak score available).

17 participants had hepatocellular carcinoma (HCC) at the time of sampling, of these 14 had cirrhosis, 1 had moderate fibrosis (Ishak stage 3), 1 (post-transplant patient) had mild fibrosis (Ishak stage 1) and 1 had no fibrosis. 14 (27%) of the 52 patients diagnosed with cirrhosis had HCC.

52% of participants had cirrhosis, which was confirmed histologically in 47/52 cases (either via a transjugular liver biopsy or from a resection specimen). In the remaining 5 participants, the diagnosis of cirrhosis was made on a combination of clinical, radiological, and clinical chemistry parameters, in these patients no transjugular biopsy or resection was performed due to the presence of unresectable hepatocellular carcinoma. The distribution of Ishak stages for participants where histology was obtained is shown in Table 2.2. 18 patients had steatohepatitis reported on their liver biopsy.

5 patients had NRH diagnosed histologically. The associated diagnoses in the 5 patients with NRH were myelofibrosis and a previous liver transplant for Budd-Chiari syndrome in 1 patient, anti-TNF therapy for Psoriasis in 1 patient, CVID in 2 patients and essential thrombocythaemia in 1 patient.

2.4.2 HVPG: The median HVPG for all participants was 7mmHg (IQR 4-14). For participants with cirrhosis (n=52) the median HVPG was 13mmHg (IQR 8-17) and for those without cirrhosis (n=48) it was 4mmHg (IQR 3-5).

The HVPG in the 5 patients with NRH were variable. In 3 patients the HVPG was below 5mmHg, in 1 patient is was 13 mmHg and in 1 patient it was 13 mmHg. The HPVG in the patient with hepatic amyloidosis was 3mmHg.

The HVPG was highest in patients with ALD or ALD/NAFLD, these patients also had high MELD and high Bilirubin levels. Of the 18 patients with steatohepatitis seen on the biopsy the median HVPG was 16mmHg.

2.4.3 ELF: The median ELF was 10.49 (IQR 8.86-12.22). In participants with cirrhosis this was 11.59 (IQR 10.08-13.27) and those without cirrhosis it was 9.00 (IQR 8.23-10.04). The ELF was highest in patients with Alcohol or combined ALD/NAFLD related liver disease and in those with steatohepatitis on the liver biopsy the median serum ELF was 14.77. The median ELF score was 10.04 in patients with NRH.

2.4.4 CPA: The median CPA was 11% (IQR 5-26%) and in participants with and without cirrhosis it was 26% (17-31%) and 6% (4-10%) respectively. The CPA was high in patients with Alcohol or ALD/NAFLD related liver disease. The highest CPA reading was in the one patient with hepatic amyloidosis, as the Sirius red protein also stains amyloid protein[374]. The lowest CPA's were 2% in a patient with CLL and no liver fibrosis, 4% in a patient with DILI and no fibrosis, and a median of 5% in 5 patients with NRH.

2.4.5 AFP: There was no difference in the AFP level in participants with or without cirrhosis. The AFP was raised in participants with cirrhosis and

Hepatocellular carcinoma (median 17.3 kU/L (IQR 5.8 – 663.7)), in comparison to those with cirrhosis and no HCC (4.1 kU/L (2.7-4.8)) p<0.01.

2.4.6 ANG1: Angiopoietin levels were determined in 83/100 participants. The levels of Angiopoietin 1 in the participants were lower than the mean values reported in healthy subjects, quoted in the ELISA product literature (37,122 pg/mL; range (14,272 – 65,570)). They are significantly lower in the participants with cirrhosis (19,690 pg/mL (10,252 – 31,115 pg/mL)), when compared to those without cirrhosis (30,090 pg/mL (20,240 – 50,990 pg/mL)); p< 0.01. The patients with Amyloid liver disease and DILI had the highest ANG1 levels as shown in table 2.5. Patients with autoimmune disease, DILI and Amyloid liver disease had high ANG1 levels.

2.4.7 ANG2: The levels of Angiopoietin 2 in the 83 participants in whom they were measured were higher than the mean values reported in healthy subjects in ELISA product literature (2494±1,341 pg/mL). The levels were higher in those with cirrhosis (5,577 pg/mL (2,721 – 9,230 pg/mL)), with respect to those without 3,300 pg/mL (,2325 – 4,262 pg/mL)); p< 0.01. The ANG2 level was lowest in the patients with NRH at 1948 pg/ml.

2.4.8 ANG2/ANG1 Ratio: The ANG2/ANG1 ratio is significantly higher in those with cirrhosis when compared to those without. There was no significant difference in the ANG2/ANG1 ratio in participants with cirrhosis and HCC (median 0.200 (IQR 0.076 – 0.513)) and those with cirrhosis, but no HCC (0.312 (IQR 0.169 – 0.774)) p=0.198.

	All participants (n=83)	Participants without Cirrhosis	Participants with cirrhosis	Reference Values*
Angiopoietin 1 (ANG1) pg/mL	23990 (15730 – 37480)	30090 (20240 – 50990)	19690 (10252 – 31115)	37122 range (14272 – 65570)
Angiopoietin 2 (ANG2) pg/mL	4017 (2386 – 6235)	3300 (2325 – 4262)	5577 (2721 – 9230)	2494 ± 1341 range (1065 – 8907)
ANG2/ANG1 Ratio	0.1688 (0.0805 – 0.3590)	0.0982 (0.0593 – 0.1724)	0.2853 (0.1683 – 0.6355)	

Table 2.3 – Median (IQR) Angiopoietin levels and ANG2/ANG1 ratio when compared to reference values. *These values are those reported in healthy subjects by R&D systems, (ELISA manufacturers).

2.4.9 Variation by Aetiology:

The results by aetiology of liver disease are reported in table 2.5 below

Aetiology of Liver Disease	n	% cirrhosis	HVPG (mmHg)	Bilirubin (umol/L)	CPA (%)	Ang-1 (pg/mL)	Ang-2 (pg/mL)	ANG2/ANG1 Ratio	ELF (HV)	ELF (Serum)	MELD	UKELD
Hepatitis C	27	33	5	11	9	23930	4492	0.1723	9.08	9.94	9	48
Alcohol	25	88	14	134	22	20576	6800	0.4140	12.33	12.61	15	54
Autoimmune	13	31	4	10	10	35485	2880	0.0976	9.32	9.73	8	46
NAFLD	9	78	11	26	21	24850	3998	0.1681	10.10	10.02	13	53
Cryptogenic	6	50	8	11	12	20480	3151	0.2403	9.96	9.74	8	46
Hepatitis B	6	50	4.5	15	9	24045	2837	0.0861	8.45	8.68	9	47
NRH / EHPVO	5	0	3	13	5	23990	1948	0.1111	10.04	10.76	6	46
DILI	3	0	3	9	4	49030	2412	0.0497	7.18	7.75	6	44
ALD/NAFLD	2	100	19	196	29	10740	23798	2.2159	14.25	16.53	26	61
Alpha 1	1	100	14	11	-	-	-	-	-	13.3	9	49
Amyloid	1	0	3	11	32	50990	4107	0.0805	9.80	10.23	15	45
Budd-Chiari	1	0	15	23	5	-	-	-	-	12.3	9	49
CLL	1	0	8	16	2	3080	1953	0.6340	9.69	10.03	10	50

Table 2.4 – Median results of HVPG, Bilirubin, CPA, ANG1, ANG2, ANG2/ANG1 ratio, ELF (HV and Serum), MELD and UKELD separated by aetiology of liver disease (all participants included).

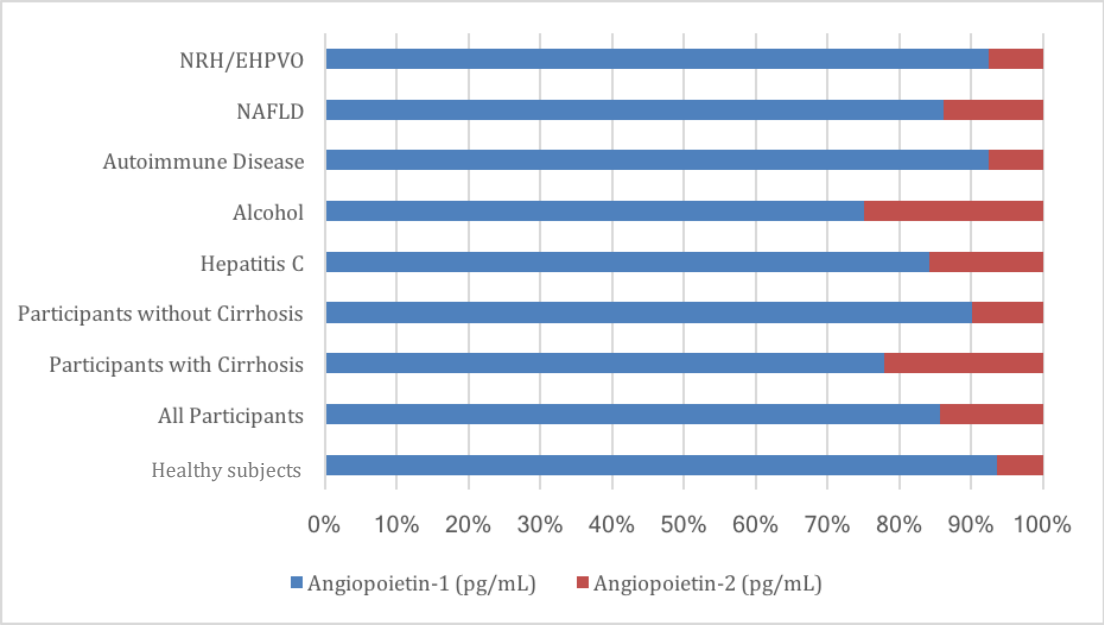

Figure 2.4 – A summary of the Angiopoietin 1 / Angiopoietin 2 ratios by aetiology of liver disease and in healthy subjects.

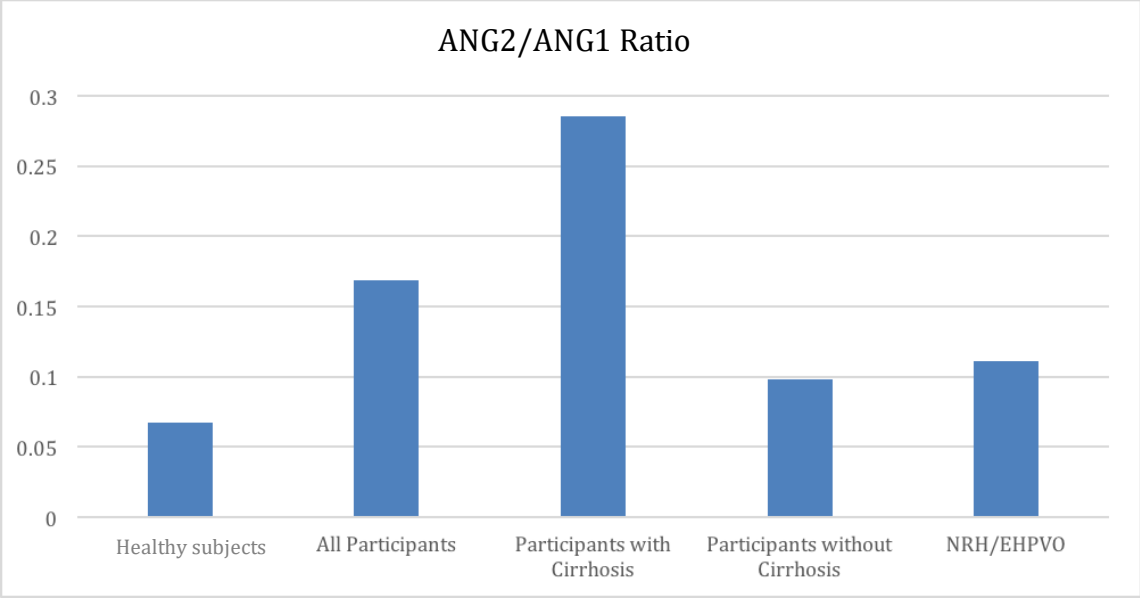

Figure 2.5 – The Angiopoietin-2 / Angiopoietin-1 ratio in participants with and without cirrhosis, with pre-sinusoidal portal hypertension (NRH/EHPVO) and in healthy subjects.

2.4.10 Angiopoietin levels in participants with HCC

The angiopoietin levels, as well as the ELF, MELD, HVPG and AFP levels are shown in the table below. There was no significant difference in HVPG, ANG1, ANG2 or the ANG2/ANG1 ratio in participants with cirrhosis, with or without HCC. The ELF and AFP were significantly different in these two groups.

	All Participants with Cirrhosis (n=52)	Cirrhosis and HCC (n=14)	Cirrhosis and no HCC (n=38)	
ANG1 (pg/mL) Median (IQR)	19690 (10253 – 31115)	21665 (13470 – 33917)	19690 (9663 – 28765)	p = 0.715
ANG2 (pg/mL)	5578 (2721 – 9230)	4120 (2564 – 7213)	6238 (3485 – 10745)	p = 0.316
ANG2/ANG1 Ratio	0.285 (0.168 – 0.636)	0.200 (0.076 – 0.513)	0.312 (0.169 – 0.774)	p = 0.198
AFP (kunits/L)	4.55 (2.88 – 8.88)	17.25 (5.80 – 663.73)	3.20 (2.48 – 4.73)	p = 0.001
HVPG (mmHg)	14 (8 – 17)	12 (7 – 15)	14 (9 – 17)	p = 0.755
MELD	13 (9 – 24)	12 (8 – 19)	14 (9 – 25)	p = 0.469
ELF	11.59 (10.08 – 13.29)	11.05 (9.49 – 11.58)	12.51 (10.33 – 14.71)	p = 0.029

Table 2.5 – Summary of the angiopoietin levels, AFP, HVPG, MELD and ELF results in participants with cirrhosis, with and without Hepatocellular carcinoma.

2.5 DISCUSSION

The median HVPG, ELF, MELD and UKELD scores, shown in table 2.2, are higher in participants with cirrhosis. Serum peripheral ELF levels are consistent with previously reported levels in patients with fibrosis and cirrhosis.[368] Previously reported cut-off values for ELF suggest <7.7 to exclude fibrosis, ≥ 7.7 to <9.8 for moderate fibrosis, ≥ 9.8 to < 11.3 for severe fibrosis and ≥ 11.3 for cirrhosis[375]. Most aetiology groups contained some patients with cirrhosis so comment on the median ELF in all aetiological groups is not possible.

The CPA levels were significantly elevated in participants with cirrhosis with a median CPA of 26%. Previous investigators have reported a cut-off value of

13.8% in patients with cirrhosis and portal hypertension.[140] The ALT and AST levels were similar in both participants with and without cirrhosis.

The Angiopoietin levels, when compared to levels previously reported in healthy subjects, provided by the manufacturer of the ELISA kits, were abnormal in participants both with and without cirrhosis. The results suggest that in participants with cirrhosis there is a tendency towards a pro-neovasculogenic Tie-2 phenotype with a reduction in the stabilising ANG1 and an increase in the destabilising ANG2. These results support a hypothesis that liver fibrosis is a pro-angiogenic condition and that angiogenesis/neo-vasculogenesis plays a role in the development and progression of portal hypertension. Previous investigators have shown that the levels of ANG2 are raised in patients and in animals with cirrhosis.[335 376 377] One recent study found a mean circulating ANG2 level in patients with cirrhosis (6230 $\pm$ 3053 pg/mL) similar to the levels we measured in our participants.[378] A second, larger investigation of 179 patients with chronic hepatitis C showed that the ANG2 levels increased significantly, and the ANG1 levels decreased, with advancing fibrosis. The same investigators showed that the ANG2/ANG1 ratio could detect liver cirrhosis with an AUROC of 0.810[379].

The clinical phenotype of portal hypertension is known to be different in patients with pre-sinusoidal portal hypertension, such as those with NRH or EHPVO. This study found that the 5 participants in this group exhibited marked variation in the pattern of angiopoietin levels with a reduction in both ANG1 and ANG2 levels. The median ANG2 in this group of participants, at 1948 pg/mL, was the

lowest of any group investigated and lower than the normal levels quoted by the ELISA manufacturers. No previous reports of serum angiopoietin levels in NRH could be identified in the literature. This observation raises questions about the pathophysiology behind the development of portosystemic collateral vessels in NRH. The histological diagnosis of NRH is challenging but is said to be characterised by the presence of micronodularity and enlarged or thickened liver cell plates in the absence of fibrous septa[380]. There may also be features of sinusoidal dilatation. NRH is associated with portal hypertension and large vessel collateralisation, and these findings suggest that a reduction in ANG1 but not an increase in ANG2 occurs in these cases. As ANG2 is also increased more generally in endothelial dysfunction, these low levels found in this condition may support a hypothesis of low levels of endothelial activation in NRH[381]. The serum ELF tests were elevated in the group of patients with NRH, which has not been previously observed and is not consistent with the histological appearances of no/minimal fibrosis. These observations of the NRH group warrant further investigations into the phenotype of fibrosis and endothelial dysfunction in patients with NRH.

In the study population, there was no significant difference in the circulating angiopoietin levels in patients with cirrhosis who did and did not have HCC. This was similar to results reported previously by Mitsuhashi, where the expression of ANG1, ANG2 and VEGF in 42 patients with HCC was evaluated.[340] Their study found a high ANG2/ANG1 mRNA ratio (and an associated reduction in survival) in resected HCC specimens, however the levels of expression were not different to that found in adjacent, non-tumour,

liver tissue. The same pattern was observed for VEGF mRNA expression. These authors concluded, that a high ANG2/ANG1 ratio was associated with a poorer survival due to increased tumour angiogenesis, however if the ratio is also related to the severity of cirrhosis, then this increased mortality may be related to worsening liver function. The data on ANG2 as a biomarker for HCC is conflicting, however, and one early study reported an increased ANG2 expression in 12 samples of resected HCC specimens and a significant difference between the tumoural ANG2 expression and the lower expression in background tissue.[341] A study of 33 patients suggested that ANG2 levels did correlate with tumour severity, however the authors also reported that the levels increased with severity of liver disease.[382] The largest population reported (by Scholz et al) suggested that serum ANG2 was elevated in 131 patients with HCC, over and above the elevation seen in 180 patients with cirrhosis.[335] Detailed information about the severity of liver disease, such as MELD score, was not available, however the investigators did try to select controls with a broad spectrum of Child-Pugh scores.

The levels of Angiopoietins have also previously been evaluated in 14 patients with focal nodular hyperplasia and it was noted that there was an upregulation of ANG1 expression and a converse downregulation of ANG2 expression. In a sample of 13 patients with cirrhosis, the same investigators did not appreciate any difference in the ANG2/ANG1 expression.[345]

The participant with a previous Budd-Chiari syndrome which required transplantation was reported to have NRH on liver biopsy but both the HVPG

and the ELF score were elevated. It is possible that the biopsy was under representative of the burden of fibrosis in the liver in this case.

There was a significantly higher ELF score in participants with cirrhosis and no HCC, in comparison to those with cirrhosis and HCC. There is no obvious basis for this and this has not been previously reported. It is likely that this is related to the small sample size.

In Summary, in this heterogenous population of patients with liver disease the ANG2/ANG1 ratio, along with the CPA and ELF were shown to be elevated in patients with cirrhosis. The ANG2/ANG1 ratio was not correlated with the presence of HCC in this population.

LIMITATIONS OF THE STUDY

The broad inclusion criteria for this study have delivered a very heterogeneous population of patients with liver disease from varying aetiologies, at various stages of fibrosis, including 17 patients with HCC and 25 patients who had received a previous liver transplant. 7/25 patients with a history of a previous liver transplant had graft cirrhosis. The advantage of this population is that the results could potentially be applied to a broad range of patients with liver disease. The heterogeneity does, however, mean that it may be difficult to identify trends or significant differences within the small sample size of 100 participants. The results show that there is considerable variation in

Angiopoietin and ELF results by the aetiology of liver disease, particularly the group with pre-sinusoidal portal hypertension, and by the stage of fibrosis for those with chronic liver disease. With such small number of participants in each cohort it is not possible to confirm patterns and further larger scale investigations are required.

FURTHER WORK

The main priority will be to repeat the sampling in larger more homogenous populations of participants in order to validate the trends seen in this study. Further work will be required to assess the role of the Tie-2 axis in patients with pre-sinusoidal portal hypertension. Whilst this study suggests that this may be a difference phenotype to portal hypertension in cirrhosis, the small number mean that additional investigations are required to confirm this finding. More work is required on the use of angiopoietins as biomarkers of hepatocellular carcinoma and future prospective studies should be designed to evaluate the contributions of both the presence and severity of HCC and the progression of liver disease to the serum levels.

Chapter 3: Comparison of peripheral and Hepatic Vein serum levels of ELF and Angiopoietins.

3.1 BACKGROUND

There is an increasing need for reliable serum markers of liver disease and accurate predictors of fibrosis portal hypertension and hepatic decompensation would be valuable tools for the clinician. While biomarkers of fibrogenesis and fibrosis regression may reflect the severity of fibrosis in the liver it is not known how well measurement of these same biomarkers in the peripheral blood might reflect their levels in the hepatic circulation. We aimed to measure the levels of angiopoietins and ELF score constituent proteins in serum taken peripherally and that obtained from hepatic vein samples, and to assess for any variability between the two sampling sites.

3.2 OBJECTIVES

1. To assess for any significant variation in serum peripheral and hepatic vein levels of ELF and angiopoietins-1 and -2.

3.3 METHODS

The study design, research and ethics committee approval, participant eligibility, consent and study procedure are as detailed in that in Chapter 2.

3.3.1 Peripheral Serum Sample collection

Each participant attended the interventional radiology unit for hepatic venous pressure measurement and transjugular liver biopsy. When the participant

entered the procedure room, and prior to the insertion of the jugular venous sheath, a peripheral venous sample of blood was taken from a forearm or antecubital fossa vein. A disposable tourniquet was used and samples were obtained in accordance with local infection control policies. 10mls of serum was obtained in two serum separator tubes using a closed vacutainer system and a 21G venesection needle.

3.3.2 Hepatic Vein Sample Collection

Once a sheath had been placed in the hepatic vein, and prior to the transjugular biopsy and HVPG measurements, 10mls of blood was aspirated from the hepatic vein and transferred into plain serum separator tubes.

3.3.3 Sample processing

Samples were processed as detailed in chapter 2.

3.3.4 ELF and Angiopoietin Level Measurement

The ELF score and Angiopoietin levels were measured using the methods described in detail in chapter 2.

3.4 RESULTS

Peripheral serum ELF score was available for 100 participants and a corresponding hepatic vein ELF score for 87 of those. Paired hepatic vein and peripheral serum samples of ANG1 and ANG2 were available for 84 participants. The levels of ELF, its constituent proteins, Angiopoietin-1 and -2

are detailed in table 3.1. There is a strong correlation between serum peripheral and hepatic vein ELF (r^2 = 0.967, p< 0.001).

	Peripheral Serum Median (IQR)	Hepatic Vein Median (IQR)	Correlation co-efficient (r^2)
ELF Score	10.49 (8.87 – 12.22)	10.04 (8.51 – 11.91)	0.967 (p<0.001)
HA (ng/mL)	108.2 (39.3 – 545.8)	98.4 (22.6 – 477.1)	0.967 (p<0.001)
P3NP (ng/mL)	10.92 (6.47 – 23.83)	9.4 (5.8 – 19.1)	0.923 (p<0.001)
TIMP-1 (ng/mL)	294.1 (188.3 – 493.7)	268.3 (173.9 – 422.5)	0.918 (p<0.001)
Angiopoietin 1 (pmol/mL)	23990 (15730 – 37480)	21600 (12000 – 34900)	0.845 (p<0.001)
Angiopoietin 2 (pmol/mL)	4017 (2387 – 6236)	3414 (2268 – 5677)	0.878 (p<0.001)

HA - hyaluronic acid, P3NP - amino terminal propeptide of type III procollagen, TIMP-1 - tissue inhibitor of metalloproteinase 1

Table 3.1 The correlation of peripheral and hepatic vein serum levels of ELF, its constituent proteins and Angiopoietins.

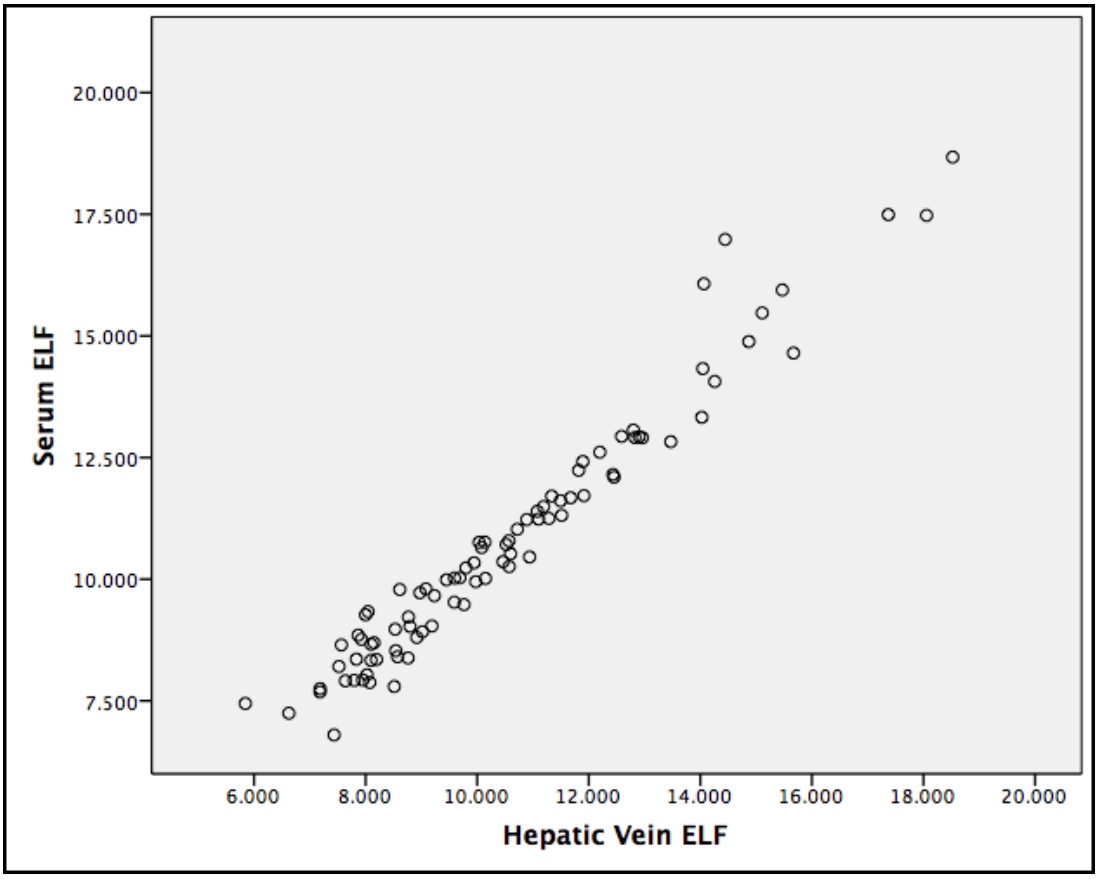

Figure 3.1 Correlation of serum peripheral and hepatic vein ELF scores. r=0.967 ($p<0.001$).

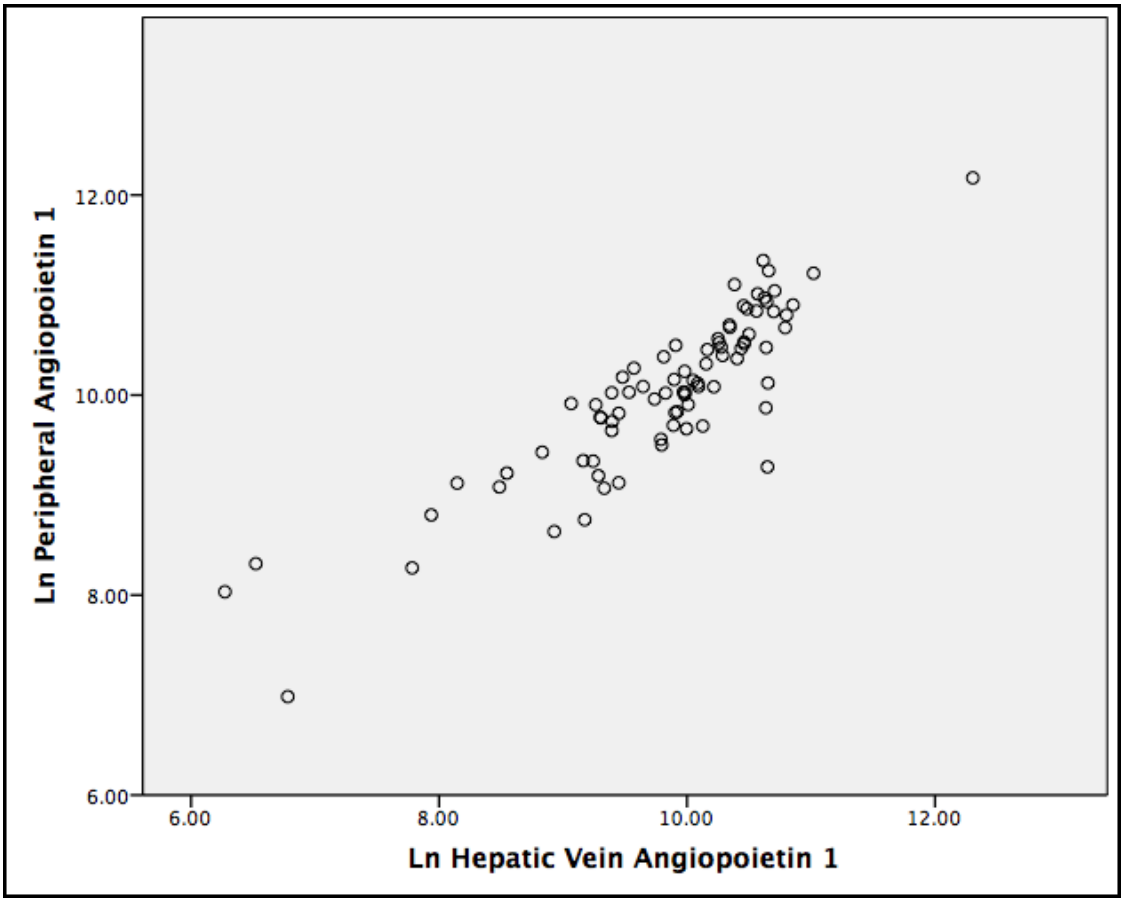

Figure 3.2 Correlation of peripheral and hepatic vein serum Angiopoietin-1 levels. $r = 0.845$ ($p<0.001$)

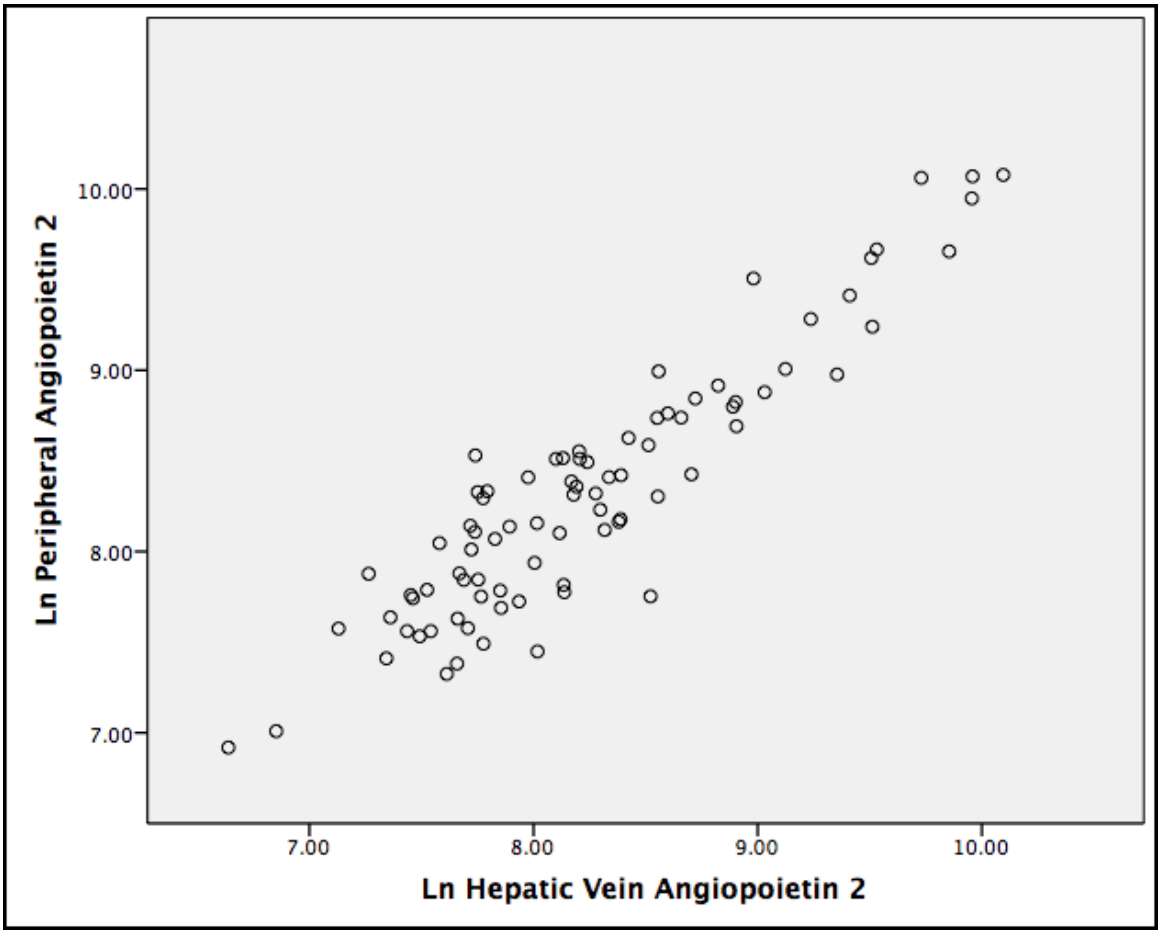

Figure 3.3 Correlation of peripheral and hepatic vein serum Angiopoietin-2 levels. r = 0.878 ($p<0.001$).

3.5 DISCUSSION

We have shown a strong and significant correlation between the hepatic vein and peripheral blood ELF scores and Angiopoietin-1 and -2 levels. These results suggest that the peripheral levels reflect the hepatic vein levels and can be used as a surrogate of the levels draining from liver sinusoids.

There is one previous publication where the constituent components of the ELF score (HA, TIMP and P3NP) were measured in paired hepatic and peripheral venous samples. In this study the authors found a significant difference in the

levels of HA and TIMP-1, but not P3NP in patients with chronic viral hepatitis. They used the same ELISA assay and performed the analysis on paired samples simultaneously, yet the results were significantly different from our own[383]. There is no straightforward explanation for the disparity obtained in the results by Suk et al, but further evaluation would be beneficial to confirm our results.

The peripheral and hepatic vein levels of angiopoietins have been assessed previously, by investigators evaluating the potential role of angiopoietins in hepatocellular carcinoma. Diaz-Sanchez and colleagues found a similar correlation in 33 patients with HCC and cirrhosis ($r^2 = 0.95$, $p < 0.001$).[382] Kuboki et al reported ANG2 levels in 21 patients with HCC undergoing surgical resection. They suggested that the levels of ANG2 were higher in the samples from the hepatic vein, however this difference was not statistically significant, and the sample size was small.[384]

Overall, these results suggest that peripheral serum samples can be used to evaluate Angiopoietins and ELF in patients with liver disease.

Chapter 4: Correlation of ELF, Angiopoietins and CPA with Hepatic Venous Pressure Gradient

4.1 BACKGROUND

Measurement of the hepatic venous pressure gradient is now the 'gold standard' tests used to assess portal hypertension. When this is not available another invasive test, Endoscopy (to assess for varices and portal hypertensive gastropathy), is used as an alternative. These tests are reliable, as discussed in Chapter 1, however, there is a need to develop biomarkers to offer minimally invasive options for diagnosis, prognostication and to assess response to therapies.

4.2 OBJECTIVES

1. To assess the correlation of markers of angiogenesis and fibrosis, measured in peripheral blood, with the degree of portal hypertension (as measured by the hepatic venous pressure gradient).
2. To correlate the serum markers of angiogenesis and fibrosis with liver histology.

4.3 HYPOTHESIS

1. Serum levels of Angiopoietin-1 (ANG1), Angiopoietin-2 (ANG2), and the ANG2/ANG1 ratio will correlate with the degree of portal hypertension with a linear correlation to HVPG.

4.4 METHODS

The study design, research and ethics committee approval, participant eligibility, consent and study procedure are described in detail in Chapter 2.

4.4.1 Hepatic Venous Pressure Gradient Measurement

All procedures were undertaken by experienced personnel operating in accordance with agreed protocols for the measurement of HVPG. The procedure for measurement is detailed below:

- Participants attended the Interventional Radiology unit for their procedure having fasted for a minimum of 6 hours.
- Participants were offered sedation with intravenous midazolam (0.5 – 2mg) in accordance with local practice.
- The participant's skin was cleaned and draped in accordance with local infection control policies.
- Ultrasound guided cannulation of the Internal Jugular Vein (usually right) was undertaken, and a single venous sheath placed into the superior vena cava.
- A guide wire was advanced into the right hepatic vein under fluoroscopic guidance (either a standard 0.35 J-wire or a hydrophilic 'Terumo' wire).
- A 1.25cc Berenstein Occlusion Balloon Catheter (Boston Scientific, USA) was then advanced into the hepatic vein.
- Pressure measurements were undertaken with a standard pressure-transducer system. The zero point was set externally at the mid-axillary line.

- The free hepatic venous pressure was measured approximately 2-3cm from the HV/IVC junction.
- The catheter balloon was inflated, and a wedge position checked by the injection of a small amount of intravenous contrast, then the wedged hepatic venous pressure was measured.
- Each measurement was recorded once the pressure had reached a steady reading and after at least 1 minute.
- A minimum of 3 repeated measurements of the free and wedged hepatic vein pressure were recorded until consistent readings were obtained.
- The gradient was calculated as the difference between the wedged hepatic vein pressure and the free hepatic vein pressure in accordance with accepted international standards[127].
- All measurements were recorded in the clinical notes.

4.4.2 Transjugular Liver Biopsy

Following HVPG measurement a transjugular biopsy was performed where clinically indicated. A biopsy was not performed via this route in patients with known hepatocellular carcinoma. Transjugular biopsy was performed using a 19G, 20mm throw, bevelled tip Bio-Cut™ semi-automatic biopsy device (Kimal PLC, Middlesex, UK). A minimum of 3 cores were obtained to ensure a sufficient quantity of tissue was obtained for accurate histological analysis. In those participants who were having HVPG measured in preparation for hepatic resection, background liver tissue obtained at the time of resection, was used for histological analysis.

4.4.3 Histological Processing

Liver biopsy samples were formalin fixed, paraffin embedded, and stained with hematoxylin & eosin, and the Gordon & Sweet method for reticulin. All samples were reviewed by the same pathologist and the Ishak stage was recorded.

ISHAK SCORE[373]

Stage	Description
0	No fibrosis
1	Fibrous expansion of some portal areas, with or without short fibrous septa
2	Fibrous expansion of most portal areas, with or without short fibrous septa
3	Fibrous expansion of most portal areas, with occasional portal to portal bridging
4	Fibrous expansion of portal areas with marked bridging (portal to portal) as well as portal to central
5	Marked bridging (portal to portal and/or portal to central) with occasional nodules (incomplete cirrhosis)
6	Cirrhosis, probable or definite

Table 4.1 – Ishak Score for assessing stage of liver fibrosis.

4.4.4 Collagen Proportionate Area (CPA)

Sections of each histological sample were stained with picro-Sirius Red for digital image analysis. This analysis was performed by one operator in the department of histopathology. After whole-section digital image capture, CPA

was measured using a visual basic script for Zeiss Axiovision (version 4.8.2.) in which binary segmentation of RGB colour channels was used to distinguish liver tissue from collagen. The CPA measurement process included a manual editing step to eliminate image artifacts, and operator-dependent thresholding to determine the stained area of the section. The CPA was calculated as the area occupied by the collagen as a proportion of the area of the whole parenchyma and expressed as a percentage.

4.4.5 Statistics

Medians were compared using the appropriate non-parametric test (Mann-Whitney U). Correlation co-efficient (r) was calculated using the Spearman's rho test and Area Under the Receiver Operator Characteristic (AUROC) curve analysis was used to predict the ability of non-invasive markers to identify portal hypertension. Sensitivity and specificity were determined using cross-tabulation, allowing the positive predictive and negative predictive values to be calculated. SPSS v22, IBM, USA was used for all statistical analysis.

4.5 RESULTS – including all participants.

4.5.1 Correlation of Angiopoietins, ELF, CPA and Ishak stage with HVPG

The correlation coefficients of ANG1, ANG2 and the ANG2/ANG1 ratio with HVPG were -0.505 ($p < 0.001$), 0.471 ($p<0.001$) and 0.670 ($p<0.001$) respectively. HVPG correlated with ELF, CPA and Ishak stage with correlation coefficients of 0.729 ($p<0.001$), 0.510 ($p<0.001$) and 0.684 ($p<0.001$)

respectively. HVPG also correlated with MELD and UKELD with correlation coefficients of 0.565 ($p<0.001$) and 0.614 ($p<0.001$). See Figures 4.1-4.4.

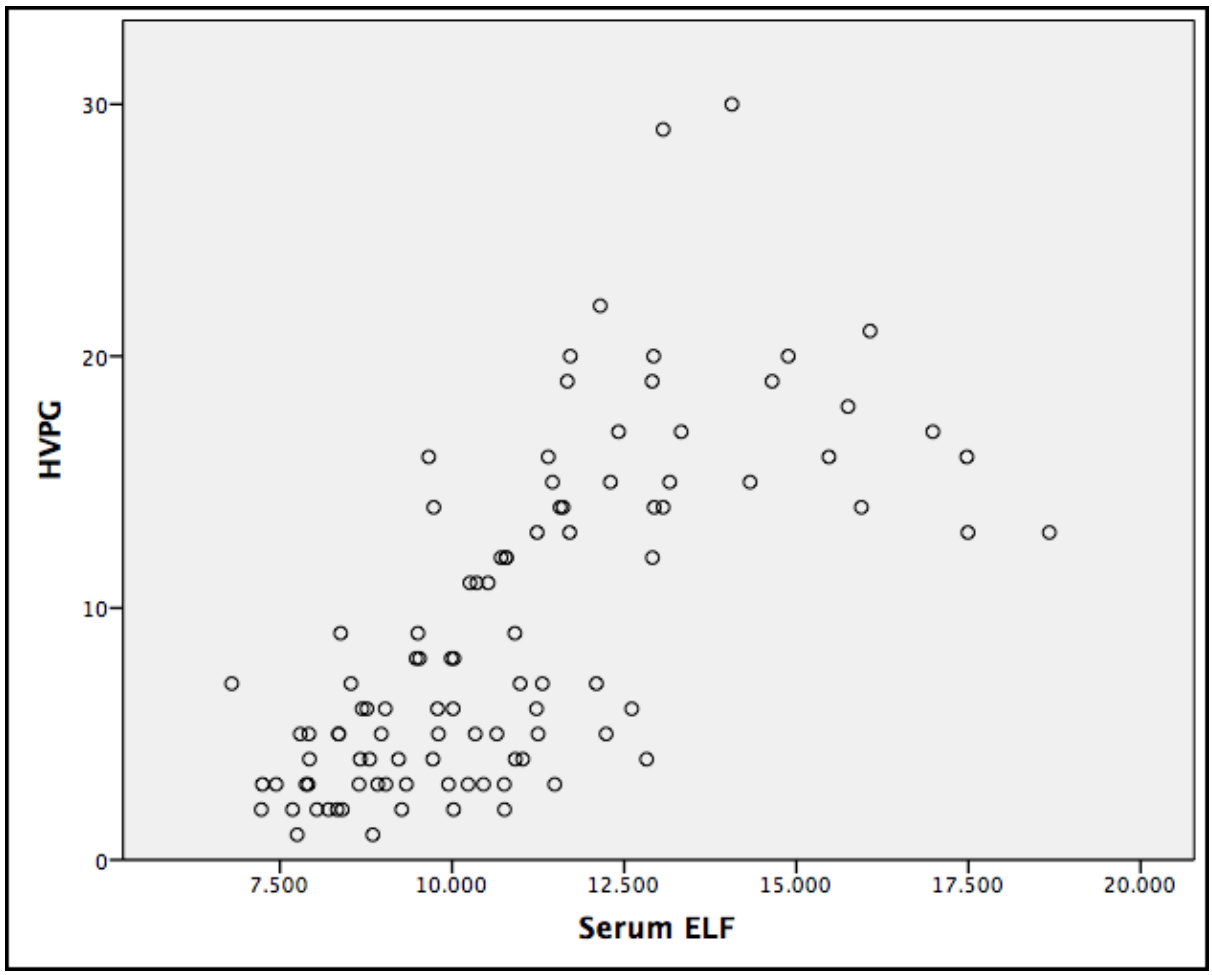

Figure 4.1. Correlation of Serum ELF and HVPG (all participants), r= 0.729 ($p<0.001$).

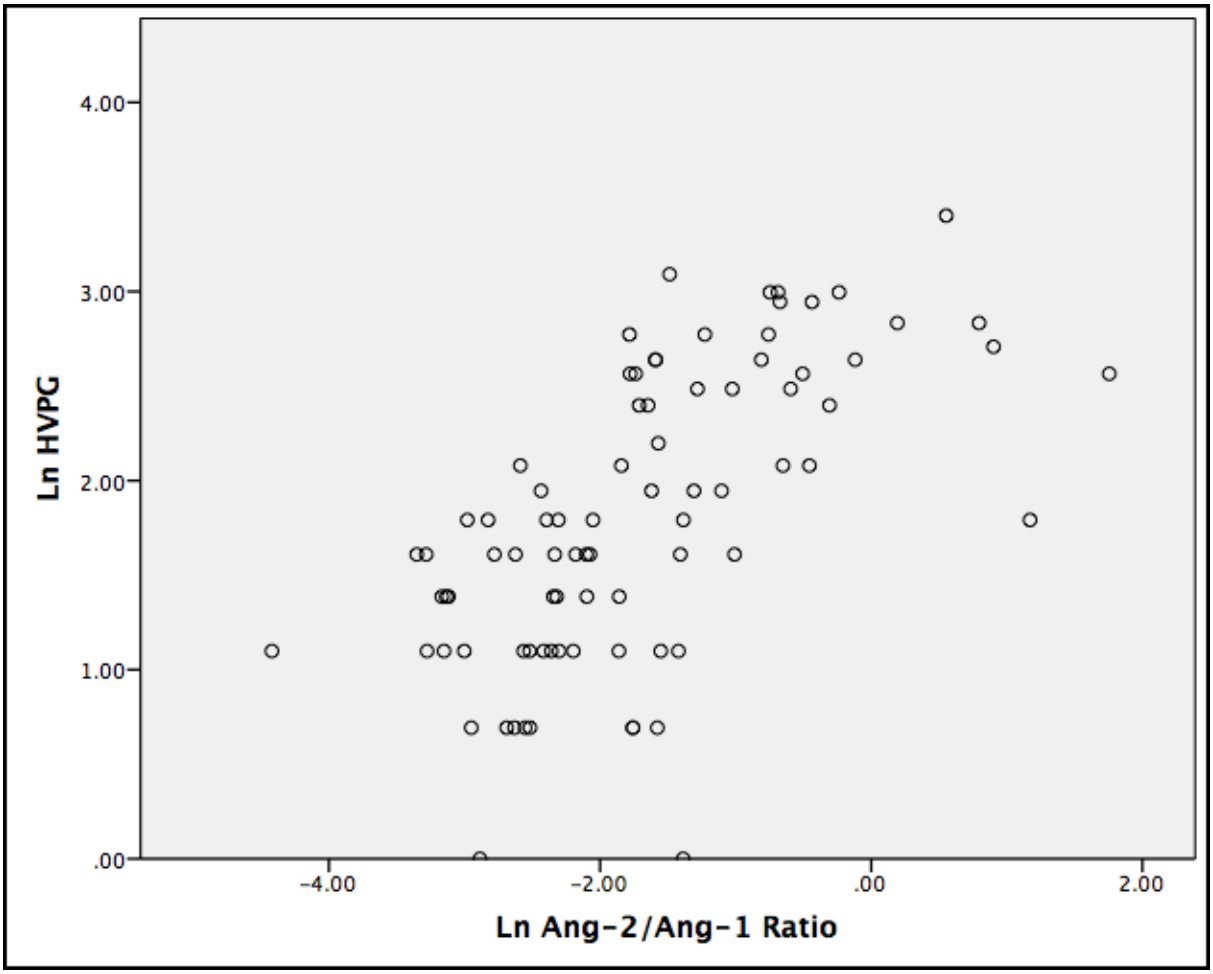

Figure 4.2. Correlation of HVPG and ANG2/ANG1 ratio (all participants), r = 0.670 ($p<0.001$).

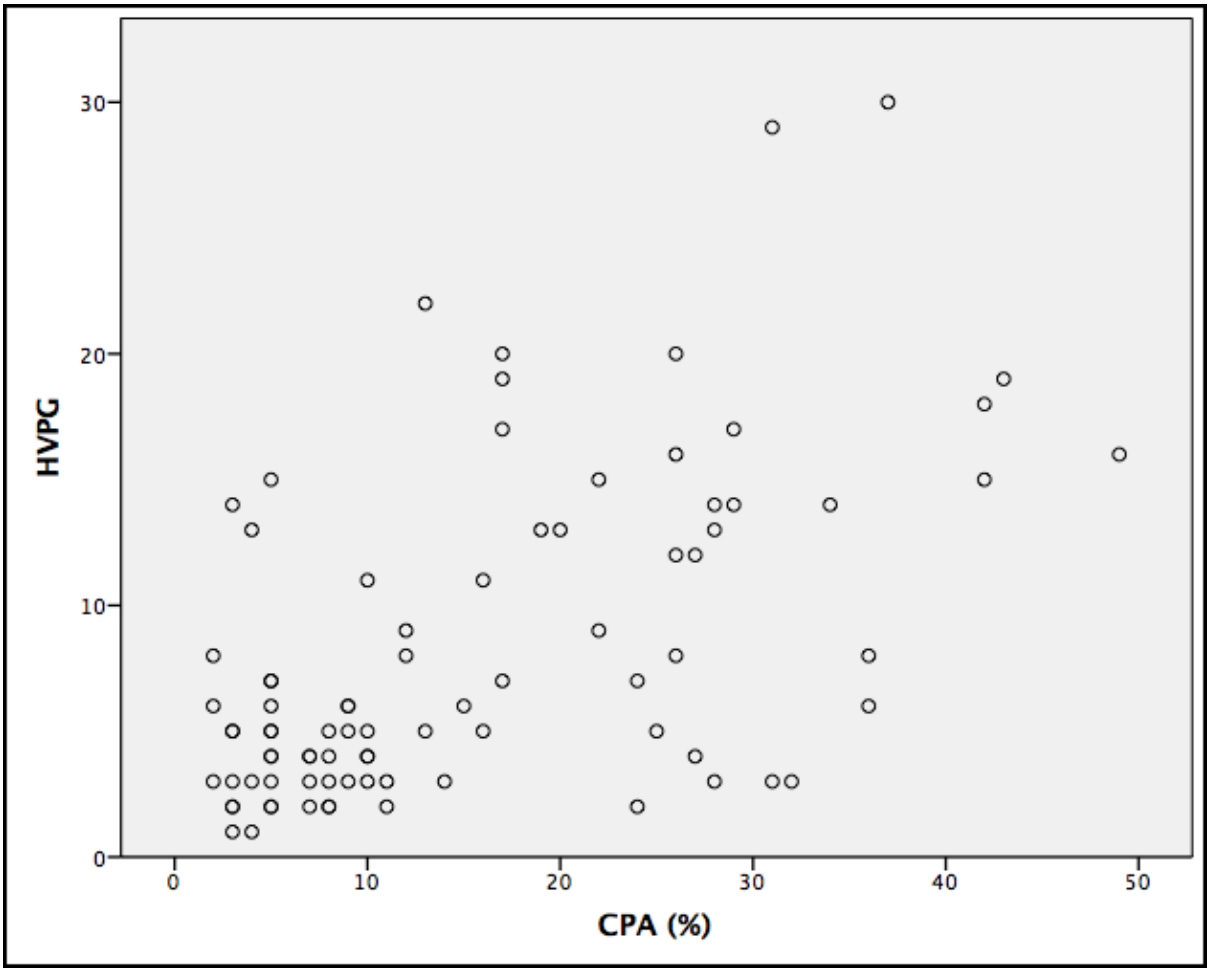

Figure 4.3. Correlation of HVPG with CPA (all participants), r = 0.510 ($p<0.001$).

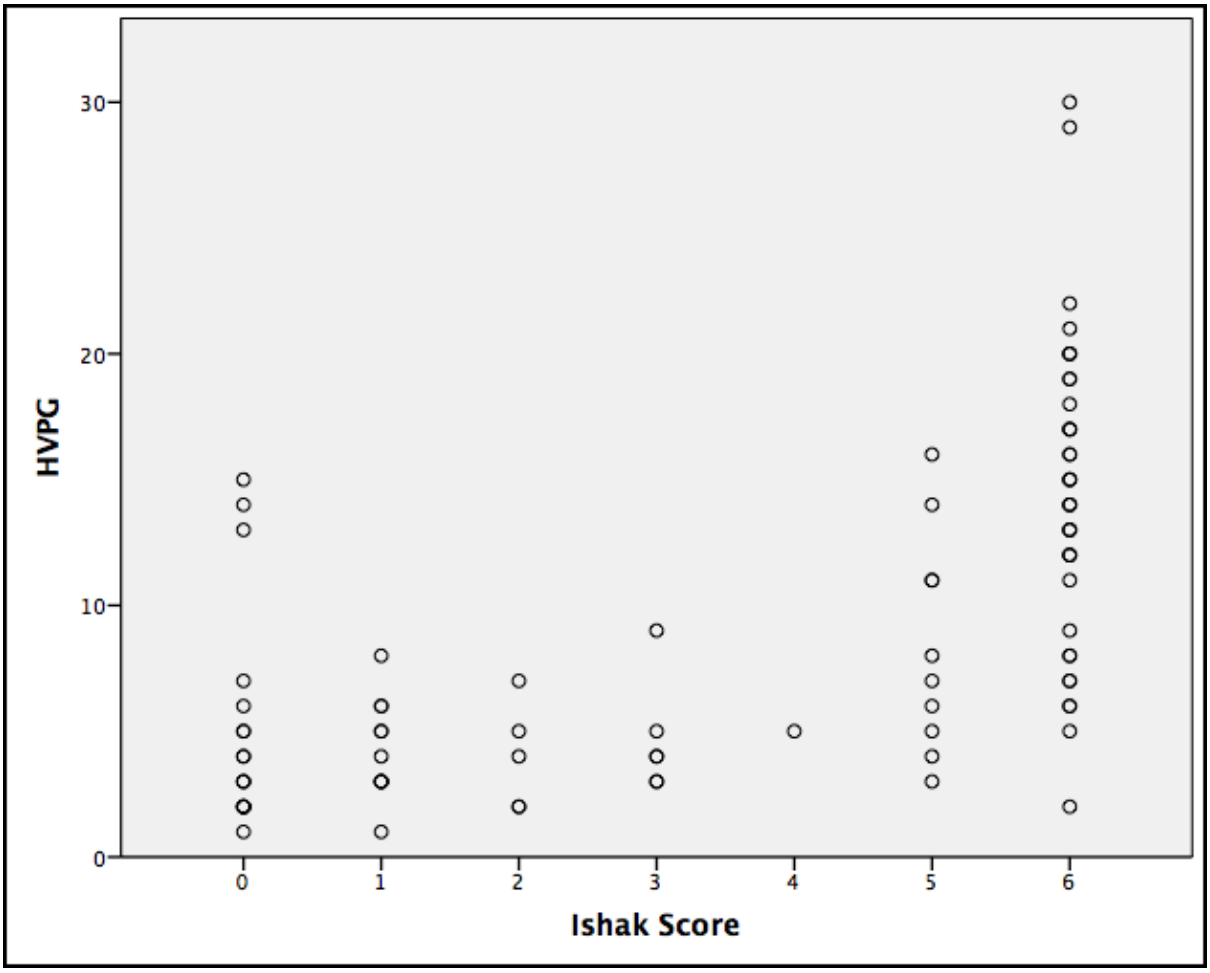

Figure 4.4. Correlation of HVPG with ISHAK stage (all participants), 0.684 ($p<0.001$) .

4.5.2 The ability of Angiopoietins, ELF and CPA to predict significant portal hypertension.

An ELF of > 9.8 predicted the presence of clinically significant portal hypertension with a sensitivity of 94.9% and a specificity of 60.7% (PPV 60.7%, NPV 94.9%).

Receiver Operator Characteristic curve analysis showed that peripheral serum ELF levels can predict the presence of portal hypertension (HVPG ≥ 6mmHg) with an AUROC of 0.840 [95% CI 0.764-0.916]; clinically significant portal hypertension (HVPG ≥ 10mmHg) with an AUROC of 0.924 [95% CI: 0.875-0.973]; and severe portal hypertension (HVPG > 12mmHg) with an AUROC of 0.940 [95% CI; 0.895-0.984].

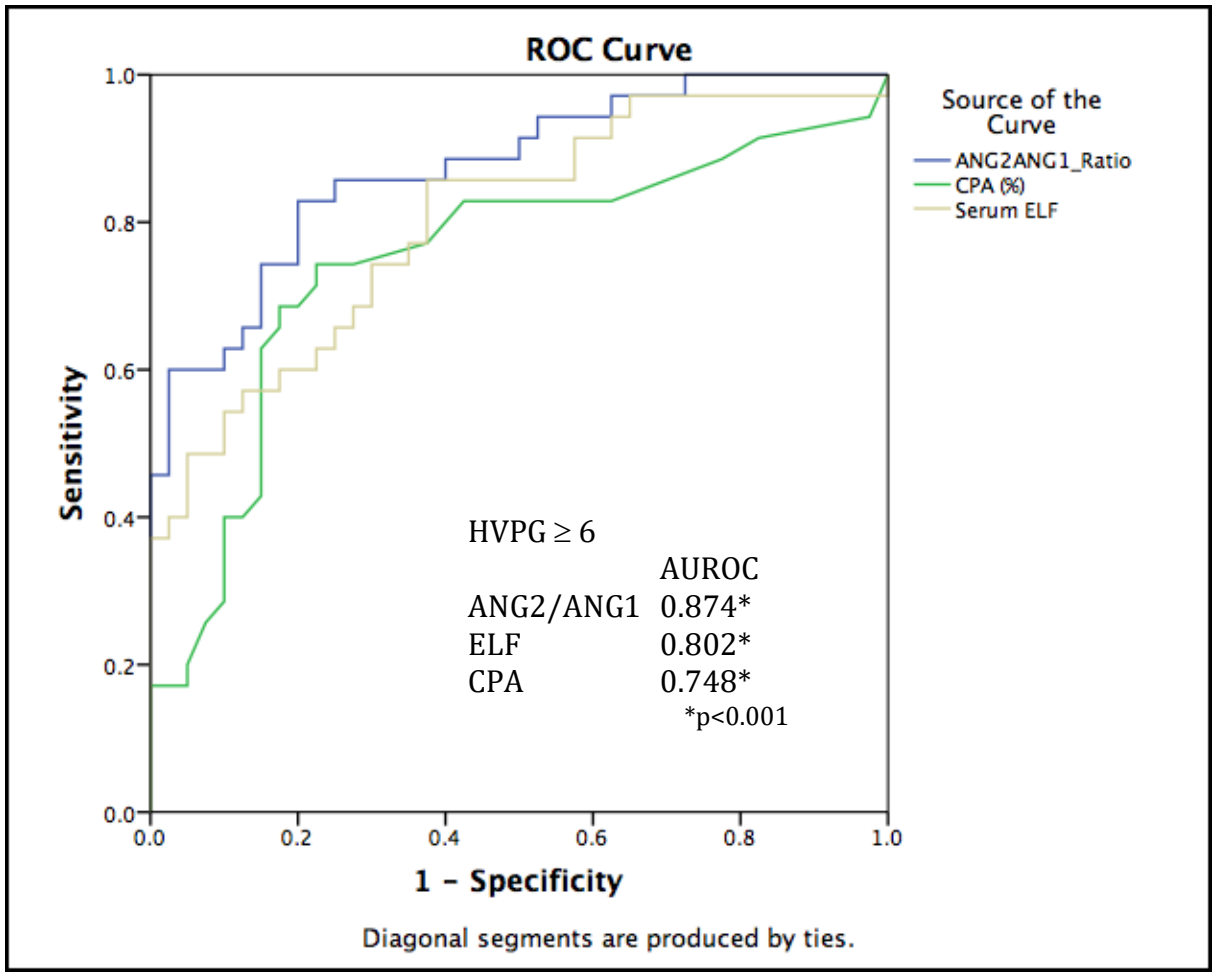

Figure 4.5. The ROC curve for the ability of ANG2/ANG1 Ratio, ELF and CPA to predict an HVPG ≥ 6 in all participants.

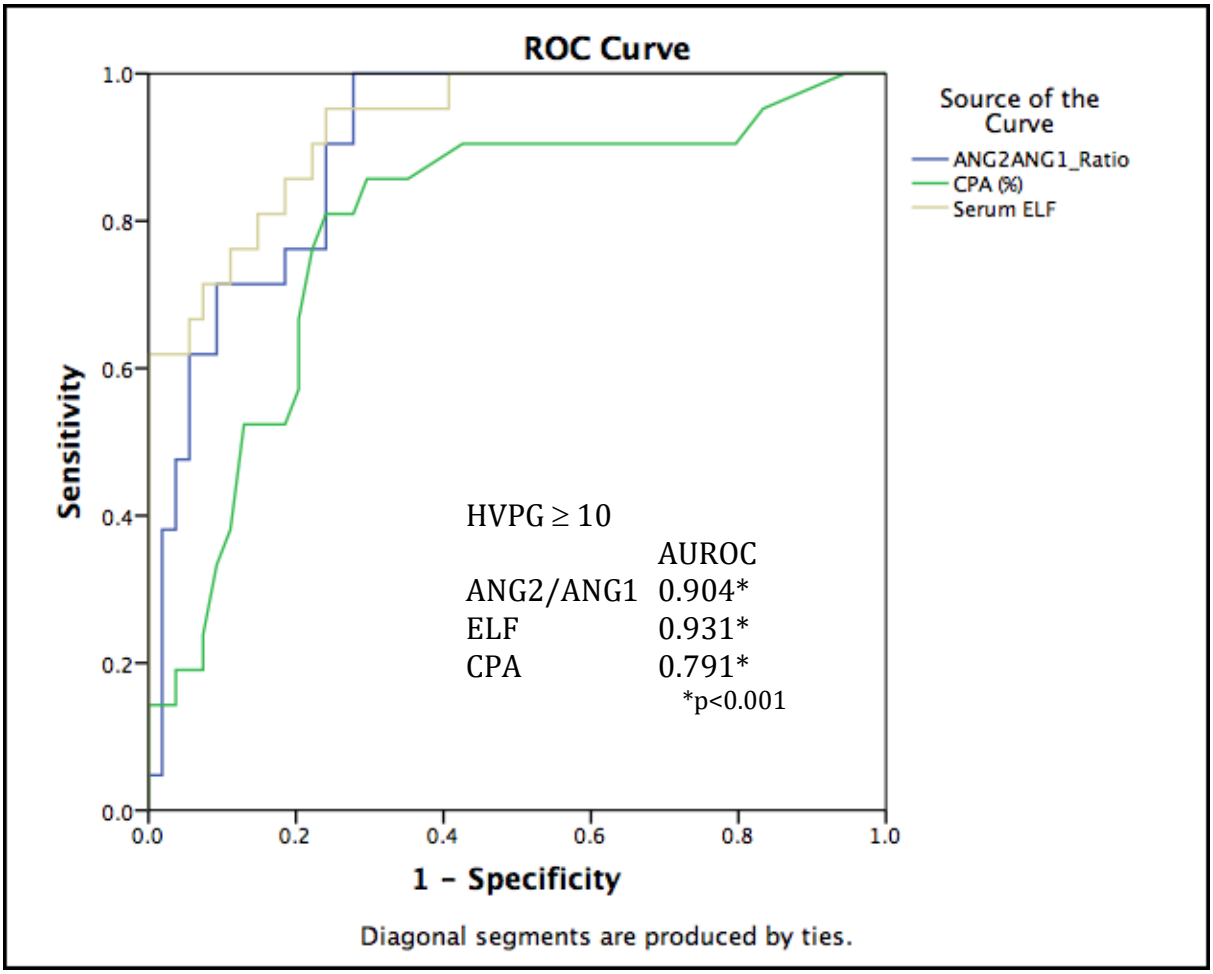

Figure 4.6. The ROC curve for the ability of ANG2/ANG1 Ratio, ELF and CPA to predict an HVPG ≥ 10 in all participants.

CPA can predict an HVPG ≥ 6mmHg, ≥ 10mmHg and ≥ 12mmHg with AUROCs of 0.756 [0.648-0.863], 0.804 [0.687-0.906] and 0.814 [0.704-0.924] respectively.

Receiver Operator Characteristic curve analysis showed that the peripheral ANG2/ANG1 ratio can predict the presence of portal hypertension (HVPG ≥ 6mmHg) with an AUROC of 0.874 [95% CI; 0.795 – 0.952]; clinically significant portal hypertension (HVPG ≥ 10mmHg) with an AUROC of 0.904 [95% CI:

0.836 – 0.964]; and severe portal hypertension (HVPG > 12mmHg) with an AUROC of 0.896 [95% CI; 0.825 – 0.966].

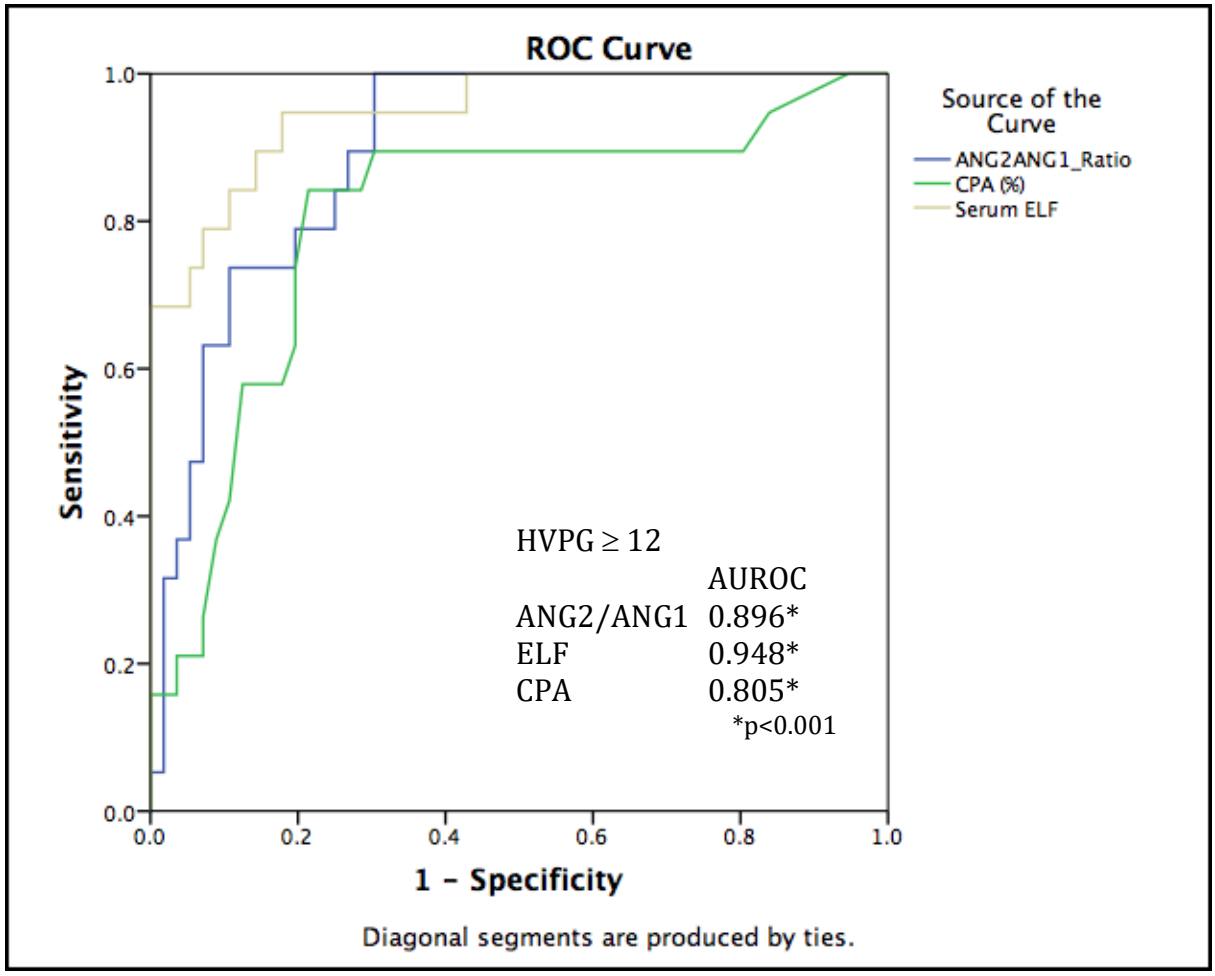

Figure 4.7. The ROC curve for the ability of ANG2/ANG1 Ratio, ELF and CPA to predict an HVPG ≥ 12 in all participants.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1 Ratio	0.1	86.05	60.00	2.15	0.23	69.81	80.00
	[95% CI]	[72.07 – 94.70]	[43.33 – 75.14]	[1.44 – 3.20]	[0.11 – 0.51]	[60.83 – 77.50]	[64.61 – 89.76]
ELF	9.8	79.3	64.3	2.22	0.32	75.41	69.2
	[95% CI]	[66.7 – 88.9]	[48.0 – 78.5]	[1.45 – 3.40]	[0.19 – 0.56]	[62.7 – 85.5]	[52.4 – 83.0]
ELF	11.3	56.9	92.9	7.97	0.46	91.7	60.9
	[95% CI]	[43.2 – 69.8]	[80.5 – 98.5]	[2.62 – 24.25]	[0.34 – 0.63]	[77.5 – 98.3]	[47.9 – 72.9]
CPA	14	74.6	81.0	3.91	0.31	83.7	70.8
	[95% CI]	[61.0 – 85.3]	[65.9 – 91.4]	[2.06 – 7.44]	[0.20 – 0.51]	[70.3 – 92.9]	[56.0 – 83.1]

Table 4.2
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 6 in all participants.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1 Ratio	0.32	62.96	91.07	7.05	0.41	77.27	83.61
	[95% CI}	[42.37 – 80.60]	[80.38 – 97.04]	[2.91 – 17.09]	[0.25 – 0.67]	[58.39 – 89.18]	[75.60 – 89.36]
ELF	9.8	94.9	60.7	2.41	0.08	60.7	94.9
	[95% CI]	[82.7 – 99.4]	[47.3 – 72.9]	[1.75 – 3.32]	[0.02 – 0.33]	[47.3 – 72.9]	[82.7 – 99.4]
ELF	10.4	89.7	73.7	3.42	0.14	68.6	91.8
	[95% CI]	[75.8 – 97.1]	[60.9 – 84.2]	[2.22 – 5.28]	[0.05 – 0.36]	[54.1 – 80.9]	[80.4 – 97.8]
ELF	11.3	76.9	90.2	7.82	0.26	83.3	85.9
	[95% CI]	[60.2 – 88.3]	[79.1 – 96.0]	[3.59 – 17.04]	[0.14 – 0.46]	[66.5 – 93.0]	[74.4 – 93.0]
ELF	12.3	56.4	96.7	17.21	0.45	91.7	77.6
	[95% CI]	[39.6 – 72.2]	[88.7 – 99.6]	[4.28 – 69.12]	[0.31 – 0.65]	[73.0 – 99.0]	[66.6 – 86.4]
CPA	14	86.1	70.5	2.92	0.20	63.3	89.6
	[95% CI]	[70.5 – 99.3]	[57.4 – 81.5]	[1.94 – 4.39]	[0.09 – 0.45]	[48.3 – 76.6]	[77.3 – 96.5]
CPA	30	44.4	88.5	3.87	0.63	69.6	73.0
	[95% CI]	[28.0 – 62.0]	[77.8 – 95.3]	[1.76 – 8.51]	[0.46 – 0.85]	[47.1 – 86.8]	[61.4 – 82.7]

Table 4.3 .
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 10 in all participants.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1 Ratio	0.32	66.67	89.83	6.56	0.37	72.73	86.89
	[95% CI]	[44.68 – 84.37]	[79.17 – 96.18]	[2.92 – 14.73]	[0.21 – 0.66]	[54.27 – 85.70]	[78.89 – 92.15
ELF	9.8	94.4	57.8	2.24	0.10	55.7	94.7
	[95% CI]	[81.3 – 99.3]	[44.8 – 70.6]	[1.66 – 3.01]	[0.02 – 0.38]	[42.5 – 68.5]	[82.7 – 99.4
ELF	10.6	96.1	69.4	3.14	0.06	76.6	94.4
	[95% CI]	[86.5 – 99.5]	[54.6 – 81.8]	[2.05 – 4.80]	[0.01 – 0.22]	[64.3 – 86.3]	[81.3 – 99.3
ELF	11.3	83.3	90.6	8.89	0.18	83.3	90.6
	[95% CI]	[67.2 – 93.6]	[80.7 – 96.5]	[4.09 – 19.31]	[0.09 – 0.38]	[67.2 – 93.6]	[80.7 – 96.5
CPA	14	87.9	68.8	2.81	0.18	59.2	91.7
	[95% CI]	[71.8 – 96.6]	[55.9 – 79.8]	[1.91 – 4.13]	[0.07 – 0.45]	[44.21 – 73.0]	[80.0 – 97.7

Table 4.4
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 12 in all participants.

4.6 RESULTS – participants with cirrhosis only

4.6.1 Correlation of Angiopoietins, ELF, CPA and Ishak with HVPG in participants with cirrhosis

The correlation coefficient of ANG1, ANG2 and the ANG2/ANG1 ratio with HVPG was -0.347 ($p = 0.028$), 0.583 ($p<0.001$) and 0.600 ($p<0.001$) respectively.

HVPG correlated with ELF and Ishak stage, but not with CPA with correlation coefficients of 0.660 ($p<0.001$), 0.411 ($p=0.04$) and 0.310 ($p=0.062$) respectively.

HVPG also correlated with MELD and UKELD with correlation coefficients of 0.465 ($p=0.001$) and 0.517 ($p<0.001$). See Figures 4.5-4.7.

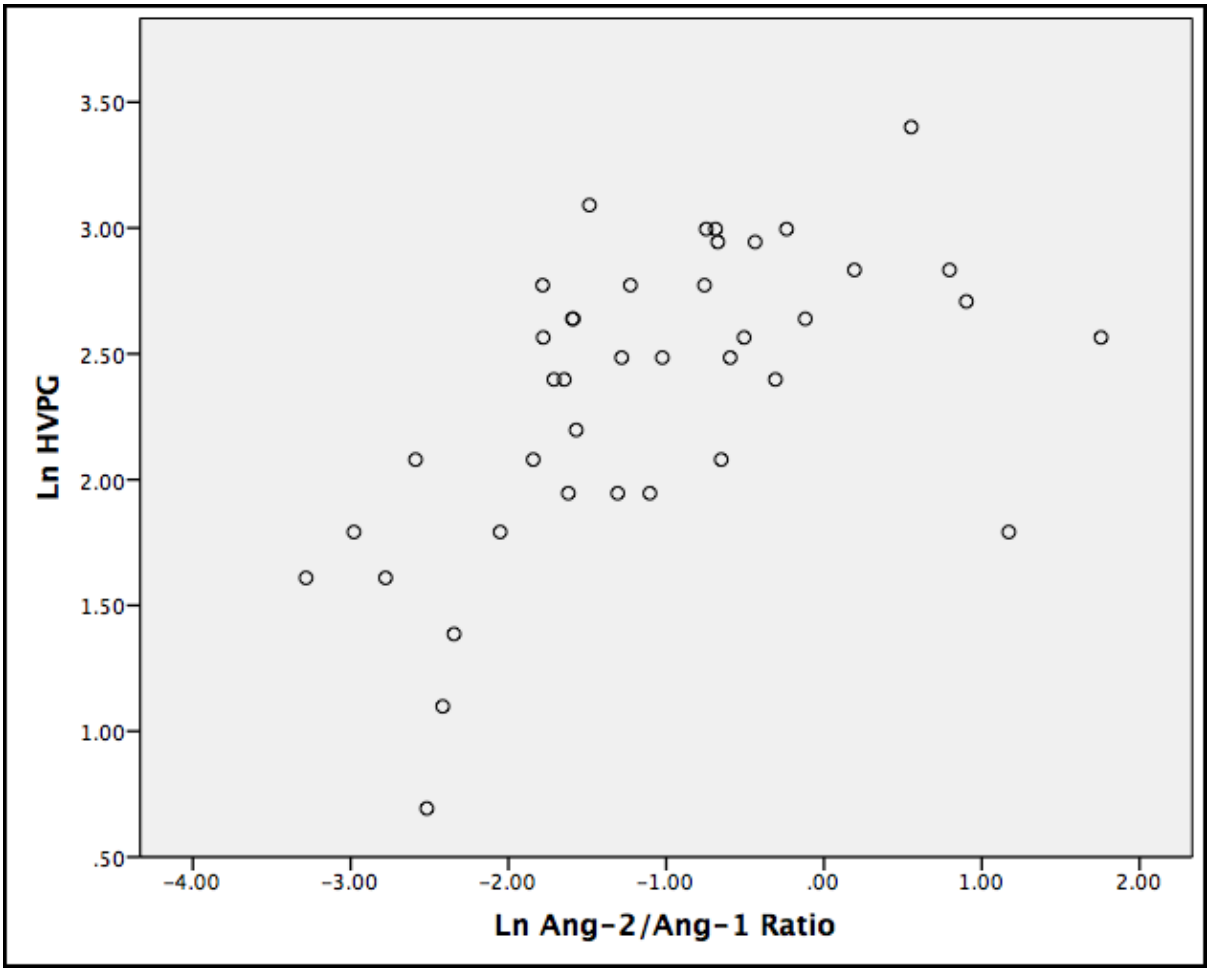

Figure 4.8. Correlation of HVPG with ANG2/ANG1 ratio in participants with cirrhosis, r = 0.600 ($p<0.001$).

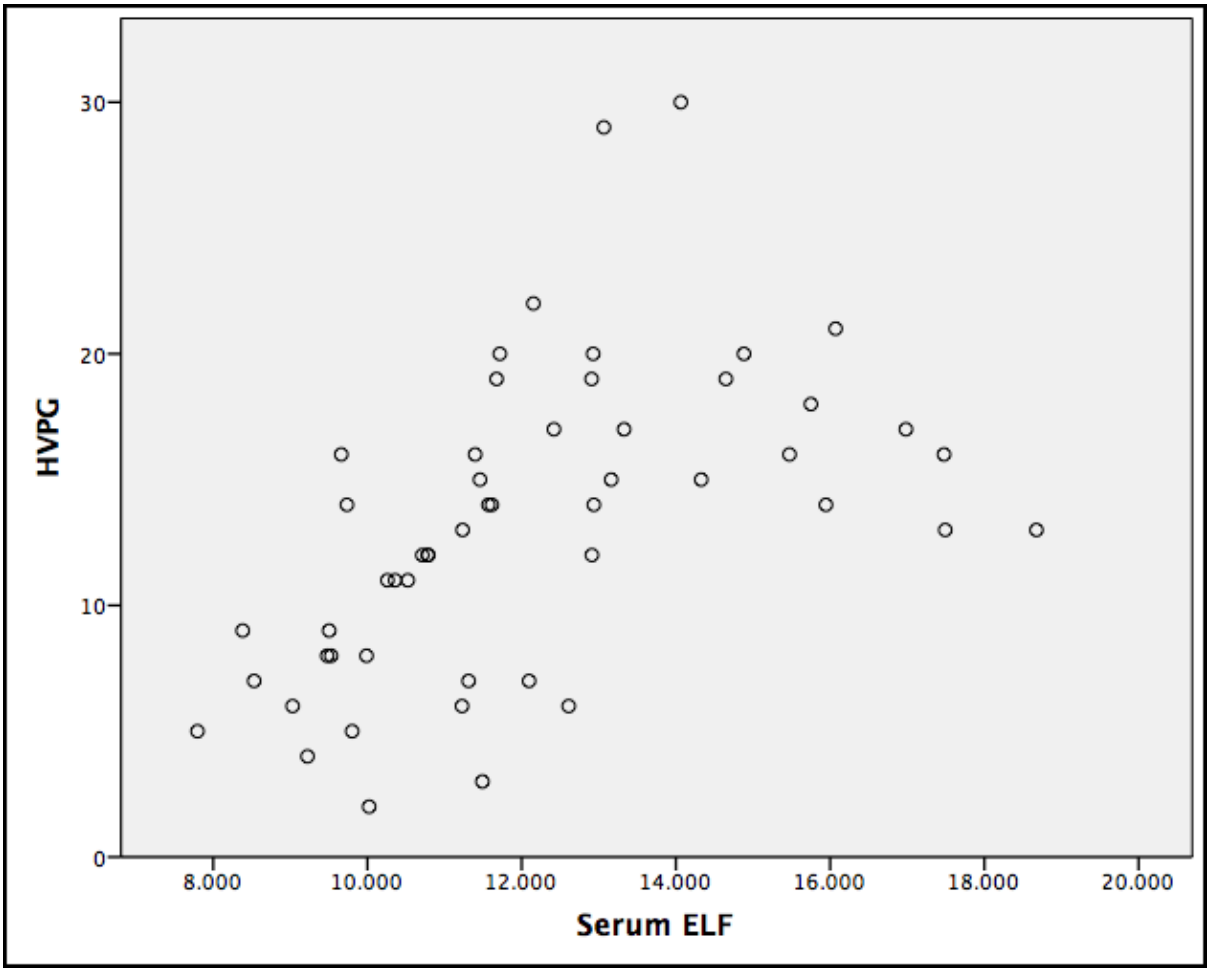

Figure 4.9. Correlation of ELF and HVPG in participants with cirrhosis, r = 0.660 ($p<0.001$).

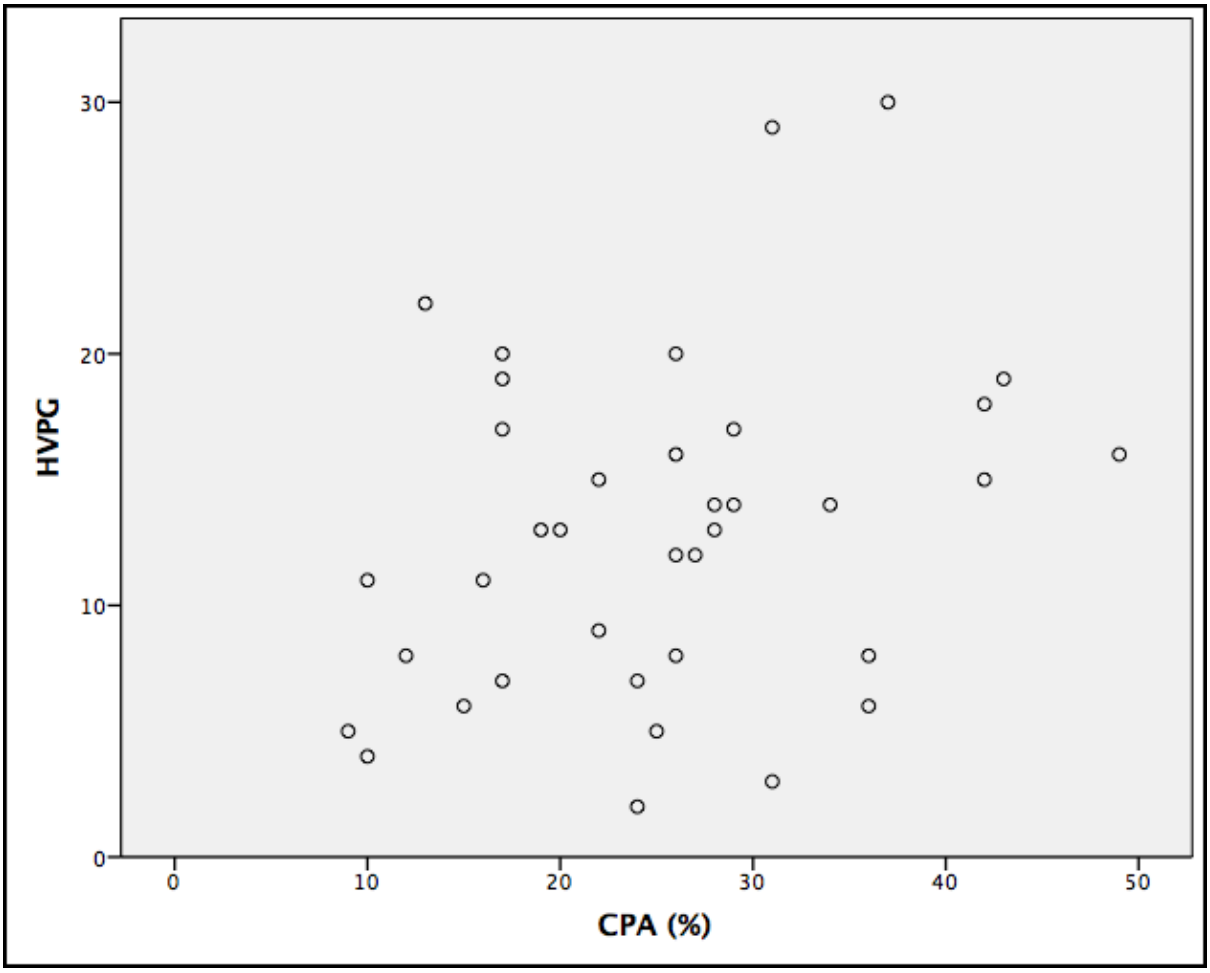

Figure 4.10. Correlation of HVPG and CPA in participants with cirrhosis, r = 0.310 (p=0.062).

4.6.2 The ability of Angiopoietins, ELF and CPA to predict significant portal hypertension in participants with cirrhosis.

A peripheral serum ELF of > 9.8 predicted the presence of clinically significant portal hypertension with a sensitivity of 94.5% and a specificity of 50% (PPV 80.1%, NPV 80%). An ELF of < 12.3 could rule out clinically significant portal hypertension with a sensitivity of 93.8%.

Receiver Operator Characteristic curve analysis showed that peripheral ELF can predict the presence of portal hypertension (HVPG ≥ 6mmHg) with an AUROC of 0.815 [95% CI; 0.644 – 0.985]; clinically significant portal hypertension (HVPG ≥ 10mmHg) with an AUROC of 0.891 [95% CI: 0.781 – 1]; and severe portal hypertension (HVPG > 12mmHg) with an AUROC of 0.918 [95% CI; 0.824 – 1].

CPA can predict an HVPG ≥ 6mmHg, ≥ 10mmHg and ≥ 12mmHg with AUROCs of 0.652 [95% CI; 0.369 – 0.934], 0.613 [95% CI; 0.410 – 0.816] and 0.702 [95% CI; 0.516 – 0.888] respectively.

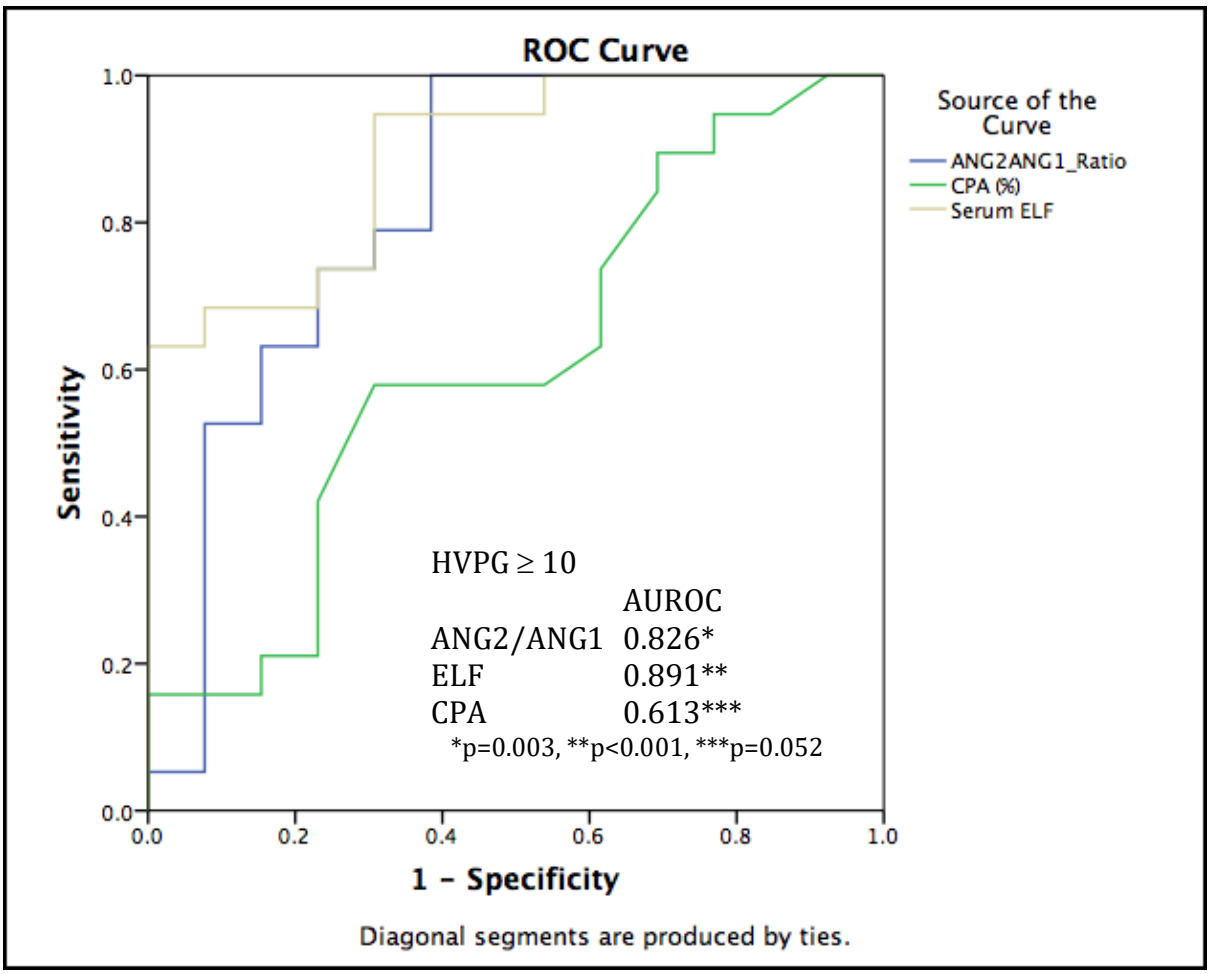

Figure 4.11. The ROC curve for the ability of ANG2/ANG1 Ratio, ELF and CPA to predict an HVPG ≥ 10 in participants with cirrhosis.

Receiver Operator Characteristic curve analysis showed that the peripheral ANG2/ANG1 ratio can predict the presence of portal hypertension (HVPG ≥ 6mmHg) with an AUROC of 0.978 [95% CI; 930 – 1.0]; clinically significant portal hypertension (HVPG ≥ 10mmHg) with an AUROC of 0.826 [95% CI: 0.663 – 0.988]; and severe portal hypertension (HVPG > 12mmHg) with an AUROC of 0.804 [95% CI; 0.644 – 0.964].

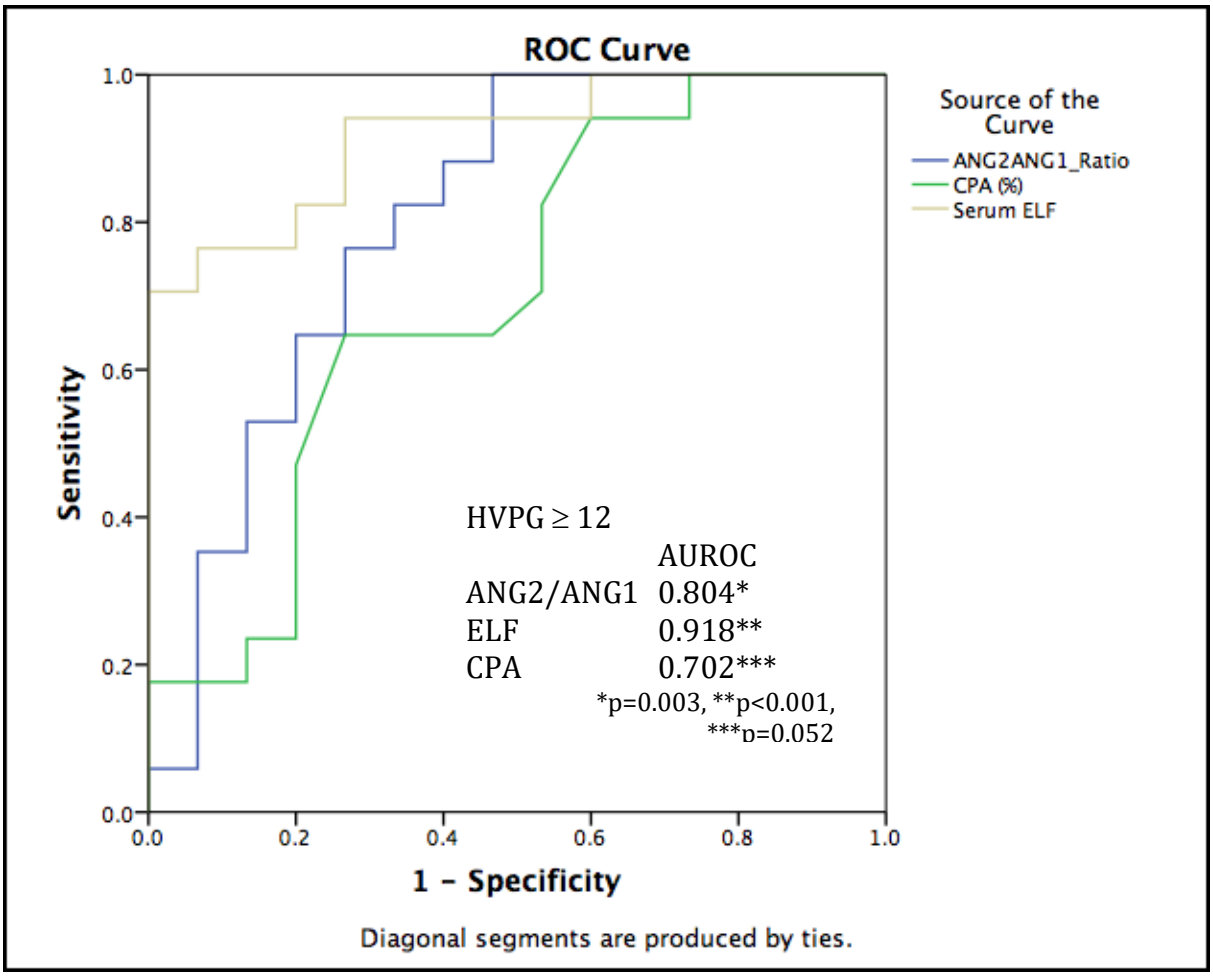

Figure 4.12. The ROC curve for the ability of ANG2/ANG1 Ratio, ELF and CPA to predict an HVPG ≥ 12 in participants with cirrhosis.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1 Ratio	0.1	94.29	100.00	-	0.06	100	71.43
	[95% CI]	[80.84 – 99.30]	[47.82 – 100.0]		[0.01 – 0.22]		[39.43 – 90.57]
ELF	9.8	92.86	20.00	1.16	0.36	82.98	40
	[95% CI]	[80.52 – 98.50]	[2.52 – 55.62]	[0.84 – 1.60]	[0.07 – 1.86]	[77.96 – 87.05]	[11.34 – 77.65]
ELF	11.3	63.83	80.00	3.19	0.45	96.77	19.05
	[95% CI]	[48.52 – 77.33]	[28.63 – 99.49]	[0.55 – 18.67]	[0.25 – 0.81]	[83.68 – 99.43]	[11.64 – 29.59]
CPA	14	93.18	40.00	1.55	0.17	89.80	40.00
	[95% CI]	[81.34 – 98.57]	[5.27 – 85.34]	[0.76 – 3.19]	[0.04 – 0.79]	[77.77 – 96.60]	[12.60 – 75.51]

Table 4.5
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 6 in participants with cirrhosis.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1	0.32	64.00	80.00	3.20	0.45	84.21	57.14
	[95% CI]	[42.52 – 82.02]	[51.91 – 95.67]	[1.12 – 9.18]	[0.25 – 0.80]	[65.02 – 93.87]	[42.73 – 70.44]
ELF	9.8	94.44	50.00	1.89	0.11	80.95	80
	[95% CI]	[81.34 – 99.32]	[24.65 – 73.55]	[1.15 – 3.10]	[0.03 – 0.47]	[72.12 – 87.47]	[48.83 – 94.37]
ELF	10.4	88.89	68.75	2.84	0.16	86.49	73.33
	[95% CI]	[73.94 – 96.89]	[41.34 – 88.98]	[1.36 – 5.94]	[0.06 – 0.43]	[75.41 – 93.04]	[50.76 – 88.00]
ELF	11.3	75.00	75.00	3.00	0.33	87.10	57.14
	[95% CI]	[57.8 – 87.88]	[47.62 – 92.73]	[1.26 – 7.16]	[0.18 – 0.63]	[73.89 – 94.15]	[41.46 – 71.51]
ELF	12.3	55.56	93.75	8.89	0.47	95.24	48.39
	[95% CI]	[38.10 – 72.06]	[69.77 – 99.84]	[1.30 – 60.64]	[0.32 – 0.70]	[74.57 – 99.27]	[38.91 – 57.98]
CPA	14	65.96	53.57	1.42	0.64	70.45	48.39
	[95% CI]	[50.69 – 79.14]	[33.87 – 72.49]	[0.91 – 2.22]	[0.38 – 1.08]	[60.38 – 78.86]	[35.64 – 61.35]

Table 4.6
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 10 in participants with cirrhosis.

	Cutoff	Sensitivity	Specificity	+ LR	- LR	PPV	NPV
Ang-2/Ang-1	0.32	68.17	77.78	3.07	0.41	78.95	66.67
	[95% CI}	[45.13 – 86.14]	[52.36 – 93.59]	[1.23 – 7.62]	[0.21 – 0.79]	[60.15 – 90.31]	[50.84 – 79.46]
ELF	9.8	93.94	42.11	1.62	0.14	73.81	80.00
	[95% CI]	[79.77 – 99.26]	[20.25 – 66.50]	[1.10 – 2.40]	[0.03 – 0.61]	[65.54 – 80.64]	[48.58 – 94.42]
ELF	10.6	93.94	73.68	3.57	0.08	86.11	87.50
	[95% CI]	[79.77 – 99.26]	[48.80 – 90.85]	[1.67 – 7.61]	[0.02 – 0.32]	[74.41 – 92.97]	[64.02 – 96.50]
ELF	11.3	81.82	78.95	3.89	0.23	87.10	71.43
	[95% CI]	[64.54 – 93.02]	[54.43 – 93.95]	[1.60 – 9.42]	[0.11 – 0.49]	[73.58 – 94.24]	[53.90 – 84.24]
CPA	14	96.67	21.05	1.22	0.16	65.91	80.00
	[95% CI]	[82.78 – 99.29]	[6.05 – 45.57]	[0.96 – 1.56]	[0.02 – 1.31]	[60.29 – 71.11]	[32.56 – 97.07]

Table 4.7
Ability of ANG2/ANG1 ratio, ELF and CPA to predict the absence of an HVPG ≥ 12 in participants with cirrhosis.

4.7 DISCUSSION

The ANG2/ANG1 ratio strongly correlated with HVPG, suggesting that this may be a useful serum marker of portal hypertension. The ANG2/ANG1 ratio, using a cut-off of 0.32, could correctly rule-out clinically significant portal hypertension in 80% of participants with cirrhosis.

Peripheral serum ELF also correlated well with HVPG and could potentially be used to predict the absence of clinically significant portal hypertension. Participants in this study with an ELF <11.3 could be 'ruled-out' from having clinically significant portal hypertension with a specificity of 90.2% (75% in the participants with confirmed cirrhosis) and hence they would be at low risk of developing complications of portal hypertension such as variceal haemorrhage or ascites. Original investigations of ELF as a serum marker in liver disease identified a level of > 9.8 as the best cut-off to diagnose significant fibrosis[368]. More recent studies have suggested that a cut-off of 11.3 had a 97% specificity to detect liver cirrhosis[369].

The correlation between HVPG and ELF, seen in figures 4.1 and 4.9 is less linear above an HVPG of 10-12 mmHg. This result was also noted in comparisons of HVPG and TE, where the liver stiffness only maintained a linear relationship with HVPG up to a value of 12mmHg. ELF is principally a marker of fibrosis, and as such may only reflect the mechanical component of portal hypertension. These findings are corroborated in a small study (n=30) investigating the use of MRE in predicting CSPH, ELF was measured and also

correlated with HVPG. Investigators found that ELF correlated with HVPG (Pearson r = 0.758, p<0.001), however for the sub-group of patients with HVPG > 10mmHg this correlation was not significant[176]. This loss of correlation at higher levels of portal pressure may indicate the ELF, predominantly a marker of fibrosis, is able to predict the mechanical component, but not the dynamic component of portal hypertension.

The association of ELF and HPVG has been previously cited by another group from Austria in patients with chronic liver disease. They reported that ELF was correlated with HVPG (r = 0.443) and that CSPH could be ruled in using an ELF ≥ 11.1 with a PPV of 81% (sensitivity 61%/specificity 92%)[385]. They also noted that at higher HVPG levels (>20 mmHg) the correlation with ELF diminished, likely related to the increased contribution of endothelial dysfunction to portal hypertension in this situation.

The correlation of HVPG and ANG2/ANG1 ratio can be seen in figures 4.2 and 4.8. The relationship between these two variables appears to be more liner, irrespective of the level of HVPG. This may suggest that this ratio is a better marker of portal hypertension than ELF. As ANG2 levels are related to the degree of endothelial activation, they may better reflect the dynamic component of portal hypertension[381].

The performance of ELF and the ANG2/ANG1 ratio is similar to the NPV reported for transient elastography (TE), another non-invasive tool used to assess portal hypertension[151 386]. TE, however, has been shown to perform

best in patients with hepatitis C related liver disease. TE can be applied to app populations of liver disease, however in aetiologies where there is greater variability in patterns of inflammation and fibrosis there is a reduction in accuracy and the manufacturers recommend the use of different disease specific thresholds in the assessment of fibrosis. ELF performs well in our mixed cohort, regardless of the underlying diagnosis and this accords with the manufacturer's recommendation to use the same thresholds in the assessment of all chronic liver diseases.

HVPG is well established as the most accurate predictor of complications and outcomes in patients with advanced chronic liver disease. There is no doubt that hepatologists would like to have access to HVPG measurement for the majority of their patients[387]. Unfortunately, due to the cost and expertise required to accurately measure HVPG, it is not available in most centres. ELF is a simple blood test which can offer a surrogate for HVPG and accurately predict clinical outcomes. We would suggest that on this performance ELF is not able replace the need for endoscopic surveillance for varices in all patients, a finding comparable to the performance of TE where a liver stiffness measurement of <20kPa has a negative predictive value of 85% to exclude varices[388]. However, the prevalence of advanced chronic liver disease is increasing and there is a need for non-invasive markers which can predict complications and direct resources for monitoring and surveillance programmes most effectively. ELF, perhaps in combination with clinical and other non-invasive assessment, may allow clinicians to target invasive surveillance where it is required, and this should be a focus of future research.

The recent Baveno VI guidelines on the management of portal hypertension have recommended the use of TE as a test which can rule-in CSPH[138]. In our cohort of 100 patients the ELF could also be used in this fashion and an ELF ≥ 12.3 could be used to rule in CSPH with a PPV of 91.7%. In a meta-analysis of 5 studies TE had a PPV of 88% to detect CSPH, but these studies used cut-offs ranging between 13.6 and 34.9kPa, predominantly in viral aetiologies[147].

We have shown that CPA correlates with HVPG and can be used as a tool to predict CSPH. Liver biopsy is a valuable tool in the assessment of liver disease and CPA gives added information to the hepatologist[142 372].

Chapter 5: ELF and Angiopoietins levels predict clinical outcomes in participants with liver disease.

5.1 BACKGROUND

Liver disease is a major cause of morbidity and mortality, and clinicians require easy, reliable indicators of the severity of disease and the likely clinical course. Biomarkers which could predict complications of liver disease would allow follow-up, interventions, and referral for specialist care to be directed to those in greatest need.

5.2 OBJECTIVE

1. To assess the correlation of markers of angiogenesis and fibrosis with outcomes in patients with liver disease.

5.3 HYPOTHESIS

Serum levels of Angiopoietin-1, Angiopoietin-2, the ANG2/ANG1 ratio and serum ELF will accurately predict 12 and 24 month survival and requirement for liver transplantation.

5.4 METHODS

The study design, research and ethics committee approval, study population, participant eligibility, consent and study procedure are as detailed in Chapter 2. Participant outcomes were recorded as outlined in the study protocol.

Serum was collected, stored and analysed for Angiopoietin and ELF as described in Chapter 2.

5.4.1 Statistics

Medians were compared using the appropriate non-parametric test (Mann-Whitney U). Kaplan-Meier plots were used to describe outcomes. SPSS v22, IBM, USA was used for all the statistical analysis.

5.5 RESULTS

	All Participants (n=100)	Participants without Cirrhosis (n=48)	Participants with Cirrhosis (n=52)	
Median (IQR) Age (years) (n=100)	55 (46 – 62)	55 (46 – 62)	55 (45 – 63)	p=0.981
Male : Female (%)	65 : 35	58 : 42	71 : 29	
Cirrhosis (%)	52	0	100	
HCC (%)	17	6	26.9	p=0.013
Post-Transplant (%)	25	38	14	
Median (IQR) HVPG (mmHG)	7 (4 – 14)	4 (3 – 5)	13 (8 – 17)	p<0.001
CPA (IQR) (%) n=85	11 (5 – 26)	6 (4 - 10)	26 (17 – 31)	p<0.001
Median (IQR) ELF (peripheral vein) n=100	10.49 (8.86 – 12.22)	9.00 (8.23 – 10.73)	11.59 (10.08 – 13.27)	p<0.001
Median (IQR) ELF (Hepatic vein) N=87	10.04 (8.51 – 11.91)	8.53 (7.86 – 10.04)	11.58 (9.94 – 14.04)	p<0.001
Angiopoietin 1 (ANG1) pg/mL n=83	23990 (15730 – 37480)	30090 (20240 – 50990)	19690 (10252 – 31115)	p<0.001
Angiopoietin 2 (ANG2) pg/mL n=83	4017 (2386 – 6235)	3300 (2325 – 4262)	5577 (2721 – 9230)	p<0.001
ANG2 / ANG1 Ratio n=83	0.1688 (0.0805 – 0.3590)	0.0982 (0.0593 – 0.1724)	0.2853 (0.1683 – 0.6355)	p<0.001
MELD Score (IQR)	9 (7 – 16)	7 (6 – 9)	13 (9 – 24)	p<0.001
UKELD Score (IQR)	48 (45 – 54)	46 (44 – 48)	53 (47 – 62)	p<0.001
Death or LT after 12 months (%)	17	2	31	p<0.001
Death or LT after 24 months (%)	20	2	36	p<0.001

Table 5.1 – Summary of baseline demographics, ELF, HVPG, Angiopoietin values and outcomes for all participants.

5.5.1 ANG2/ANG1 Ratio Predicting Survival for all participants:

Overall the median survival for all participants during a maximum of 45 months of follow-up was 27 months. The survival of participants for those with ANG2/ANG1 ratios above and below 0.32 is shown in table 5.2 below.

	Mean ± SD	Median (IQR)
All Participants	25.2 ± 1.5	27 (15 – 40)
ANG2/ANG1 ≥ 0.32*	20.0 ± 3.0	23 (5.5 – 29.5)
ANG2/ANG1 < 0.32*	32.1 ± 1.6	35 (24 – 42)

Table 5.2 – Survival in months for all participants stratified by ANG2/ANG1 Ratio, *(p<0.001)

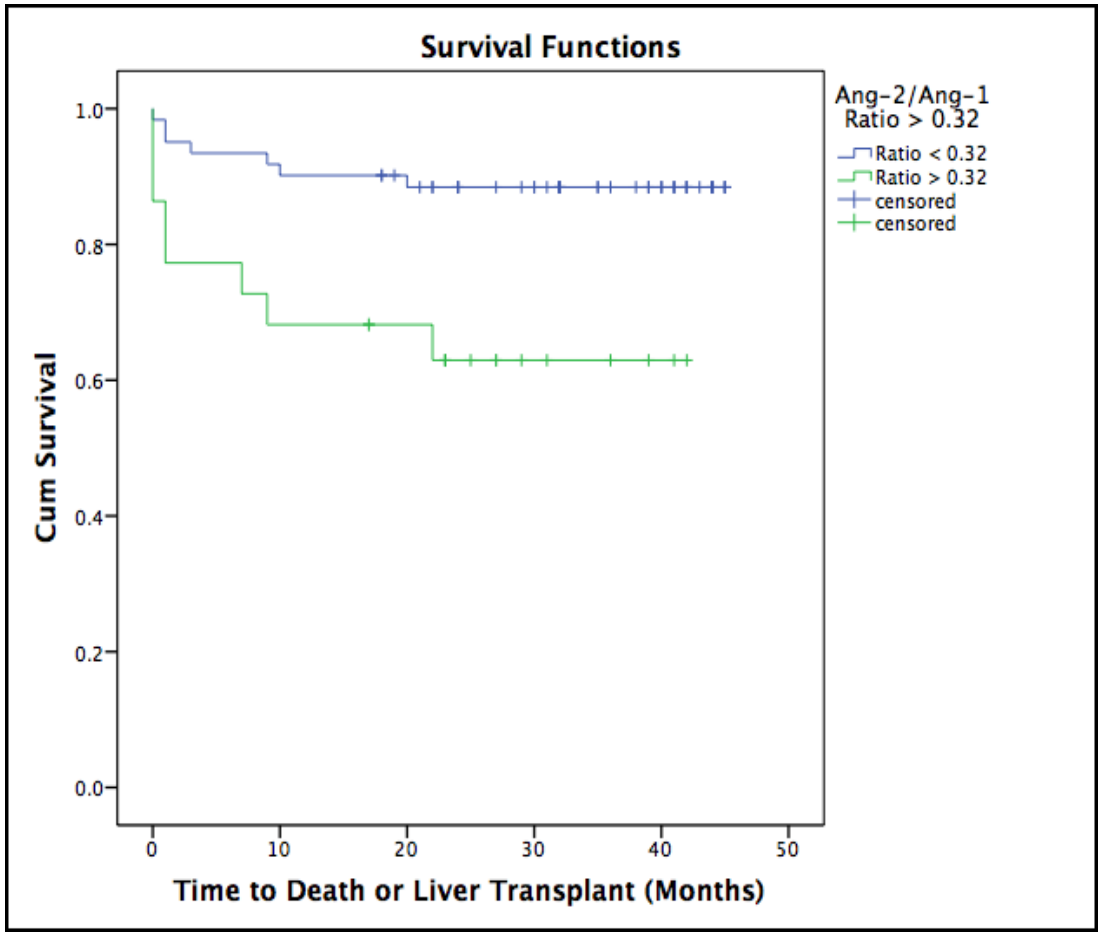

Figure 5.1 – Kaplan-Meier plot of time to death or liver transplant for all participants, stratified by ANG2/ANG1 ratio.

5.5.2 ANG2/ANG1 Ratio Predicting Survival for participants with cirrhosis:

The survival for participants with cirrhosis stratified by ANG2/ANG1 ratio are shown in Table 5.3 and figure 5.2.

	Mean ± SD	Median (IQR)
Participants with cirrhosis (n=52)	19.3 ± 2.0	18.5 (6 – 31)
ANG2/ANG1 ≥ 0.32* (n=19)	17.5 ± 3.1	22 (1 – 27)
ANG2/ANG1 < 0.32* (n=21)	26.6 ± 3.2	31 (15 – 42)

Table 5.3 Survival in months for participants with cirrhosis stratified by ANG2/ANG1 Ratio, *(p=0.034)

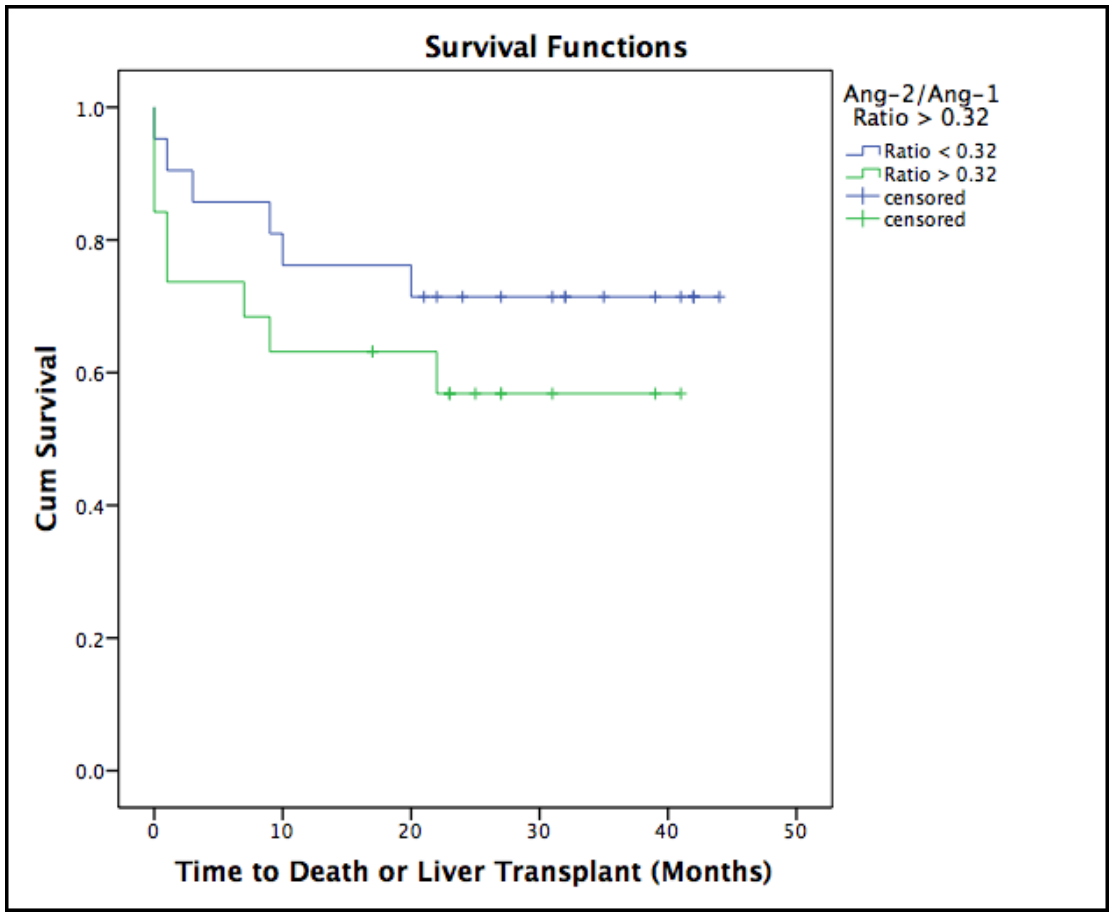

Figure 5.2 – Kaplan-Meier plot of time to death or liver transplant for participants with cirrhosis, stratified by ANG2/ANG1 ratio.

5.5.3 ELF predicting Survival

The cohort was separated into 3 groups using established ELF cut-offs[369]. The median (IQR) transplant free survival in months for patients with a low (<9.8), intermediate (9.80-11.29) and high (>11.3) ELF score was 38 (30-42), 29 (21-39) and 13 (2-25) respectively (p<0.001).

Alternatively, using a single ELF cut-off of 9.8 the median (IQR) survival in months for those with a lower and higher ELF were 38 (30-42) and 22 (8-32) respectively (p<0.001).

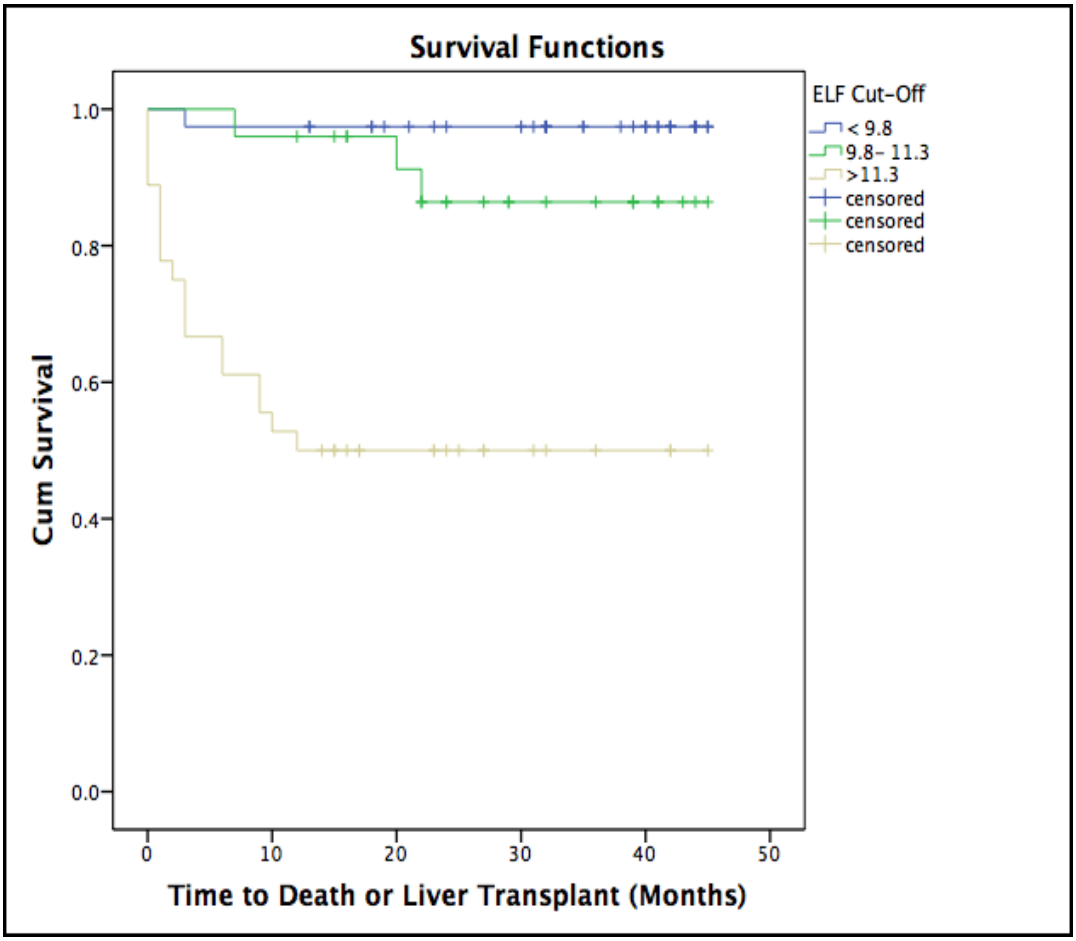

Figure 5.3 – Kaplan-Meier plot of survival for all participants stratified by ELF score.

5.6 DISCUSSION

This study found that both a higher ELF and ANG2/ANG1 ratio are associated with reduced short-term prognosis. Participants with an ANG2/ANG1 ratio of < 0.32 had a better prognosis but, although still statistically significant, the difference was less marked when evaluating only participants with cirrhosis. Previously studies have suggested that the levels of ANG2 increase over time in patients with cirrhosis, but there have been no direct correlations between the angiopoietin levels and disease outcomes.

An increased serum ELF levels was also able to predict either death or the need for liver transplant and, as demonstrated in figures 5.3 and 5.4, an ELF score > 11.3 was associated with significantly reduced short term survival in this population. An ELF score of > 10.43 has previously been associated with an increased risk of mortality[389]. Follow-up for patients with ACLD is currently focused on regular clinical review, usually with 6 monthly appointments, imaging and blood tests. The aim of review is to try and identify the risk of a patient developing a decompensating event and whether any specific intervention is required to try and prevent this. Ultimately, in more advanced disease a clinician and patient must make a decision about when to refer to liver transplantation. Recent BAVENO VII guidance has suggested the use of liver stiffness measurements for this purpose, and as the LSM increases then further intervention to prevent decompensation is recommended[10]. The ELF score, as shown in our participant population above would help to guide (either to reassure, or by prompting action) longer term strategy in these patients.

Chapter 6: General Discussion and Conclusions

6.1 Discussion

This study found that peripheral serum ANG2/ANG1 ratio and ELF score were elevated in a cohort of patients with a range of different aetiologies of chronic liver disease. This is in keeping with previous findings and with the hypothesis that both fibrosis and angiogenesis/neo-vasculogenesis are contributory to the progression of liver disease. It is important to remember that ANG2 levels have previously been shown to be elevated in other inflammatory diseases, such as ARDS, sepsis and pancreatitis, so rather than being a true marker of portal hypertension, it may be a marker of the angiogenetic component of inflammation and should be interpreted cautiously when assessing patients with other conditions associated with endothelial activation.

We have shown that peripheral serum and hepatic vein samples are comparable, and that peripheral serum levels of angiopoietins and ELF can be used as a reliable measure of hepatic vein levels. There are some conflicting previous data in this area and this result should be validated.

This study also suggests that the serum ANG2 levels cannot be used as a marker of HCC and their increase is likely to be related to the severity of liver disease rather than the presence of hepatic malignancy. Again, one large study has found conflicting results and these results should be validated.

The ANG2/ANG1 ratio and ELF score correlated well with the measurements of HVPG. We have also shown that CPA, MELD and UKELD scores, all predictors of prognosis, correlate with HVPG in the present study.

We have shown a linear relationship of ANG2/ANG1 levels and HVPG irrespective of the degree of portal hypertension, and a loss of linear relationship between ELF and HVPG above and HVPG of 10-12 mmHg. This may reflect the different aspects of portal hypertension assessed by these two tests. The ELF test is predominantly a marker of serum fibrosis, and thus the mechanical portal hypertension, and the Angiopoietins are a marker of endothelial activation and angiogenesis/neo-vasculogenesis.

Results obtained from participants with NRH and EHPVO differed markedly from patients with other aetiologies of CLD, having low levels of ANG2 and ELF suggesting that these disease processes exhibit a different phenotype to liver disease, or has a lesser degree of endothelial activation.

Finally, we have demonstrated that both ANG2/ANG1 ratio and ELF can be used to predict the risk of death or the need to liver transplantation after up to 24 months follow-up. This may be the most useful clinical application of these biomarkers.

6.2 Conclusions

1. Levels of Angiopoietin-1 are decreased, and Angiopoietin-2 increased in participants with advanced liver disease and their levels correlate with the degree of portal hypertension. This suggests that angiogenesis and neo-vasculogenesis may contribute to the pathophysiology of portal hypertension.

2. Levels of Angiopoietins 1 and 2 and levels of ELF were very similar in the peripheral blood and hepatic vein blood, with a high coefficient of correlation. This suggests that levels of these proteins are stable in the peripheral circulation.

3. This study suggests that ELF and the ANG2/ANG1 ratio correlate well with HVPG, and have a similar NPV for 'ruling-out' clinically significant portal hypertension to transient elastography.

4. ELF and the ANG2/ANG1 ratio were are able to predict clinical outcomes (death or need for liver transplantation) and may be useful prognostic markers of serious end-points of ACLD.

5. The ANG2/ANG1 ratio did not correlate with the degree of portal hypertension in patients with NRH and EHPVO.

6.3 Suggestions for further work

The following questions are raised, and further investigations are required to further assess the potential usefulness of this study.

- This investigation needs to be validated in a larger more homogeneous cohorts of patients with ACLD to assess the real-world application of both ELF and the ANG2/ANG1 ratio in assessing the severity of portal hypertension and negating the need to HVPG or endoscopy.

- Further work should aim to use these markers in combination with other minimally invasive methods of assessing liver disease severity, such as elastography or simple serum tests. A combined algorithmic approach may be better able to identify a clinically applicable approach.

- Further consideration should be given to the potential role of anti-angiogenic agents, such as Angiopoietin inhibitors in liver disease. Evidence of the use of multipotent tyrosine kinase inhibitors suggest that these agents can have some effect in reducing portal pressure in later stages of disease. However, given that fibrosis and angiogenesis/neo-vasculogenesis occur simultaneously they may have a beneficial role earlier in the disease process to prevent disease progression.

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