Challenges and Opportunities

In the

Management of Portal Hypertension

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Portal hypertension and Gastro-Oesophageal varices (GOV) can occur in early stage Primary Biliary Cirrhosis (PBC) and are associated with a poor prognosis. Screening with endoscopy however, is only recommended in advanced disease. Transjugular-intrahepatic-portosystemic shunting (TIPS) is a life saving procedure in patients with decompensated portal hypertension. Serial TIPS patency checks using a venogram, in stable patients, offers a unique opportunity to sample portal venous blood, and in doing so to study the role of the human intestinal mucosa in bio-transforming essential nutrients.

The aim of this work was to create a non invasive, inexpensive, externally validated screening tool to identify PBC patients with GOV and to use in-situ TIPS as a novel route of access to sample portal venous blood to define the exact site of bio-transformation of folates in humans.

A cross-sectional retrospective study of 330 PBC patients who underwent an OGD at Newcastle was used to create a predictive tool that was externally validated in PBC patients from Cambridge and Toronto. 48% of the Newcastle, 31% of the Cambridge and 22% of the Toronto cohorts of PBC patients had GOV. 25% (95% CI 18–32%) of the Newcastle cohort had GOV diagnosed at an index variceal bleed. Of the others, 37% (95% CI 28–46%) bled after a median of 1.5 years (IQR 3.75). Transplant-free survival was significantly better in those without GOV vs. those with GOV (p <0.001), but similar in patients with GOV that bled and those that did not (p = 0.1). The NVP score (%Probability of GOV) = 1 / [1+exp ^ − (9.186 + 0.001 * alkaline phosphatase in IU − 0.178*albumin in g/L − 0.015*platelet×10^9)] was validated in external cohorts
(AUROC 0.86). Cost consequences analyses revealed the NVP score to be as accurate as, but more economical than using either OGD directly or other risk scores for screening.

A prospective cross-over study of portal and peripheral venous labelled folate concentrations following oral dosing with physiological doses of stable-isotope-labelled folic acid (FA) or 5-formyltetrahydrofolic acid (5-FTHF) in six subjects with a TIPS in situ was set up. At 15 minutes, a median 86% [range 60-88%] of labelled folate in the hepatic portal vein following a dose of FA was unmodified FA. In contrast, following a dose of 5-FTHF, only a median 3% [range 2-6%] of labelled folate in the portal vein was unmodified 5-FTHF; the rest being methylated to 5-MTHF, suggesting limited gut wall dihydrofolate reductase capacity and suggesting that the liver in humans, rather than the intestinal mucosa as previously thought, is the organ responsible for this process.
STATEMENT OF ORIGINALITY

I declare the work presented in this thesis is, to the best of my knowledge and belief, original and my own work, except as acknowledged in the text, and that the material has not been submitted either in whole or in part, for a degree at this or another university.

STATEMENT OF CONTRIBUTION TO JOINTLY PUBLISHED WORK

For the published manuscripts relating to the thesis I have contributed to the conception and design of the projects, acquired the relevant data, carried out the clinical aspects of the study including obtaining informed consent, obtained the relevant samples for laboratory analysis, assisted with external quality assurance of the sampling process, completed data analysis and interpretation (Portal hypertension / PBC study) or contributed to the analysis and interpretation (Folic Acid Study), wrote the first drafts or contributed to the preparation of the manuscript, and approved final versions for publication.

STATEMENT OF CONTRIBUTION BY OTHERS

Professor David Jones: Participated in developing all study concepts and in designing all studies. Co-PI on BBSRC project grant which funded the folic acid study. Assisted with blood sampling and participated in data analysis. Principle research supervisor.

Dr. Mark Hudson: Participated in developing all study concepts. Clinical lead co-investigator on BBSRC project grant which funded the folic acid study. Assisted with portal venous blood sampling and participated in data analysis. Principle clinical supervisor.
Dr. Maria King and Dr. Mark Philo: Undertook laboratory sample analysis and participated in data analysis at the Institute of Food Research Norwich.

Professor David Barrett: Developed techniques for analysis, supervised the sample analysis for folic acid study and participated in data analysis.

Dr. John Rose and Dr. Ralph Jackson: Carried out TIPS venography and enabled portal venous catheterisation.

Dr. Jack Dainty: Assisted in developing study experimental protocol and led the data analysis in the folic acid study.

Dr. Anthony Wright: Developed study concept and co-investigator on BBSRC project grant which funded the folic acid study. Participated in data analysis.

Dr. Paul Finglas: Developed study concept. Co-PI on BBSRC project grant which funded the folic acid study. Participated in data analysis.

Dr. Peter Mcmeekin: Designed health economics outcomes analyses tool

Dr. George Mells, Dr. Hemant Shah, Dr. Catalin Coltescu, Professor Graeme Alexander and Dr. Gideon Hirschfield assisted with providing data for external validation.

Professor Julia Newton: Assisted with design for the portal hypertension study.

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LIST OF PUBLICATIONS

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1. The International Liver Congress™ 2012, EASL, Berlin, Germany
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2. The Liver Meeting® 2011, AASLD, San Francisco, USA


4. British Association for the Study of the Liver Meeting 2010, Edinburgh, UK
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“At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.”

*Albert Schweitzer*

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Sampa, Mum, Dad, Aaliya and Anaaya—Everything I do is tinged with your unwavering love, understanding and encouragement. Your sparks keep my flame burning. I dedicate this work to you.
ABBREVIATIONS

AASLD – American Association for the Study of Liver Diseases

AE – Adverse Event

AIC – Akaike’s Information Criterion

ALB - Albumin

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AMA – Anti mitochondrial Antibody

a-PBC – Asymptomatic PBC

AST – Aspartate Aminotransferase

AUROC – Area under the ROC

BBSRC – Biotechnology and Biological Sciences Research Council

BIC- Bayesian Information Criterion

BIL – Bilirubin

BMI – Body Mass Index

CI – Confidence Intervals

CO – Carbon Monoxide

CP – Child Pugh Score

CPG – Child Pugh Grade
CRP – C Reactive Protein

CT – Computed Tomography

DHFR – Dihydrofolate Reductase

DNA – Deoxyribonucleic Acid

EASL – European Association for the Study of the Liver

ELISA – Enzyme-Linked Immunosorbent Assay

FA – Folic Acid

FHVP – Free Hepatic Venous Pressure

FR – Folate Receptor

FTHF – Formyl-Tetrahydro Folate

GI – Gastro Intestinal

GOV – Gastro-Oesophageal Varices

GPI – Glycosyl Phospho Ionisitol

GWAS - Genome Wide Association Study

HCC – Hepato-Cellular Carcinoma

HE – Hepatic Encephalopathy

HLA – Human Leucocyte Antigen

HNU – Human Nutrition Unit

HPLC – High-Performance Liquid Chromatography
HVPG – Hepatic venous pressure gradient

IFR – Institute of Food Research

INR – International Normalised Ratio

IQR – Inter Quartile Range

IU – International Units

LC-MS – Liquid Chromatography Mass Spectrometry

LLN – Lower Limit of Normal

L-LOLA – L-Ornithine-L-Aspartate

LSPS – Liver Stiffness Platelet Spleen Size

MABPT – Male gender, Albumin, Bilirubin, PT

MELD – Model for End Stage Liver Disease

MRP – Multi Drug Resistance Protein

MRS – Mayo Risk Score

MTX – Methotrexate

NASH – Non Alcoholic Steato Hepatitis

NIH – National Institute of Health

NO – Nitric Oxide

NPV – Negative Predictive Value

NVP – Newcastle Varices in PBC Score
NVP-S - Newcastle Varices in PBC Score - Splenomegaly

OGD – Oesophageo-Gastro Duodenoscopy

OLT – Orthotopic Liver Transplant

PBC - Primary Biliary Cirrhosis

PDC – Pyruvate Dehydrogenase Complex

PPV – Positive Predictive Value

PT – Prothrombin Time

PteGlu – Pteroyl Glutamic Acid (Folic Acid)

PTFE – Poly Tetra Fluoro Ethylene

QoL – Quality of Life

RCF – Red Cell Folate

RFC – Reduced Folate Carrier

ROC – Receiver Operating Characteristic

Sen – Sensitivity

s-PBC – Symptomatic PBC

Spe – Specificity

THF – Tetrahydrofolate

TIPS – Transjugular Intrahepatic Stent Shunt

UDCA – Ursodeoxycholic Acid
ULN – Upper Limit of Normal

US – United States

US FDA – United States Food and Drug Administration

USS – Ultrasound Scan

UV – Ultraviolet

WHVP – Wedged Hepatic Venous Pressure
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GENERAL INTRODUCTION
Chronic Liver Disease is the 5th largest cause of mortality in the UK. A large proportion of this mortality is attributable to the development of portal hypertension, a progressive complication of cirrhosis that causes life threatening complications such as bleeding from gastro-oesophageal varices (GOV), ascites and spontaneous bacterial peritonitis, hepatorenal syndrome and hepatic encephalopathy. Of these, the development of GOV is one of the most severe complications; bleeding from GOV associated with a mortality of around 20%. (N. Chalasani et al., 2003; Carbonell et al., 2004) Progressive liver disease ultimately results in decompensation and liver failure in cases where modifiable causal factors, such as alcohol, are not addressed adequately or when disease modifying interventions are ineffective. Identifying patients with chronic liver disease who are at risk of developing complications such as portal hypertension, end stage liver failure who would not respond to standard of care remains an area of intense research. Being able to address this challenge would result in improved outcomes by allowing early initiation of pharmacological therapy and where these fail, early consideration for liver transplantation leading to improved survival.

Primary Biliary Cirrhosis (PBC) is a chronic liver disease of cholestatic aetiology which lends itself well to studying prediction models that aim to identify patients at risk of developing complications. Portal hypertension and GOV can develop early in PBC, independent of the presence of cirrhosis, (Nakanuma, 2003; Ali et al., 2011) and herald a poor prognosis. (Rydning et al., 1990; D.E.J. Jones et al., 2002b; Takeshita et al., 2003) Invasive screening of all PBC patients by means of Oesophagoscop — Gastro-duodenoscopy (OGD) is associated with risk, which is unnecessary in those who can be stratified as being unlikely to have GOV, in addition to being expensive. Current guidelines are unclear as to when
screening with an OGD should be initiated in early disease. A non invasive, cost effective, externally validated tool to predict the presence of GOV in patients with PBC, especially in early disease would have a beneficial impact outcome by allowing pharmacotherapy to be initiated to reduce the risk of bleeding. Finding such a tool remains a challenge and its creation would be an important development in the study of the evolution of portal hypertension in these patients.

The liver plays an important role in the post absorptive metabolism of a number of macro and micronutrients as well as drugs and toxins. Accepted hypotheses about the bio availability and metabolic pathways of essential nutrients and vitamins in humans have often been derived using animal models or in vitro human cell culture studies. To study such pathways in humans would require portal venous blood sampling from human volunteers after the oral ingestion of the nutrient in question. Portal venous sampling, however, is only possible through trans-jugular or trans-hepatic routes which is invasive and associated with significant risk to the individual and therefore not ethically justifiable. Experimental animal models and in-vitro studies may not accurately replicate the human metabolic milieu and may not therefore reliably answer important yet basic questions about the exact site of biotransformation, exact bio availability or metabolic pathways of essential micro nutrients and vitamins in humans.

In selected chronic liver disease patients who present with complications of portal hypertension such as bleeding from GOV not adequately treated with endotherapy or in those with refractory ascites, a transjugular intrahepatic stent shunt (TIPS) can be a life saving and quality of life improving procedure. A significant proportion of such patients have minimally progressive or non
progressive disease especially when the iterative aetiological factors (usually
alcohol) have been adequately addressed. TIPS dysfunction secondary to stent
occlusion remains a serious concern, especially in patients who have
uncovered stent prosthesis. In an effort to detect occlusion early, most centres
offering TIPS have follow up protocols that include regular TIPS patency checks
as a part of the long term surveillance of such patients to ensure that clinical
benefit is maintained. These patency checks can be undertaken by either an
ultrasound doppler scan or a TIPS venogram. Following up well compensated
patients with in-situ TIPS by means of a venogram offers a unique opportunity
to access portal venous blood in humans and define metabolic pathways and
the exact site of biotransformation of orally ingested tagged micronutrients or
vitamins. Folic acid is one such vitamin where controversy about the exact site
of biotransformation into bioactive folate metabolites is a hotly debated topic.
Currently accepted wisdom, based on animal studies, is that the site of
biotransformation is in the human small intestine. However, an increasing body
of evidence suggests that the liver rather than the small intestine may be
involved in the biotransformation of folic acid in humans. Portal venous
sampling in patients with in-situ TIPS who have ingested tagged stable folic acid
offers an opportunity to study the exact site of biotransformation of this
important vitamin in humans.

This piece of work aims to rise up to the clinical challenge of predicting the
presence of portal hypertension in patients with PBC and to utilise the
opportunity presented at routine TIPS venography to answer important
questions about the actual role of the human small intestine and liver in the
metabolism of folic acid.
CHAPTER 1: NON INVASIVE PREDICTION OF PORTAL HYPERTENSION IN PBC
1.1 Introduction

Current EASL and AASLD guidelines recommend screening with Oesophago-Gastro-Duodenoscopy (OGD) in advanced (Stage IV) disease although it is recognised that histology may underestimate disease severity and that the evidence regarding the selection of patients and timing of screening is contradictory especially in patients with early stage disease. The default of indiscriminate screening is associated with increased cost and risk to patients. There is therefore a need to support the development of validated and more rational ways to stratify risk of developing GOV. Alternative triggers are therefore needed to decide when to screen for varices. These triggers should ideally be independent of histology in PBC as a liver biopsy is no longer needed to make the diagnosis. Several non-invasive tools based on laboratory parameters alone, or in combination with imaging, help identify patients with PBC and GOV appropriate for OGD screening. However, they have not been comprehensively validated outside the derivation cohort. It is also not clear if such approaches are cost effective.

1.1.1 Portal Hypertension

Portal hypertension can occur in the presence or absence of chronic liver disease. When it occurs in the setting of chronic liver disease, portal hypertension is usually associated with poor outcomes and is a poor prognostic marker in these patients. The most significant endpoints in the natural history of portal hypertension are the development of and bleeding from Gastro Oesophageal Varices (GOV), the occurrence of hepatic encephalopathy and the occurrence of hepatorenal syndrome. Understanding the development of portal hypertension requires an understanding of hepatic blood flow and portal vascular anatomy and the pathophysiological changes that occur with the
advent and progression of liver cirrhosis. Diagnostic modalities including non-invasive and invasive techniques allow risk stratification and the initiation of prophylactic therapies which can reduce the risk of life-threatening events and improve outcomes.

**Hepatic blood flow and portal vascular anatomy**

The liver is a highly vascular organ and receives about a quarter of the cardiac output per minute. About 2/3rd of the blood supplied to the liver is via the portal vein and the remainder via the hepatic artery. The portal vein receives blood from the splanchnic organs, omentum and the spleen and is formed by the confluence of the splenic vein and the superior mesenteric vein behind the neck of the pancreas. The liver therefore receives blood from the entire alimentary tract. The portal vein and the hepatic artery enter the liver at the porta hepatitis which also serves as the exit portal for bile ducts and lymphatics. After repeated branching in the liver, terminal portal venules and hepatic arterioles supply hepatic sinusoids to form metabolic micro-hubs in the liver. Blood from the sinusoidal and inter sinusoidal spaces is collected by terminal hepatic or central venules which empty into larger hepatic veins and these finally empty into the inferior vena cava. The direction of blood flow in the portal vein in normal circumstances is towards the liver. When portal pressure increases low pressure collaterals located in anatomical areas of extra-hepatic portosystemic venular communication open up leading to the formation of varices. The anatomical sites of varix formation include the confluence of the oesophageal branches of the left gastric vein and the oesophageal veins, the superior rectal vein and the middle and inferior rectal veins, para umbilical veins and the superficial abdominal wall veins, retroperitoneal veins and some colonic veins,
the bare area of the liver and finally (and rarely) the ductus venosus between
the left hepatic vein and the inferior vena cava.

**Definition of portal hypertension**

The term portal hypertension was first coined by Gilbert and Carnot in 1902.
(Sandblom, 1993) The hepatic venous pressure gradient (HVPG) in the portal
vein is normally between 1-5 mmHg. Any increase in this pressure is termed
portal hypertension. Clinically significant portal hypertension leading to the
development of GOV, however, usually occurs when the portal pressure is ≥10-
12 mmHg. (Garcia-Tsao *et al.*, 1985; Groszmann *et al.*, 2005) Depending on the
cause of portal hypertension and the site of the pathological obstruction, portal
hypertension may be classified (**Table 1**) as being pre-sinusoidal, sinusoidal or
post-sinusoidal in origin.

**Table 1:** Classification and causes of portal hypertension

<table>
<thead>
<tr>
<th>Type of Portal Hypertension</th>
<th>Causes</th>
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| Pre sinusoidal              | **Pre Hepatic**Portal vein thrombosis, Splenic vein thrombosis (Left
  sided portal hypertension), Idiopathic tropical splenomegaly          |
|                             | **Hepatic**Schistosomiasis, Nodular Regenerative Hyperplasia, Tuberculosis, Sarcoidosis, Amyloidosis, Polycystic liver disease, Infiltrative diseases such as myeloproliferative disorders and certain glycogen storage diseases |
| Sinusoidal                  | **Hepatic**Liver cirrhosis, Alcoholic hepatitis, Acute viral hepatitis, Acute fatty liver of pregnancy |
| Post sinusoidal             | **Hepatic**Veno-occlusive liver disease                                |
|                             | **Post Hepatic**Budd Chiari Syndrome, Increased right sided cardiac pressure, Severe pulmonary hypertension and Infiltrating malignant diseases of the inferior vena cava and or hepatic veins |
**Measurement of portal pressure**

Portal venous pressure measurements were first reported by Thompson in 1937 during laparotomy. (Sandblom, 1993) Direct measurement of portal venous pressure through transjugular or trans-hepatic portal venous catheterisation is possible but associated with significant risks and therefore not performed routinely. (Juan Rodes *et al.*, 2007a) Indirect measurement of portal venous pressure can be carried out by measuring the pressure in the hepatic vein after wedging it, either by inflating a balloon or by advancing the tip of the catheter into a small branch of the hepatic vein, the former being the preferred method. This yields the Wedged Hepatic Venous Pressure (WHVP) which represents the pressure in the column of blood between the hepatic sinusoids and the wedge which represents the hepatic sinusoidal pressure. In the absence of liver pathology, this is lower than the portal pressure due to the equilibrating effect of inter sinusoidal communications. (Kumar *et al.*, 2008) In cirrhosis, where the normal architecture of the liver is distorted, these connections are lost and in such cases, WHVP correlates with portal pressure. (Lin *et al.*, 1989; Escorsell *et al.*, 1999; Thalheimer *et al.*, 2005) The portal pressure is most accurately expressed, however, as the Hepatic Venous Pressure Gradient (HVPG), which is the difference between the WHVP and the free hepatic venous pressure (FHVP). This is because measuring the HVPG eliminates any effects of raised intra abdominal pressure on the WHVP and the FHVP, as can be observed in ascites or after large volume paracentesis, resulting in erroneous results. (Juan Rodes *et al.*, 2007a) A schematic showing the measurement of the HVPG using a balloon to act as a wedge using a radiologically guided pressure transducer and an example of the pressure tracing obtained whilst measuring HVPG is shown in Figure 1.
Figure 1: Measurement of HVPG

Figure 1A and 1B are schematic diagrams showing the use of a radiologically guided pressure transducer in the measurement of FHVP and WHVP in health and in sinusoidal disease respectively. Figure 1C shows a pressure transducer tracing from a typical balloon catheter. Abbreviations: IVC, Inferior Vena Cava; PVP, Portal Venous Pressure; WHVP, Wedged Hepatic Venous Pressure; HVPG, Hepatic Venous Pressure Gradient; FHVP, Free Hepatic Venous Pressure (Figure adapted from Parikh S. Dig Dis Sci 2009; 54: 1178–1183)
The measurement and recording of the WHVP, FHVP and the HVPG can help classify the cause of portal hypertension as pre-sinusoidal, sinusoidal or post sinusoidal (Figure 2). This classification is useful in pointing to the aetiology of portal hypertension and in doing so allowing appropriate management to be initiated.

**Figure 2:** Identifying the site of portal hypertension using portal pressure studies

Using free hepatic venous pressure (FHVP), wedged hepatic venous pressure (WHVP) and the hepatic venous pressure gradient measurements (HVPG) to identify the nature of pathology causing portal hypertension. (A) Pre-sinusoidal hypertension where FHVP and WHVP are not raised but HVPG is raised (B) Sinusoidal hypertension where WHVP is increased but not FHVP and (C) Post Sinusoidal hypertension where both FHVP and WHVP are raised.
1.1.2 Pathogenesis of portal hypertension

The pressure in any vascular bed depends on its vascular resistance and the volume of blood flowing through it. In the context of hepatic portal circulation therefore, portal pressure is a product of the vascular resistance of the hepatic vascular bed and the volume of splanchnic blood received by it. Increased vascular resistance or increased blood flow or a combination of both factors (as is commonly encountered clinically) results in portal hypertension. Whereas the cause for pre sinusoidal and post sinusoidal portal hypertension is predominantly increased vascular resistance due to true pre or post sinusoidal anatomical obstruction to the flow of portal venous blood, the cause for cirrhotic (sinusoidal) portal hypertension is far more complex and includes both a mechanical structural component and a dynamic vasoactive component. Cirrhosis of the liver is a condition in which the liver undergoes architectural distortion due to the formation of nodules as a result of fibrosis. Nodular regeneration and vascular changes are associated with but not required to make the diagnosis of cirrhosis. The process of fibrosis is driven by iterative injury and ultimately leads to cirrhosis. When the aetiology causing the iterative injury is addressed by treatment (for example successful treatment of viral hepatitis or iron overload in haemachromatosis) or in the case of alcohol, by abstinence, there is increasing evidence that the fibrosis might regress. (J. Rodes et al., 2007b) The Child Pugh Turcotte Score and the Child Pugh Grade (Table 2) were initially used to stage the severity of liver disease in patients with cirrhosis. The Chid Pugh system, though useful, includes parameters that depend on clinical judgment of severity and hence include subjective components. The Model for End Stage Liver Disease (MELD) and the UK End Stage Liver Disease (UKELD) scores are regression based mathematical tools
that use lab parameters to predict outcome thereby eliminating subjectivity in staging and prognostic risk stratification. The MELD and UKELD are preferentially used to stage severity of liver disease especially with regards to prioritising liver transplantation listing. In addition to predicting outcome, in most cases, advancing scores correlate well with an increased risk of developing portal hypertension and GOV.

**Table 2:** Child Pugh Scoring and Grade to stage liver cirrhosis

<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td><strong>Ascites</strong></td>
<td>None</td>
<td>Mild</td>
<td>Moderate to Severe</td>
</tr>
<tr>
<td><strong>Hepatic Encephalopathy</strong></td>
<td>None</td>
<td>Grade I-II (or suppressed with medications)</td>
<td>Grade III-IV (refractory)</td>
</tr>
<tr>
<td><strong>Bilirubin (μmol/L)</strong></td>
<td>&lt;34</td>
<td>34-50</td>
<td>&gt;50</td>
</tr>
<tr>
<td><strong>Albumin (g/L)</strong></td>
<td>&gt;35</td>
<td>28-35</td>
<td>&lt;28</td>
</tr>
<tr>
<td><strong>INR</strong></td>
<td>&lt;1.7</td>
<td>1.7-2.3</td>
<td>&gt;2.3</td>
</tr>
</tbody>
</table>

**Child Pugh Grade** = Sum of individual scores (Survival at 24 months): A = 5-6 points (85%); B = 7-9 points (57%); C= 10-15 points (35%)

*Structural distortion*

As mentioned above, the pathogenesis of portal hypertension in cirrhosis is a combination of increased vascular resistance and increased portal blood flow. The increased vascular resistance occurs as a result of both mechanical changes in hepatic vascular anatomy and a dynamic component that causes increased vascular tone. The mechanical component in cirrhosis is caused due to the distortion of the normal vascular architecture of the liver by progressive fibrosis and regenerative micro nodule formation leading to sinusoidal obliteration and increased anatomical vascular resistance.
Vasoactive mediators

A separate dynamic component (Shibayama and Nakata, 1985) contributing up to 30% of the pressure in cirrhotic portal hypertension (Bhathal and Grossman, 1985) has been identified and is an ideal target for therapeutic interventions in portal hypertension. This “dynamic” component is caused by increase in hepatic vascular tone which is caused due to a disruption of the balance between vasodilator and vasoconstrictor substances in the portal circulation that are responsible for maintaining normal hepatic perfusion in health. Important vasoactive mediators involved in the pathogenesis of portal hypertension are shown in Table 3. (Maruyama and Yokosuka, 2012)

**Table 3**: Vasoactive mediators involved in maintaining normal hepatic perfusion in health and in the development of portal hypertension in cirrhosis

<table>
<thead>
<tr>
<th>Vasodilators</th>
<th>Vasoconstrictors</th>
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<tbody>
<tr>
<td>Nitric Oxide</td>
<td>Endothelin</td>
</tr>
<tr>
<td>Carbon Monoxide</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Nor epinephrine</td>
</tr>
<tr>
<td>Endocannabinoid</td>
<td>Vasopressin</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td></td>
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**Nitric Oxide**

Amongst the various vasoactive mediators, Nitric oxide (NO) appears to be the most important mediator of portal hypertension. A powerful vasodilator, NO is produced from the amino acid L-arginine by endothelial NO synthase (eNOS). In rats, NO inhibition increases portal pressures and increases markedly the hepatic response to epinephrine. (Wiest and Groszmann, 1999) In cirrhosis hepatic NO induced vasorelaxation is impaired due to insufficient hepatic NO
production. (Gupta et al., 1998) This occurs despite normal expression of eNOS mRNA and normal levels of eNOS protein. Though the exact mechanism of reduction in NO production in cirrhosis is unclear, the decreased activity of hepatic eNOS may in part be due to increased expression of caveolin, as Akt (a serine threonine kinase)-induced phosphorylation of eNOS reverses inhibitory conformation of eNOS in association with caveolin-1. (Garcia-Cardena et al., 1997; Maruyama and Yokosuka, 2012) This reduction in hepatic NO results in increased portal pressure through its direct effect on the vascular endothelium and also, to a degree its effect on the hepatic stellate cell (HSC). Under normal physiologic conditions, NO exerts paracrine effects on HSCs. However, in cirrhosis, when NO generation is impaired, along with concomitant HSC activation and changes in sinusoidal structure, portal pressures can increase significantly resulting in portal hypertension. (Langer et al., 2008) Not only is the intrahepatic NO production reduced in cirrhosis, there is also an increase in extrahepatic NO production resulting in splanchnic vasodilatation. (Pizcueta et al., 1992; Wiest and Groszmann, 1999) The increase in extrahepatic splanchnic NO production seen in cirrhosis is caused by increased expression and activity of splanchnic eNOS due to a combination of the effects of shear stress, the increase circulating vasoactive factors (e.g., endothelin, angiotensin II, vasopressin, and norepinephrine) and an over expression of the angiogenic factor vascular endothelial cell growth factor (VEGF). (Maruyama and Yokosuka, 2012) The increased intra hepatic resistance is unable to cope with increased portal venous blood flow brought about by splanchnic vasodilatation resulting in portal hypertension.
Carbon Monoxide

Another important but less potent vasoactive mediator is carbon monoxide (CO). CO is a product of haeme group oxidation by haeme oxygenases. It is a smooth muscle relaxant. In cirrhosis its production in the liver is reduced leading to vasoconstriction whilst at the same time systemic production is increased causing increased cardiac output and a hyperdynamic circulation. (Tarquini et al., 2009; Maruyama and Yokosuka, 2012)

Glucagon

Glucagon is a humoral vasodilator which works by relaxing vascular smooth muscle by decreasing its sensitivity to endogenous vasoconstrictors, such as nor epinephrine, angiotensin II and vasopressin. (Wiest et al., 2001) Increased circulating insulin and glucagon levels are commonly observed in patients with cirrhosis as well as in animal models of portal hypertension. (Gomis et al., 1994) The role of glucagon in causing splanchnic hyperaemia provides a rationale for the use of somatostatin and its synthetic analogs in the treatment of portal hypertension. (Maruyama and Yokosuka, 2012)

Endocannabinoids, Prostacyclins and other mediators

The endogenous cannabinoid anandamide and activation of vascular cannabinoid CB1 receptors have been implicated in the development of portal hypertension in endotoxaemic cirrhotic patients. (Batkai et al., 2001) Prostacyclins and COX derived prostanoids including Thromboxane A2 play an important role in modifying the vascular response of the cirrhotic liver to vasoactive agents suggesting a role in the development of portal hypertension. (Graupera et al., 2003) Endothelins (ETs) are a family of homologous 21 amino
acid peptides which include the peptides ET-1, -2, -3, and -4. They exert various biological effects and cause vasoconstriction and stimulate cell proliferation. Plasma levels of ET-1 and ET-3 are increased in cirrhotic patients. (Moller et al., 1995) The actual mechanism by which ETs modulate vascular tone in cirrhosis is unclear. (Maruyama and Yokosuka, 2012) In addition to these factors, angiotensin II, epinephrine, vasoactive endothelial growth factor (VEGF) and endothelial dysfunction have additional roles in the development and propagation of portal hypertension. Neo-angiogenesis occurs through a VEGF mediated pathway leading to the formation of portosystemic collaterals. These collaterals further perpetuate portal hypertension by maintaining increased portal pressures and increasing splanchnic vasodilatation. (Fernandez et al., 2005) A schematic representation of the role of various factors involved in the pathogenesis of portal hypertension in cirrhosis is shown in Figure 3.
Figure 3: Schematic representation of factors involved in the pathogenesis of portal hypertension in cirrhosis

Vasoactive plasticity of the portal vein

Vasoactive plasticity of the portal vein may be involved in the development of portal hypertension. When contractile responses were studied in isolated tubal segments of branches of the rat portal vein and hepatic artery, portal vein branches were approximately three times more sensitive to nor epinephrine than hepatic arteries. Vasopressin effectively contracted hepatic arteries, whereas it had no effect on portal veins. Both vessel types responded to prostaglandin F2 alpha with contractions although the effect was less potent.
than the other vasoactive agents. (Hogestatt et al., 1986) This suggests that the portal vein in addition to the hepatic artery and the intrahepatic sinusoidal bed through the presence of vasoactive receptors is involved in the development of portal hypertension and in maintaining normal portal flow in health. Catecholamine modulation of the intrinsic rhythmical contractions of the portal vein are complemented by SP, released from intramural nerves, and vasoactive intestinal polypeptide (VIP), released from adventitial nerves, which extend into the circular and longitudinal muscle layers of the portal vein. (Barja and Mathison, 1982)

1.1.3 GOV – a significant complication of portal hypertension

Complications of portal hypertension include the development of portosystemic collaterals, ascites and hepatic encephalopathy. Clinically the most significant site of portosystemic collateral formation is at the distal oesophagus and proximal stomach resulting in the formation of GOV. Other important but clinically less relevant sites of portosystemic collateral formation include the rectum, where the systemic inferior mesenteric vein communicates with the internal pudendal vein resulting in the formation of rectal varices; the umbilicus, where the vestigial umbilical vein communicates with the left portal vein and gives rise to prominent collaterals externally visible around the umbilicus resulting in the caput medusae appearance and; the retroperitoneum, where collaterals, especially in women, communicate between ovarian vessels and iliac veins. Prevalence of GOV in cirrhosis is high and almost half of all patients with cirrhosis have GOV. (Garcia-Tsao et al., 2007) Prevalence increases with liver disease severity and ranges from 40% in patients with Child Pugh A cirrhosis to 80% in those with Child Pugh C disease. Cirrhotic patients develop varices at the rate of 8% per year (Groszmann et al., 2005) with a similar rate of
increase in size from small varices to large varices per year. The risk of bleeding from GOV ranges from 5-15%. This risk is directly proportional to the HVPG. Mortality from GOV bleeding is high and approaches 20% at 6 weeks. [reviewed by (Garcia-Tsao et al., 2007)]

1.1.4 Diagnosis of Gastro-Oesophageal Varices

The gold standard test for the diagnosis of GOV is Oesophageal Gastro Duodenoscopy (OGD). This involves the use of a flexible fibre-optic endoscope to visualize the upper gastrointestinal lumen and look for bluish tinged mucosal elevations in the upper GI tract. Oesophageal varices are seen in columns in the lower oesophagus whilst gastric varices are classically seen as a “bunch of grapes” in the fundus (Figure 4). The former are further classified as small (<5 mm) or large (>5mm) depending on their size or small (just visible varices), medium (occupying < 1/3rd of the oesophageal lumen) or large (occupying >1/3rd of the oesophageal lumen) depending on how much of the oesophageal lumen is compromised by their presence. (The Northern Italian Endoscopic Club for the study and treatment of Esophageal Varices, 1988) The management of medium and large varices is identical and therefore current AASLD guidelines recommend that the 2 stage classification be used. (Garcia-Tsao et al., 2007) Gastric varices are classified by location, which correlates with their risk of haemorrhage. Varices in direct continuity with the oesophagus along the lesser and greater curves of the stomach are called gastrooesophageal varices (GOV) types 1 and 2 respectively. Isolated gastric varices in the fundus (IGV1) occur less frequently than GOVs (10% versus 90%) but are most likely to bleed. (Toubia and Sanyal, 2008) On endoscopy, blood spurting from a varix confirms a bleed. However, in 40% of cases GOV bleeding settles spontaneously, and therefore stigmata of recent haemorrhage which
include red wale marks (longitudinal red streaks on varices that resemble red corduroy wales), cherry red spots, haematocystic spots (raised discrete red spots on the surface of varices that appear as blood blisters) and white nipple spots indicating a fibrin /platelet plug are sought at endoscopy where recent bleeding from GOV is suspected (Figure 4).

Figure 4: Endoscopic views of Gastro Oesophageal Varices (GOV)

A) Oesophageal varices which appear as columns of elevated oesophageal mucosa with a bluish hue with stigmata of recent haemorrhage which in this case is a red spot at the 3'O Clock and 4'O Clock position and B) Gastric varices seen in the fundus as a lobular / bunch of grape like mucosal elevations on retroflexion (Figure used under the commons open licence – author Samir)

Given the high mortality associated with a bleed and the possibility of primary prophylaxis using betablockers in reducing this risk, both EASL and AASLD guidelines recommend endoscopic screening in all patients with cirrhosis. However, endoscopy is an invasive procedure accompanied by a risk of complications including bleeding and perforation. It is also not preferred by patients due to the perceived discomfort caused during intubation and gastric insufflation. A large proportion of patients with early stage cirrhosis undergoing
screening will not have varices. All of these factors have led to a number of studies that aim to develop or find non invasive markers or tests which would help predict the risk for having GOV and focus endoscopic screening in patients in whom the yield would be high or avoid it completely.

1.1.5 Non Invasive Prediction of GOV

Non invasive methods previously studied to predict GOV have included the use of lab based parameters such as the platelet count either on its own or in combination with known complications of cirrhosis such as splenomegaly (Giannini et al., 2003), increased liver stiffness as demonstrated by transient elastography (Vizzutti et al., 2007), presence of imaging signs of portal hypertension such as multidetector CT scanning (Perri et al., 2008) and / or increased spleen stiffness measured by transient elastography (Colecchia et al., 2012) or MR elastography. (Jayant A. Talwalkar et al., 2009) An example of a composite predictive score is the liver stiffness, spleen size and platelet count (LSPS) score. (Berzigotti et al., 2013) Though most show promise in derivation cohorts, none have been universally validated in large cohorts of cirrhotic patients and indeed some have found to be lacking in discriminating power when validated independently. (Riggio et al., 2002; Burton et al., 2007; Kemp and Roberts, 2009; Schwarzenberger et al., 2010) Due consideration should also been given to the fact that the aetiology of chronic liver disease in predictive modelling cohorts have typically under represented cholestatic liver disease, focusing more on alcohol and viral hepatitis (in particular hepatitis C). This limits the universal applicability of these predictive tools and highlights the need for further better validated and inexpensive tools to be developed especially in chronic cholestatic diseases such as PBC and PSC.
1.1.6 Medical Management of Gastro-Oesophageal Varices

Reducing portal pressure during the evolution of cirrhosis or maintaining it at thresholds below clinical significant levels would reduce the risk of developing GOV and this would be the ideal management of cirrhotic patients. Though theoretically attractive, there is currently no effective therapy for the prevention of varices in patients with cirrhosis and portal hypertension. Identifying patients with a high risk of bleeding from GOV and attempting to reduce the risk of bleeding by reducing portal pressure has been shown to be effective and is discussed below. Reducing the risk of bleeding relies on successfully reducing the HVPG either non-invasively by pharmacotherapy or by endotherapy when pharmacotherapy is not tolerated. Porto-systemic shunting is not recommended in the prophylactic setting. When bleeding does occur, vasopressin analogues such as Terlipressin, nitrates, somatostatin analogues and antibiotics are used to reduce portal pressure in the acute setting in combination with endotherapy to achieve haemostasis. Porto-systemic shunting, in this setting, is effective and can be a life saving measure as rescue therapy when endotherapy fails.

Beta blockers

Both selective and non selective beta blockers can reduce portal pressure. The latter are more potent because NSBBs cause vasoconstriction of the splanchnic circulation by β2-receptor inhibition whilst also decreasing cardiac output by β1-receptor blockade. This results in unopposed alpha-1 activity, leads to a decrease in portal venous inflow, and thereby lowers portal pressure. (Westaby et al., 1984) Other effects of beta blocker therapy include the reduction of azygos blood flow and a decrease in bacterial translocation from the gut, both of which may play a role in the prevention of variceal haemorrhage. (Biecker, 2013) Studies have shown that reducing the portal pressure to below 12mm Hg
or by at least 20% of baseline results in a significantly reduced risk of bleeding from varices and indeed this reduction has been associated with improved outcomes. (Groszmann et al., 1990; Feu et al., 1995; Casado et al., 1998) Meta-analyses suggest the reduction in risk of bleeding from GOV with NSBBs is about 40%. (Hayes et al., 1990; Bernard et al., 1997) Though Propranolol, Nadolol and Timolol have all been shown to successfully reduce portal pressure, more recently, Carvedilol with its mild intrinsic anti-α (1)-adrenergic activity has been shown to be more effective than conventionally used NSBBs. (Reiberger et al., 2012; Aguilar-Olivos et al., 2014)

Timolol was unable to reduce the risk of developing varices in patients with cirrhosis and portal hypertension (Groszmann et al., 2005) and therefore NSBB use for prevention of GOV development is not recommended. (Ge and Runyon, 2014) Though one trial suggested Nadolol prevented the growth of small oesophageal varices with a small increase in adverse events (Merkel et al., 2004) there is insufficient evidence to recommend the use of NSBBs in patients with small varices, who have not bled and who do not have clinical risk factors associated with a high risk of bleeding. In patients with small varices and Child Pugh B or C cirrhosis and / or stigmata of haemorrhage at endoscopy the benefits of NSBBs outweigh the risks and therefore they should be prescribed to reduce the risk of bleeding. All patients with medium to large varices however should receive prophylaxis with NSBBs.

Not all patients receiving beta blockers respond with a reduction of the HVPG. (Turnes et al., 2006) A reduction in the resting heart rate by 25% of baseline, but not less than 55, is clinically predictive of efficacy of betablocker therapy in reducing portal pressures to desired levels. However, only a third of patients
manage to achieve this endpoint and about 20% of patients do not tolerate
NSBBs. (Toubia and Sanyal, 2008) In addition, beta-blocker therapy can result
in both cardiac as well as non-cardiac adverse effects. The decrease in cardiac
output from β-1 antagonism may cause major cardiac side effects such as
worsening or induction of heart failure especially in patients with
cardiomyopathy which can occur in cirrhosis and negative chronotropy. (Ge and
Runyon, 2014) Non cardiac side effects include bronchospasm and reduced
effort tolerance, exacerbation of peripheral arterial disease, poor recovery from
insulin induced hypoglycaemia in diabetics, depression, fatigue, and sexual
dysfunction. (Ge and Runyon, 2014)

There is emerging evidence which suggests that NSBBs may only be effective
within a “therapeutic window” in the natural progression of cirrhotic liver
disease. (Krag et al., 2012) In advanced cirrhosis, in response to effective
hypovolaemia, peripheral vasodilatation and arterial hypotension, a number of
circulatory changes occur, including the up-regulation of the sympathetic
nervous system and of the renin-angiotensin-aldosterone system which along
with the development of sodium and water retention and the formation of
ascites, are aimed at maintaining adequate cardiac output and organ perfusion.
With progression of cirrhosis, this compensatory ability is lost and the
maintenance of blood pressure and cardiac output is important in prolonging
overall survival. Median survival was significantly lower in patients with cirrhosis
and refractory ascites treated with propranolol (5 months, with 19% probability
of survival at 1 year), as compared to the median survival in patients who were
not treated with propranolol (20 months, with 64% probability of survival at 1
year) (p<0.0001). (Sersté et al., 2010) Based on this data, NSBBs should not be
prescribed to patients with advanced cirrhosis with refractory ascites and should
be used with caution in patients with cirrhosis at any stage with hypotension. (Ge and Runyon, 2014)

**Nitrates and other agents**

Nitrates cause splanchnic vasodilatation and can reduce portal pressure. However, they are non-selective, and also cause systemic vasodilatation and hypotension. Their use in combination with betablockers has been shown to be associated with significant adverse effects without demonstrating a significant benefit when compared to using NSBBs alone. (Garcia-Pagan *et al.*, 2003)

Statins, beyond their cholesterol lowering actions, have been shown to increase NO bioavailability, modulate HSC contractile functions, inhibit both ET-1 expression and release and have antioxidant/anti-inflammatory properties all of which play a role in reducing portal pressure. (Ramarez *et al.*, 2012) In one study, Simvastatin significantly decreased HVPG (-8.3%) without affecting systemic haemodynamic effects and appear to be promising. (Juan G. Abraldes *et al.*, 2009) Further randomised studies are needed to confirm these effects prior to their routine use in the management of portal hypertension.

1.1.7 **Endoscopic management of Gastro oesophageal varices**

Endoscopic therapy in the form of sclerotherapy or band ligation has been explored as an alternative to pharmacotherapy as primary prophylaxis against bleeding from GOV. In one study, endoscopic sclerotherapy, which involves the injection of a sclerosant such as ethanolamine into varices, was found to be associated with increased mortality. (The Veterans Affairs Cooperative Variceal Sclerotherapy Group, 1991) Endoscopic band ligation has been shown to significantly reduce the risk of variceal bleeding when compared to no treatment (Shiv K. Sarin *et al.*, 1996; Lay *et al.*, 1997) or propranolol. (S. K. Sarin *et al.*, 2009)
However, band ligation has not been shown to be associated with improved survival when compared with NSBBs and therefore its role in prophylaxis is limited to patients who express a preference for banding due to NSBB intolerance or those in whom NSBBs are contraindicated. Combined therapy with betablockers and band ligation has not been shown to be superior to treatment with betablockers alone. (Toubia and Sanyal, 2008) This might be reflective of poor trial design and further studies are needed to assess combination pharmacologic and endoscopic treatment on the development of GOV in cirrhosis.

1.1.8 Management of acute variceal haemorrhage

General Measures
The initial treatment of acute variceal bleeding relies on resuscitative and supportive measures to maintain systemic perfusion whilst taking care to avoid over transfusion that might result in rebound increase in portal pressures. (McCormick et al., 1995; Castaneda et al., 2001) A recent randomised trial compared a restrictive transfusion strategy with a liberal transfusion strategy in 277 cirrhotic patients who presented with an upper GI bleed, 190 of which were related to GOV bleeding. The probability of survival was significantly higher in the group who were randomised to the restrictive transfusion strategy as opposed to the liberal strategy in patients with Child Pugh A or B disease (hazard ratio, 0.30; 95% CI, 0.11 to 0.85), but not in those with cirrhosis and Child–Pugh class C disease (hazard ratio, 1.04; 95% CI, 0.45 to 2.37). Within the first 5 days, the portal-pressure gradient increased significantly in patients assigned to the liberal strategy (P=0.03) but not in those assigned to the restrictive strategy. (Villanueva et al., 2013) It is therefore suggested that the
threshold Haemoglobin level for transfusion in acute variceal bleeding should be ≤7g/dL with a target post transfusion range of 7-9 g/dL. Antibiotic prophylaxis has been shown to improve short term survival by reducing the incidence of infective complications resulting from gut bacterial translocation and by reducing the risk of rebleeding. (Hou et al., 2004; Garcia-Tsao et al., 2007) Though proton pump inhibitors are commonly used to maintain clot stability and prevent post endotherapy complications due to gastric acid, their role in this setting lacks evidence. Omeprazole, in a randomised controlled trial did not demonstrate a beneficial effect on post sclerotherapy ulcers. (Garg et al., 1995) Pantoprazole was shown to reduce the size but not the number of post band ligation ulcers in a randomised placebo controlled trial. (Shaheen et al., 2005) However no trial has studied the role of acid suppression on prophylaxis or rebleeding risk in the setting of GOV bleeding.

Specific Measures

Treatment with splanchnic vasoconstrictors such as vasopressin and its synthetic analogues such as Terlipressin (which has a longer half life) improve survival in patients with acute variceal bleeding by increasing the probability of achieving haemostasis when used along with endoscopic treatment. (Escorsell et al., 2000; Bañares et al., 2002) Definitive haemostasis can be achieved by endoscopic therapy. Band ligation has been found to be superior to sclerotherapy and is recommended first line. (Laine and Cook, 1995; de Franchis, 2010) Band Ligation has been shown to be associated with early reduction in portal pressures to baseline levels post therapy resulting in fewer re-bleeds when compared with endoscopic sclerotherapy, which is associated with persistently raised portal pressures post therapy. (Avgerinos et al., 2004) Band ligation is started at the oesophago gastric junction and then continued
in a spiral manner every 2 cm upwards. If successful, band ligation is continued 1-2 weekly until varices are too small to treat or are eradicated. A further endoscopy in 3 months with enhanced endoscopic surveillance 6-12 monthly thereafter is recommended for follow up. (Garcia-Tsao et al., 2007) Endoscopic band ligation is effective in achieving haemostasis in 80-90% of patients. (D'Amico et al., 1995) Intraluminal balloon tamponade with a Sengstaken Blakemore tube (Figure 5) or a Linton tube may be used in cases where endoscopic therapy is technically difficult, for example due to poor views, or where it is unsuccessful. Balloon tamponade is successful in achieving haemostasis in 80-90% of cases. Though deploying the gastric balloon usually suffices, a separate oesophageal balloon may be used when adequate haemostasis is not achieved. This oesophageal balloon must be intermittently deflated to prevent pressure necrosis of the oesophagus. The insertion and deployment of the Sengstaken tube can be associated with complications and cannot be used for longer than 48 hours. It is therefore used temporarily - either as a bridge whilst another attempt at therapeutic endoscopy is made or whilst arrangements to allow the definitive reduction in portal pressure by a radiologically inserted or surgically created porto-systemic shunt can be made. Self expanding metal stents can also be used to achieve tamponade when bleeding from oesophageal varices does not respond to endotherapy. (Hubmann et al., 2006) However, data on safety and efficacy from larger cohorts is needed prior to their use being recommended outside of a controlled trial setting.
The Sengstaken Blakemore tube is used to control variceal bleeding as a rescue / bridging option when endotherapy fails or is unavailable. The SB tube is shown in yellow and has a gastric balloon which is inflated and wedged at the oesophago gastric junction to achieve haemostasis. An oesophageal balloon shown in red can also be inflated when gastric balloon insufflation is unable to control bleeding and blood is aspirated from the oesophageal suction port. A separate gastric aspiration port can be used to check if intragastric bleeding is persistent. (Figure used under the commons open licence – author Olek Ramesz)
1.1.9 Porto systemic shunts in the management of GOV

Portosystemic shunting to decompress the portal system can be achieved surgically or percutaneously under radiological guidance. Portosystemic shunting is associated with increased risk of hepatic encephalopathy (HE) in patients with pre-existing cirrhosis and increases mortality and therefore it is not recommended for primary prophylaxis. (D'Amico et al., 1995; Garcia-Tsao et al., 2007) When used early, surgical (porto-caval or distal splenorenal shunt surgery) or radiologically placed transjugular intrahepatic portosystemic shunts (TIPS) are effective at controlling bleeding and have been shown to improve survival. (Rossle et al., 1994; Sanyal et al., 1996; Banares et al., 1998; Garcia-Pagan et al., 2010; Orloff et al., 2012) One group has reported better long term outcomes and cost effectiveness of surgical porto-caval shunting when compared to TIPS. The higher intervention needed for post TIPS complications of stent occlusion / stenosis when compared to surgery were cited as disadvantages. (Orloff et al., 2012) However, the outcomes reported by this centre have yet to be replicated elsewhere and might be due to the use of uncovered Wallstents®, which as described below, are associated with a higher incidence of post TIPS dysfunction. Furthermore, the incidence of post TIPS shunt occlusion is much lower with PTFE covered stents which are currently the preferred prosthesis used for this purpose. (Rossle et al., 2006; Yang et al., 2010) A further advantage of TIPS is the opportunity to embolise the bleeding vessel visualised during venography offering superior control of bleeding and haemostasis. This along with the lack of availability of portosystemic shunt surgery as a treatment option, especially as salvage therapy due to limited surgical experience in most centres makes TIPS the preferred portosystemic shunt option to treat complications of severe portal hypertension.
1.1.10 Transjugular Intrahepatic Portosystemic Shunt (TIPS)

TIPS is a non surgical method of reducing portal pressure by radiologically guiding the insertion of a stent between the hepatic and portal veins performed via the transjugular route. (Figure 6) The first TIPS was performed in animal experiments by Rösch in 1969 (Rosch et al., 1969) and its use was first described clinically in 1982 by Colapinto and colleagues. (Colapinto et al., 1982) Through improvements in stent design, especially the PTFE covered stents, refinements in techniques and better understanding and management and surveillance of post stent dysfunction / occlusion and complications, TIPS has presently become the preferred management option (to surgery) to treat life threatening complications of portal hypertension and chronic liver disease. (Rössle, 2013)
**Figure 6:** A Transjugular Intra-hepatic Shunt Stent (TIPS) in situ.

TIPS placed through the right hepatic vein into the right branch of the portal vein using an expandable PTFE covered mesh stent. The procedure is usually carried out under general anaesthesia. The internal jugular vein is cannulated and a catheter is passed through the right atrium selectively into the right hepatic vein branch. A tract is created between the hepatic vein and a branch of the portal vein and this is dilated with a balloon. A stent, usually PTFE covered, is then placed in the tract and dilated to the required diameter. Pre and post stent HVPG measurements are taken to ensure clinically meaningful reduction in the gradient has occurred and the sheath removed after radiological patency using venography is confirmed. Figure used with permission from VIR, Chicago.
Indications for TIPS

TIPS can be a life saving measure in carefully selected patients with severe complications of portal hypertension who do not or will not meet criteria for liver transplantation and in those awaiting liver transplantation - the so called “bridge to transplantation”. Apart from bleeding from GOV, as discussed above, indications for TIPS include refractory ascites, (F. Salerno et al., 2007) hepatic hydrothorax, (Strauss et al., 1994; Jeffries et al., 1998; Spencer et al., 2002) Budd Chiari Syndrome, (Olliff and Olliff, 2006; Corso et al., 2008) portal vein thrombosis, (Blum et al., 1995; Senzolo et al., 2006) hepatorenal syndrome (Guevara et al., 1998) (Rössle and Gerbes, 2010) and sinusoidal obstruction syndrome (Azoulay et al., 2000; Jacobi et al., 2006). A small study and a critical review of literature reported a significant improvement in body composition and body mass in sarcopaenic cirrhotic patients post TIPS with one study reporting an increase in muscle grip strength, (Camci et al., 2009; Dasarathy et al., 2011) suggesting a role in improving sarcopenia, although this benefit needs further investigation in larger, better designed studies with standardised endpoints. Improvements in quality of life have been reported in a number of studies after TIPS. Nazarian and colleagues reported significant improvement in quality of life scores when assessed on a Karnofsky scoring scale when patients were followed up for 24 months (pre TIPS 48% post TIPS 86%). (Nazarian et al., 1996) However, this scoring scale was developed in patients on chemotherapy for cancer where a score of 0 equated to death and 100% to normal health. Zhuang et al used a Rand 36 item health survey to study the impact of TIPS on quality of life. Though a significant improvement was seen in limitations due to emotional problems, emotional well being, energy and fatigue and health change domains, no difference was seen in general health, physical function,
limitations due to physical health, social functioning and pain domains. (Zhuang et al., 2002).

Procedural considerations

Pre-procedural broad spectrum antibiotics have been shown to reduce the incidence of infective complications after TIPS (Gulberg et al., 1999) and are offered to all patients. Patency of the portal vein, confirmation of anatomy and exclusion of multifocal tumours is excluded by pre procedural imaging usually in the form of a CT Scan. Though the procedure can be performed using conscious sedation, in our centre, general anaesthesia is used as majority of procedures are carried out as rescue therapy after unsuccessful endotherapy for bleeding GOV. Stents that can be used for TIPS include uncovered mesh stents such as the Wallstent ® (Boston Scientific) or PTFE covered Nyitinol mesh stents such as the Viatorr® stent (W.L.Gore & Associates Inc.). The latter (Figure 7) has been shown to be associated with fewer complications long term and has become the preferred stent for this procedure. PTFE covering prevents bile permeation and intimal growth into the stent. The Viatorr® endoprosthesis additionally has an unlined bare portal venous section to allow unobstructed portal perfusion whilst at the same time allowing better anchorage of the stent.

Using a transjugular approach the right hepatic vein is cannulated and a wedge pressure along with a portal venogram using CO₂ is obtained. Thereafter portal venous cannulation is achieved, confirmed by portal venography and the portosystemic pressure gradient is determined. The tract hence formed is dilated to 8-10 mm using a balloon and a stent is then deployed across the dilated tract which is also dilated to 8-12 mm to achieve an appropriate reduction in portal pressure (<12 mm Hg). If the pre stent deployment pressures are not found to be elevated to clinically significant levels, a parallel
decompressing shunt might be responsible and this is isolated and embolised prior to stenting. A final venogram is obtained prior to the completion of the procedure. A post procedural baseline doppler ultrasound is requested prior to discharge to allow documentation of baseline shunt flow and hepatic vein flow.

**Figure 7:** The Viatorr® PTFE covered endoprosthesis stent

![Diagram of Viatorr stent endoprosthesis]

*The Viatorr stent endoprosthesis has an uncovered bare portal section which allows unobstructed portal perfusion whilst at the same time allowing better anchorage of the stent whilst the covered portion prevents bile permeation and intimal hyperplasia which can result in stent occlusion. Adapted from www.goremedical.com/viatorr.*

**TIPS related complications**

TIPS is an invasive procedure associated with risks and complications both short term and long term. Short term complications are related to the procedure itself and physiological changes that result from hepatic bypass of portal venous blood into the systemic circulation. In the longer term, problems encountered are those of stent occlusion and dysfunction. Technical complications occurring at the time of TIPS placement include capsular perforation and intraperitoneal haemorrhage (1-2%), which can be minimised if portal sonography is used at the time of portal venous puncture; pulmonary embolism, mainly if additional
variceal embolisation is performed; stent migration / misplacement and finally infection, which can be minimised with the use of pre-procedural antibiotics. (Gulberg et al., 1998; reviewed by Rössle, 2013) A clinically significant complication of TIPS is the occurrence of hepatic encephalopathy (HE) which occurs in 30-55% of patients. (Nolte et al., 1998; ter Borg et al., 2004a; Tripathi et al., 2004; Masson et al., 2008; Riggio et al., 2008) Pre-existing hepatic encephalopathy is the most important predictor of its recurrence post procedure. A significant proportion of these patients can be managed by pharmacotherapy using lactulose, Rifaximin and L-Ornithine L aspartic acid (L-OLA). One study found the incidence of HE to be lower when using PTFE covered stents. (Tripathi et al., 2006) However, about 10% of patients will develop severe pharmacotherapy refractory HE after a TIPS which may only respond to stent revision with reduction in stent diameter or stent occlusion. (Riggio et al., 2008) Other complications of TIPS include hepatic dysfunction and stent occlusion which again occur less frequently with PTFE covered stents, which are now most commonly used for this procedure. (Haskal et al., 1997b; Bureau et al., 2004; Barrio et al., 2005; Tripathi et al., 2006)

Patient selection criteria for TIPS

Given the clinical significance of complications, patient outcomes are determined by sound patient selection criteria which include accurate staging of liver disease and consideration of other concomitant co morbidity. Older age, (ter Borg et al., 2004b) high pre TIPS bilirubin >50 μmol/L, (Rajan et al., 2002) pre existing renal impairiment (Naga Chalasani et al., 2000; M. Schepke et al., 2003b; ter Borg et al., 2004b; Yoon et al., 2005) and pre existing HE (Jalan et al., 1995; Masson et al., 2008) have all been found to be independent predictors of poor outcome. The Child Pugh score has also been used to predict outcome.
after TIPS (Encarnacion et al., 1995) but was found to be inferior to a composite score, using three variables – bilirubin, creatinine and INR, called the Model for End Stage Liver Disease (MELD) score. A MELD > 18 was found to be associated with a significantly high mortality at 90 days. (Malinchoc et al., 2000; Francesco Salerno et al., 2002; Angermayr et al., 2003; Michael Schepke et al., 2003a) Non hepatological predictors of poor outcome include cardiac dysfunction and severe pulmonary hypertension. TIPS insertion causes an increase in the left atrial diameter, the pulmonary capillary wedge pressure, and total pulmonary resistance. This may unmask coexisting subclinical cardiomyopathy. (Van der Linden et al., 1996; Huonker et al., 1999; Naritaka et al., 2004) Diastolic dysfunction as predicted by an E/A ratio ≤1 (Rabie et al., 2009) has been shown to accurately predict mortality in patients undergoing TIPS. Therefore, the presence of cardiac systolic or diastolic dysfunction and clinically significant pulmonary hypertension are contraindications to TIPS. A small hard and shrunken liver, polycystic liver disease with large cysts, multifocal liver tumours and untreated active hepatic and systemic infection are other factors that relatively contraindicate TIPS placement both due to technical difficulties and higher rates of complications. As suggested by Rossle, predictors of outcome should only serve to guide choice and should not be used as absolute contraindications to carrying out the procedure. The risk of exsanguination and definite death in the absence of this life saving alternative should always remain an important consideration when faced with this decision. (Rössle, 2013)

Long term follow up of TIPS patency and outcome

Given the long term complications of shunt occlusion, periodical check of shunt patency has been advocated. This can be carried out by means of TIPS
venography or doppler USS every 6-12 months. (Foshager et al., 1995; Latimer et al., 1998; Bureau et al., 2007) TIPS venography remains the gold standard modality of evaluating shunt patency. An advantage of venography is the potential to offer treatment with angioplasty or re-stenting when shunt occlusion is detected. TIPS venography is carried out using a transjugular approach and is therefore invasive. Non invasive monitoring with the help of Doppler USS measurements of blood flow velocity in the shunt and/or portal vein have been used as surrogate markers of shunt patency. (Dodd et al., 1995; Ferguson et al., 1995; Foshager et al., 1995; Haskal et al., 1997a; Murphy et al., 1998; Zizka et al., 2000; J. G. Abraldes et al., 2005; Carr et al., 2006; Bureau et al., 2007; Pan et al., 2008) Though studies have reported on various doppler ultrasound criteria to predict patency with reasonable accuracy and high sensitivity, (Dodd et al., 1995; Foshager et al., 1995; Zizka et al., 2000; J. G. Abraldes et al., 2005) these have not been validated independently. When used in non derivation cohorts, these criteria have not shown similar results, questioning the use of doppler ultrasound in the long term follow up for the early detection of shunt occlusion. (Haskal et al., 1997a; Murphy et al., 1998; Carr et al., 2006; Pan et al., 2008). One reason for this discordance may be that the measurement of shunt velocity using USS doppler might only be useful for simple mid shunt stenosis rather than complex neo intimal proliferation with variable occlusion along the shunt. (Rössle, 2013) With this in mind and given the low incidence of shunt occlusion seen with currently used PTFE covered stents, it might be reasonable to do away with USS doppler and venographic surveillance. (Pan et al., 2008) However, at the present time, this cannot be universally recommended as a proportion of patients under follow up continue to have uncovered wallstents and remain well compensated and need long term
follow up for the early detection of shunt dysfunction. In our own centre, patients are allowed to make an informed choice on the type of follow up, if any, they would prefer and follow up decisions jointly made by the patient and liver team.

1.1.11 Endoscopic surveillance for GOV

Current guidelines recommend endoscopy in all cirrhotic patients at diagnosis. However, these guidelines do acknowledge that in patients with Primary Biliary Cirrhosis (PBC) GOV can occur during early stage disease and that stage at which surveillance should be carried out is unclear. This is detailed in the section that follows. In patients without GOV and well compensated chronic liver disease (Child Pugh A disease), endoscopies should be repeated at 2-3 yearly intervals. In patients with small GOV that have never bled, surveillance endoscopies should be repeated at 1-2 yearly intervals. Patients with decompensated disease with no varices or small varices should have annual endoscopies. (Garcia-Tsao et al., 2007)

1.1.12 Primary Biliary Cirrhosis (PBC)

Primary biliary cirrhosis is characterized by progressive non-suppurative destruction of small intrahepatic bile ducts resulting in cholestasis, portal inflammation and fibrosis which may ultimately result in cirrhosis and portal hypertension. The name itself is a misnomer as not all patients with PBC develop or have cirrhosis at diagnosis. It was first described by Addison and Gull in 1851 though the first clear description of the disease was given by Ahren and colleagues from the Rockefeller Institute in New York in 1950. (J. Rodes et al., 2007b)
1.1.13 Pathophysiology of PBC

PBC is considered to be a paradigm of autoimmune mediated liver disease with over 95% of patients (Oertelt et al., 2007) having highly specific M2 anti-mitochondrial antibodies (AMA) directed against mitochondrial 2-oxo-acid dehydrogenase complexes. These include the pyruvate dehydrogenase complex (PDC-E2), the branched chain 2-oxo-acid dehydrogenase complex, and the 2-oxo-glutaric acid dehydrogenase complex. These enzymes catalyze oxidative decarboxylation of keto acid substrates and are located in the inner mitochondrial membrane. (K. D. Lindor et al., 2009) The cause for auto-reactivity against these antigens is unclear and combination of genetic and environmental factors are thought to be involved. Studies have identified associations between smoking, urinary tract infections, hair dye and nail polish use, previous obstetric cholestasis, xenobiotic use (in animals), familial predisposition and PBC. (Zhang et al., 1994; Parikh-Patel et al., 2001; Selmi et al., 2004; Gershwin et al., 2005; R. Poupon, 2010; M.I. Prince, Ducker, S.J., James, O.F.W., 2010) One study reported a reduced risk of PBC with the use of oral contraceptives. (Corpechot et al., 2010) Recent studies have highlighted the role of impaired nuclear receptor signalling in the pathogenesis of cholestasis. (Halilbasic et al., 2013) However, the greatest insight into the genetics of PBC comes from a number of recent Genome Wide Association Studies (GWAS) and iChip studies which have identified a number of Human Leucocyte Antigen (HLA) associations associated with the condition. Risk haplotypes associated with PBC include those carrying DRB1*08 and DRB1*04 alleles and protective haplotypes include those carrying DRB1*11 and DRB1*15 alleles. Several non-HLA genetic loci associated with PBC listed in Table 4 have also been identified and may have implications for targeted treatment of the disease. (George F.
Mells and Hirschfield, 2001; Marco Carbone et al., 2014) These genetic studies have made it clear that PBC is a disease of immune dysregulation and have provided a conceptual framework to link augmented and impaired immunity of the Th-1 phenotype with conventional autoreactivity in the face of an impaired response to a possible pathogen. (David E. J. Jones and Mells, 2011; G.F. Mells et al., 2011)

**Table 4** Non HLA risk loci for PBC identified at genome-wide level of significance in at least one genome-wide association study or iCHIP study of primary biliary cirrhosis (Adapted from Marco Carbone et al., 2014)

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP</th>
<th>Odd ratio</th>
<th>p-value</th>
<th>Candidate gene</th>
<th>Diseases sharing risk loci with PBC</th>
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</table>

For each locus, results are from the study with strongest evidence of association.

a. Phenotypically-associated disorders that share risk loci with PBC are listed.
b. AITD, autoimmune thyroid disease; CD, Crohn's disease; CeD, celiac disease; T1DM, diabetes mellitus type 1; MS, multiple sclerosis; PS, psoriasis; RA, rheumatoid arthritis; SARC, sarcoidosis; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UC, ulcerative colitis; VIT, vitiligo.
1.1.14 Auto-antibodies in PBC

95% of sera of patients with PBC are positive for AMAs. Depending on the test used, 5-10% of sera of PBC patients are negative for AMA. (Michieletti et al., 1994) AMAs represent a heterogeneous group of auto-antibodies directed against antigens located on the inner mitochondrial membrane. According to their immunochemical structure, these antigens have been classified into different types from M1 to M9. Only anti-M2 antibodies (specific for E2 subunit of the pyruvate dehydrogenase complex, a multienzymatic complex localized within the inner mitochondrial membrane), anti-M4 antibodies (specific for solphito-oxidase), anti-M8 and anti M9 (specific for glycogeno-phosphorylase) are specific for PBC. Among these, anti-M2 antibodies have diagnostic importance and clinical significance as they are present in high titres in almost all the patients with PBC, while the other three are present in low titres and always accompany anti-M2 antibodies. Although the predominant autoreactive antibodies in PBC are AMA, a subgroup of PBC patient sera are positive for antibodies direct toward nuclear components (ANA) that at indirect immunofluorescence produce homogeneous, nuclear dot, speckled, centromere, or rim-like patterns. These antibodies occur in up to 43% of patients with PBC and their prevalence is shown in Table 5. During the last two decades, a number of nuclear structures have been recognized as specific targets of ANA in PBC. Two typical patterns of nuclear immunofluorescence staining (best described using Hep2 cells rather than the composite tissue blocks used in AMA immunofluorescence) have been described in PBC, one giving a membrane like pattern of staining (M-ANA) such as the anti gp210 and anti p65 antibodies and the other giving multiple nuclear dot (MND-ANA) staining such as anti sp100 and anti PML antibodies. Patients with AMA
negative PBC have significantly higher ANA detectable than patients with AMA positive PBC patients and although Anti gp210 and Anti SP100 antibodies lack sensitivity their high specificity allows the diagnosis of PBC to be made in the absence of AMA especially when a liver biopsy is not helpful or cannot be obtained. (Zeman and Hirschfield, 2009) Upto 50% of patients with PBC have extractable nuclear antigen antibodies (ENA) such as the anticentromere antibodies in the absence of coexisting rheumatological disorders. Their presence in AMA negative patients should raise the suspicion of PBC in patients with cholestasis. (Granito et al., 2006) The clinical significance of the presence of non AMA antibodies in PBC is unclear. Recent studies have suggested a role in defining the nature of progression of liver disease in these patients. The presence of the anti gp210 in 276 patients with biopsy proven PBC was associated with an increased risk of liver associated death or the need for liver transplantation [odds ratio (OR) = 33.777, 95% CI: 5.930, 636.745] suggesting their presence confers a poorer hepatological long term outcome and hence a need for enhanced follow up. In the same study, the presence of the anti-centromere antibody was associated with an increased risk of portal hypertension (OR = 4.202, 95% CI: 1.307, 14.763) therefore suggesting a need for screening of such patients for GOV early in the disease. (Nakamura et al., 2007)
Table 5: Autoantibodies in PBC - prevalence and sensitivity / specificity in PBC
(Adapted from Zeman and Hirschfield, 2009)

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>Prevalence, %</th>
<th>PBC diagnosis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AMA +</td>
<td>AMA -</td>
</tr>
<tr>
<td>ANA-positive</td>
<td>47–48</td>
<td>68–100</td>
</tr>
<tr>
<td>Multiple nuclear dot-like</td>
<td>12–24</td>
<td>38–41</td>
</tr>
<tr>
<td>Perinuclear/rim-like membranous</td>
<td>6–14</td>
<td>31–50</td>
</tr>
<tr>
<td>Speckled</td>
<td>24</td>
<td>41–46</td>
</tr>
<tr>
<td>Anticentromere</td>
<td>14–20</td>
<td>14–23</td>
</tr>
<tr>
<td>Anti-Sp100</td>
<td>24–31</td>
<td>38–54</td>
</tr>
<tr>
<td>Antiglycoprotein 210</td>
<td>16–18</td>
<td>15–45</td>
</tr>
</tbody>
</table>

1.1.15 Natural History of PBC

PBC is a slow and variably progressing disease mostly affecting women aged between 40-59 yrs over decades. (Danielsson et al., 1990; Witt-Sullivan et al., 1990; James et al., 1999; M. Carbone et al., 2013) The estimated prevalence of PBC in the UK is about 35/100000, and the number of patients in UK with PBC is estimated at about 20000. (James et al., 1999) More recently, it has been suggested that there are 3 forms of PBC with a “typical form” with progression to cirrhosis if untreated over 10-20 years, a crossover syndrome type with fluctuating or persistent co existent features of autoimmune hepatitis characterised by rapid progression of disease and a premature ductopaenic
variant which presents with jaundice and progresses to cirrhosis rapidly over 5 years. (R. Poupon, 2010) In addition, based on the presence or absence biochemical cholestasis and symptoms PBC may be further classified as asymptomatic and symptomatic PBC. Recent evidence suggests that the classification of PBC should include the clinical phenotype in relation to response to treatment, more specifically to UDCA (as detailed below), into “Responders” and “Non Responders” as there is an emerging body of evidence that this defines the long term outcome in these patients.

**AMA positive patients without other features of PBC**

AMA can be found in patients without any clinical or biochemical abnormalities consistent with PBC – sometimes referred to as “latent” PBC. Many of these patients have histological changes consistent with PBC when biopsied and a significantly large proportion demonstrate liver test abnormalities and symptoms during follow up over decades. (Metcalf *et al.*, 1996) None of these patients, however, develop portal hypertension or cirrhosis on follow up and therefore patients with “latent” PBC are considered to have very slowly progressing disease with survival similar to the general population. (Abe and Onji, 2008)

**Asymptomatic PBC**

With increasing awareness and the widespread use of generic autoimmune screening in the investigation of abnormal LFTs, a group of patients with positive serology and mild cholestatic liver tests without any symptoms of the disease have been identified. These are referred to as the asymptomatic PBC (a-PBC) patients. This cohort was first described by Long and colleagues in 1977 who noted that a decade was required for symptoms to develop in these patients. (Long *et al.*, 1977) Various groups have shown that these patients
have worse survival when compared to age and sex matched healthy controls. (Balasubramaniam et al., 1990; Mahl et al., 1994; Springer et al., 1999). In one of the largest studies of its kind, 469 asymptomatic PBC patients from the North East of England were followed for a median of about 7 years. The proportion of a-PBC patients developing symptoms was 50% at 5 years and 70% at 10 yrs post diagnosis. This study did not find any difference in overall survival between patients with a-PBC and symptomatic PBC (s-PBC) at diagnosis. The proportion of patients with a-PBC presenting with bleeding varices was 3% and 9% at 5yr and 10 yrs respectively in this study. A fifth of all patients presenting with a-PBC died from liver disease or needed transplantation during the follow up period contradicting previous suggestions that this was a milder form of the disease (Mahl et al., 1994) and highlighting the importance of close monitoring and the need for long term surveillance in this cohort. (M. I. Prince et al., 2004)

**Symptomatic PBC**

The clinical course and natural history of patients with PBC who present with symptoms has been altered by a better understanding of the disease and improved management strategies including the use of Ursodeoxycholic Acid (UDCA) at doses now recognised to be therapeutic. Patients with s-PBC have accelerated progression of their liver disease compared with those who have a-PBC. Mean survival time in these patients, in the pre-UDCA, era varies between 6-10 years; (Abe and Onji, 2008) time to progression to histologically advanced stage of fibrosis is 2 years and the probability of remaining in early stage over a period of 4 years is 29%. (R. Poupon, 2010) The development of liver failure was noted to be 15% in a large community based cohort of PBC patients followed up for 5 years in the North-East of England. (M. Prince et al., 2002)

One third of patients in a large cohort (a third of whom were cirrhotic) developed
varices when followed up over approximately 6 years. These patients had poorer 3 year survival (60%) and this was further worsened (45%) after an episode of GOV bleeding. (G.J. Gores et al., 1989a) UDCA delays histological progression in PBC with a 5 fold lower progression per year when compared to placebo. (Corpechot et al., 2000) UDCA treatment also improves transplant free survival in patients with PBC, the magnitude of this effect being 24% - 32% reduction in the risk of transplantation or death. (R.E. Poupon et al., 1994; K. D. Lindor et al., 1996) In one study, UDCA improved transplant free survival rates to 84% and 66% at 10 and 20 years, respectively, which were better than the spontaneous survival rate as predicted by the updated Mayo model, a prognostic scoring tool used in PBC discussed below (RR, .5; P < .01). The survival rate of patients in early stage disease was similar to that in the control population whereas the probability of death or OLT remained significantly increased in treated patients in late histologic stages (RR, 2.2; P < .05). (Corpechot et al., 2005)

**AMA negative PBC**

A separate group of patients who are negative for AMA but who present with symptoms, signs, lab biochemistry and histology similar to patients with PBC who are AMA positive have been described. (Michieletti et al., 1994; Goodman et al., 1995; Lacerda et al., 1995) 8% of 297 patients with clinical and histological features of PBC in an Italian cohort were found to be AMA negative. This group had a higher prevalence of Anti Nuclear and Anti Smooth Muscle antibodies than those with AMA positive disease. No significant differences were noted between the two groups with regards to complications of cirrhosis and development of liver failure resulting in death or referral for liver
transplantation. (P. Invernizzi et al., 1997) A discussion of various antibody profiles and their clinical significance is presented below.

### 1.1.16 Prognostic Predictors and Prognostic Scoring Systems in PBC

A number of clinical, laboratory biochemical variables and histological staging have been used to predict prognosis in PBC. Bilirubin is the most powerful predictor of outcome in PBC (Shapiro et al., 1979; R. Poupon, 2010) and a component of various prognostic scoring systems used in PBC. These scoring systems most commonly use regression to create mathematical formulae to help predict prognosis. Common variables included in these scores include age, bilirubin, albumin, prothrombin time, cholesterol, degree of cholestasis, severity of fibrosis, features of the overlap syndrome with autoimmune hepatitis and the presence of peripheral oedema. (Roll et al., 1983; E.R. Dickson et al., 1990; Inoue et al., 1995) The Mayo Risk scoring system, (E. Rolland Dickson et al., 1989) the updated Mayo model (Murtaugh et al., 1994) and the and the revised Autoimmune Hepatitis Group Score (J. A. Talwalkar et al., 2002) are the most commonly used scoring systems in PBC. The Mayo scores do not seem to be affected by the use of UDCA (P. Angulo et al., 1999c) and include age, serum levels of bilirubin and albumin, prothrombin time and presence or absence of peripheral oedema, including response to diuretic therapy. The advantage of the Mayo score is that it does not need a liver biopsy. Its predominant use currently is to predict the survival in untreated patients and to help compare this with patients receiving an intervention for PBC in trial settings. (Y. M. Lee and Kaplan, 2005) A number of scoring systems based on regression have been also developed to help predict prognosis in patients based on their response to UDCA. These are discussed in detail below.
**1.1.17 Clinical Manifestations of PBC**

Fatigue and pruritus are the main symptoms of PBC and described in detail below. Xanthomas and hyperlipidaemia are commonly seen in patients with PBC. (Longo *et al.*, 2002) Symptoms and signs of coexistent autoimmune conditions such as the Sicca syndrome, Sjogren’s syndrome, Raynaud’s syndrome, hypothyroidism and arthritis may be seen. PBC patients may be at a higher risk of developing osteoporosis, with severity of bone disease correlating with advanced PBC stage and disease severity. (Menon *et al.*, 2001; Mounach *et al.*, 2008) Signs and symptoms of portal hypertension can occur both in early and late disease as described below. Finally, signs of established chronic liver disease may be seen in advanced stages.

**Fatigue**

Fatigue was first reported as a symptom in PBC in the early 80s’ (Christensen *et al.*, 1980) and has been shown to be associated with decreased overall survival. (D.E Jones *et al.*, 2006; D.E.J. Jones *et al.*, 2010) It is present in over 2/3rd of PBC patients, with prevalence being reported to be as high as 85% in some studies, and has been shown to have a significant impact on perception of quality of life whilst not being associated with clinical, biochemical, or histological criteria suggestive of advanced liver disease. (Cauch-Dudek *et al.*, 1998; P.M. Huet *et al.*, 2000; Younossi *et al.*, 2000; Goldblatt *et al.*, 2002; R.E. Poupon *et al.*, 2004; Stanca *et al.*, 2005; G. F. Mells *et al.*, 2013) Recent evidence suggests that autonomic dysfunction is highly prevalent in patients with PBC and correlates with severity of fatigue. (Newton *et al.*, 2004; Newton *et al.*, 2007) Increased daytime somnolence, (Newton *et al.*, 2011) accelerated myocardial senescence (Hollingsworth *et al.*, 2012) and undiagnosed
hypothyroidism (Elta et al., 1983) may be contributory and worsen fatigue experienced by PBC patients.

**Pruritus**

Pruritus is another common and significant symptom of PBC occurring in up to 55% of all patients. (J. A. Talwalkar et al., 2003) Its mechanism is poorly understood and it is thought to be a combination of increased opioidergic tone (E.A. Jones and Bergasa, 1999) and the result of increased levels of bile acids in skin tissue. (Ghent et al., 1977) A recent study has suggested increased levels of serum lysophospholipase, autotaxin (ATX), and its product, lysophosphatidic acid (LPA), as potential mediators of cholestatic pruritus. (Kremer et al., 2012) Once pruritus develops, it does not go away completely unless treated. (K. D. Lindor et al., 2009) Patients with AMA negative disease have been shown to have lesser pruritus than those with AMA positive disease. (Sakauchi et al., 2006)

**1.1.18 Diagnosis and histological staging of PBC**

The diagnosis of PBC requires the presence of cholestasis (with preferentially raised alkaline phosphatase levels) in the presence of either compatible serology with anti mitochondrial auto antibodies with M2 specificity detected by ELISA or immunoblot assays and / or histological demonstration of characteristic signs suggesting non suppurative destructive cholangiopathy. (K. D. Lindor et al., 2009; R. Poupon, 2010) Patients with compatible serology without biochemical or symptomatic features consistent with PBC may have latent PBC as described above. A markedly raised ALT or AST and raised immunoglobulin G levels may occur in patients with the autoimmune hepatitis / PBC overlap syndrome and in these patients a liver biopsy is needed to
ascertain the diagnosis. Biopsies may also be needed if a coexistent pathology such as non alcoholic steatohepatitis is suspected. Histologically, the severity of PBC can be graded using scoring systems proposed by Scheuer or Ludwig. (P.J. Scheuer, 1967; Ludwig et al., 1978) Histological staging of PBC is shown in Table 6. Given the patchy nature of lesions (P. J. Scheuer, 1998) and the fact that severity of underlying fibrosis might be underestimated at needle biopsy, (Garrido and Hubscher, 1996) a biopsy with at least 10 – 15 portal tracts (K. D. Lindor et al., 2009; R. Poupon, 2010) and a tissue width of at least >1 mm (Colloredo et al., 2003) have been suggested to reduce the incidence of sampling error.

Table 6: Histological staging system for PBC

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>Florid lesion. Bile duct damage with inflammatory reaction. (Portal hepatitis)</td>
</tr>
<tr>
<td>Stage II</td>
<td>Vanished bile ducts, portal and periportal inflammation and ductular proliferation (Ductular reaction and periportal hepatitis). Periportal fibrosis.</td>
</tr>
<tr>
<td>Stage III</td>
<td>Portal fibrosis with periportal expansion and bridging fibrosis, typically without bile ducts. Septal fibrosis.</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Cirrhosis</td>
</tr>
</tbody>
</table>

1.1.19 Treatment of PBC

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/Kg/day is currently recommended as the first line therapy for Primary Biliary Cirrhosis and should be considered for all patients with abnormal biochemistry. (European Association for the Study of the, 2009; K. D. Lindor et al., 2009; R. Poupon,
2010) It is the only drug approved by the US FDA for treatment in PBC. UDCA is found in large quantities in bear bile (hence the name) and has been used in Chinese medicine for over 5000 years. Commercially available UDCA is a synthetic 24 carbon steroid bile acid which is hydrophilic and replaces the endogenic lipophilic bile acid pool by 40-50%. In PBC, UDCA acts by increasing the hydrophilic nature of endogenous bile acid pool thereby increasing choleresis. It also acts by immunomodulation and has cyto protective effects against cytokine and bile acid mediated damage. (Raoul Poupon, 2012) A number of studies have shown the beneficial effect of UDCA in PBC on improving liver biochemistry, (R. E. Poupon et al., 1991; Battezzati et al., 1993; Heathcote et al., 1994; Combes et al., 1995) delaying histological progression, (Paul Angulo et al., 1999a; Corpechot et al., 2000; R.E. Poupon et al., 2003) and improving survival. (Corpechot et al., 2005) In a combined study using data from 3 clinical studies, PBC patients with moderate to high risk (as defined by serum bilirubin levels and those with stage IV disease) treated with UDCA at a dose of 13-15 mg/Kg/day had a transplant free survival which was significantly better than those treated with placebo. (R.E. Poupon et al., 1997) UDCA may also have a role in lowering portal pressures in PBC. In one prospective trial, UDCA use was shown to reduce the risk of varices in patients with PBC. Over 4 years 16% of patients on UDCA versus 58% of those on placebo developed varices (p<0.001). (K.D. Lindor et al., 1997b) The effect of the use of UDCA on the natural history of disease has been to reduce the number of patients with PBC undergoing liver transplantation. (D.E.J. Jones et al., 1995; J. Lee et al., 2007; Milkiewicz, 2008) Some meta-analyses, however, have questioned this beneficial effect. In one meta-analysis, no significant difference was found in the incidence of death (odds ratio 1.21, 95% CI 0.71-2.04), liver related death (0.72,
(0.22-2.32), liver transplantation (1.27, 0.78-2.07), death or liver transplantation (1.26, 0.87-1.82), and in the development of complications of liver disease (1.11, 0.64-1.92) when compared with placebo. (Goulis et al., 1999) 2 recent Cochrane reviews seemed to support these findings. (Gong et al., 2007b; Gong et al., 2008; Rudic et al., 2012) However, it is generally accepted that the results of these meta-analyses might have been affected by the inclusion of trials with too short a duration of follow up and suboptimal UDCA doses to observe a beneficial effect on long term outcomes. (K. D. Lindor et al., 2009; R. Poupon, 2010) Furthermore, these results do not take into consideration the fact that not all patients with PBC respond to UDCA and that non responsive PBC may be a separate clinical phenotype when compared to responsive PBC. This is discussed in more detail in the next chapter. A number of alternative treatment modalities have been considered for the treatment of PBC in those who do not respond adequately to UDCA.

**Budesonide**

Budesonide along with UDCA has been shown to improve biochemistry and histology in patients with PBC. (Leuschner et al., 1999) In a 3 yr prospective open labelled randomised trial 77 patients were randomised to receiving UDCA at a dose of 15mg/Kg/day alone or in combination with Budesonide at a dose of 6 mg/day. Fibrosis decreased by 25% in the combined group but increased by 70% in the monotherapy group (p=0.0009). Bilirubin values however increased in the combination group and were lowered in the monotherapy group. However, endogenous steroid response blunting was noted at 2 years suggesting systemic exposure after long term use. (Rautiainen et al., 2005) In another study when 22 patients with suboptimal response to UDCA were given budesonide at a dose of 9mg/day for 1 year a significant, but transitory
improvement in bilirubin was seen and a significant, but marginal improvement in serum alkaline phosphatase was seen with combination therapy. The Mayo risk score however increased significantly (p =0.02) and there was a significant loss of bone mass (p <0.001) in the lumbar spine. Budesonide-induced hyperglycaemia and cosmetic adverse effects were noted in 10% of the study cohort. (P. Angulo et al., 2000) Budesonide use was associated with the development of portal vein thrombosis in patients with stage 4 disease. (Hempfling et al., 2003) Given the lack of long term data on improving outcomes in PBC and concerns about its safety in patients with advanced disease, current guidelines recommend the use of budesonide only in patients with early stage disease where autoimmune overlap is clinically suspected.

**Fibric acid derivatives**

Fibric acid derivatives such as fenofibrate and bezafibrate in combination with UDCA (Hazzan and Tur-Kaspa, 2010) have been shown to improve liver biochemistry, IgM levels and histology in those with incomplete response. (Kanda et al., 2003; Dohmen et al., 2004; Itakura et al., 2004; Walker et al., 2009; C. Levy et al., 2011) Most of these studies are of short duration with variable end points and therefore further longer term studies are warranted before fibric acid derivatives can be routinely recommended.

**Other treatments for PBC**

No difference in survival as predicted by the Mayo Score was observed when methotrexate or colchicine were added to UDCA when compared to UDCA alone in a 10 year study of these medications in patients with PBC. (Kaplan et al., 2004) Other trials have also not demonstrated any benefit of methotrexate in the treatment of PBC. (Bach et al., 2003; Combes et al., 2005) Cyclosporine
has been trialled in PBC with a few early reports suggesting a benefit. (Wiesner et al., 1990; Lombard et al., 1993) However significant side effects greatly limit its use and a recent Cochrane review found no evidence supporting or refuting the hypothesis that Cyclosporine delays progression, death or liver transplantation in PBC. (Gong et al., 2007a) Obeticholic acid, a farnesoid X receptor ligand has recently been shown to improve liver biochemistry in PBC. Significant pruritus was however noted in these patients. (A. Mason et al., 2010) Various other therapies, in phase I or II trials have been shown to be effective in PBC. These including the anti CD20 agent Rituximab, (Tsuda et al., 2012) antiretroviral agents (A. L. Mason et al., 2004) and tamoxifen (Pietro Invernizzi et al., 2004; Reddy et al., 2004). Long term studies have yet to be performed to routinely recommend these agents in the management of PBC. Mycophenolate Mofetil, (J. A. Talwalkar et al., 2005) chlorambucil, (Li et al., 2012) thalidomide (McCormick et al., 1994) and D- penicillamine (Gong et al., 2004) have not been found to be useful for the treatment of PBC. In patients with end stage liver disease or severe pruritus liver transplantation should be considered and is associated with excellent graft and long term patient survival. (Jacob et al., 2005) Fatigue responds poorly to transplantation and therefore it should not be considered as the sole indication for listing. Post transplant surveillance for recurrence is not needed given that it is rarely clinically significant. (Neuberger, 2003)

1.1.20 Portal hypertension and GOV in the context of PBC

Incidence and Prevalence of GOV in PBC

Early reports suggested that the incidence of portal hypertension in PBC was low and that GOV were a characteristic of advanced disease. (Sherlock, 1959)
Both of these presumptions have been challenged in recent years. Incidental finding of GOV on OGD or a de novo bleed from GOV prior to the onset of jaundice or cirrhosis have been reported as a presenting feature in PBC in a number of studies. (Zeegen et al., 1969; Kew et al., 1971; Navasa et al., 1987; Colina et al., 1992; Vlachogiannakos et al., 2009) In a report on the development of portal hypertension in PBC, 47% of patients with PBC were found to have GOV and the development of GOV was associated with significantly reduced survival. One of the most comprehensive reports on GOV development included 265 patients without GOV who were enrolled in a trial of Penicillamine D for the treatment of PBC and followed up for a median of 6 years. 31% of patients in this cohort developed GOV. Almost half (48%) of those that developed GOV in this study experienced a variceal bleed and survival in those that bled was 63% and 43% at 1 and 3 years respectively. Histological stage and bilirubin levels were the only predictors of time to development of GOV. (G. J. Gores et al., 1989b) Similar point prevalence has been reported in other cohorts. Point prevalence of endoscopically proven GOV and bleeding from GOV were 25% (42/166), 8% (13/166) and 1% (2/166) respectively in the Newcastle PBC cohort. (D.E.J. Jones et al., 2002a) GOV were found to be present in 23% of PBC patients entering a trial of UDCA at the Mayo Clinic in USA (K. D. Lindor et al., 1997a) and in 30% of PBC patients in a Canadian study. (Bressler et al., 2005) In a recent trial designed to evaluate the effect of UDCA treatment on PBC that included HVPG measurements, portal hypertension as defined by a pressure of >6mm Hg was found in 37% of unselected patients with PBC and clinically relevant portal hypertension as defined by a pressure of >12mm Hg was found in 20% of the cohort. (Pierre-Michel Huet et al., 2008)
**Pathogenesis of portal hypertension in PBC**

The pathogenesis of portal hypertension in PBC patients with advanced stage IV disease (cirrhosis) is similar to that seen in non PBC cirrhotic patients and has been described in detail above. In patients with early stage PBC, portal inflammation and portal venulitis are thought to result in portal venous microthrombi formation. This vascular injury is then thought to result in nodular regenerative hyperplasia causing architectural distortion and leading to presinusoidal portal hypertension. (J. Rodes *et al.*, 2007b) Nodular regenerative hyperplasia was reported in up to half of all patients with early stage disease in one study. (Colina *et al.*, 1992) Nodular regenerative hyperplasia was also seen in all PBC patients presenting with portal hypertension and in 60% of those with PBC who developed GOV within the first 2 years after diagnosis in another study. (Kew *et al.*, 1971) In addition to nodular regenerative hyperplasia, portal phlebosclerosis has been demonstrated in patients with early PBC and might be an additional factor in the development of portal hypertension in these patients. (Nakanuma, 2003) Navasa et al showed that though the prevalence of portal hypertension was similar in stage II and stage III disease (57% vs. 55% respectively), pre sinusoidal hypertension was only seen in patients with stage II disease. This further adds credence to the hypothesis that portal hypertension in PBC starts with a presinusoidal component which is added onto by a sinusoidal component as the disease progresses. (Navasa *et al.*, 1987) The presence of anti centromere antibodies is associated with a higher risk of progression to portal hypertension in patients with PBC. (Nakamura *et al.*, 2007; Gao *et al.*, 2008) In this subset of patients, a ductular reaction is more pronounced and would seem to suggest an additional autoimmune mediated pathogenic mechanism for GOV development that has yet not been defined.
**GOV in early stage and asymptomatic PBC**

GOV development can occur in early stage and asymptomatic PBC patients. In the study by Gores et al, 7% and 12% of patients with histological stage I and II PBC had developed varices at 3 years and 5 years respectively. (G. J. Gores et al., 1989b) Similar finding have been reported in other studies. 7% of a-PBC patients and 8% of patients with histological stage I and II (early stage PBC) were found to have varices in a Japanese study. (Takeshita et al., 2003) In a more recent study, from the Mayo clinic, 6% of patients with Stage I and II disease and 16% of patients with Stage III disease were found to have GOV. (Ali et al., 2011) The Japanese study noted that the survival in a-PBC patients with GOV was significantly poorer than in a-PBC patients without GOV, however the numbers of patients included in the analysis was small. (Takeshita et al., 2003)

**Impact of GOV on outcomes in PBC**

As discussed above, the development of GOV and portal hypertension in patients with PBC heralds a poor prognosis. Though the development of GOV carries a poor prognosis in general, patients with normal bilirubin levels and bleeding from GOV as the presenting feature leading to the diagnosis have a survival similar to those who have never bled. (M R Biagini et al., 1990b; Vlachogiannakos et al., 2009) There remains some controversy about whether bleeding from GOV affects survival when GOV develop in patients with established PBC. Though Gores et al reported a poorer survival after a bleeding episode, (G. J. Gores et al., 1989b) Biagini et al reported no difference in the mean survival between bleeders (79 months) and non bleeders (90 months) in patients with an established diagnosis of PBC. (M. R. Biagini et al., 1990a)
The role of UDCA therapy on development of GOV

The role of UDCA therapy on the development of GOV is unclear. In bile duct ligated rats, hepatocyte and sinusoidal volume fractions were significantly higher whilst portal pressure, portal tributary blood flow and cardiac index were significantly lower in rats receiving UDCA for 4 weeks when compared with rats receiving placebo. Serum aminotransferase and alkaline phosphatase activities, and total serum bile acids and individual bile acid concentrations were not significantly different between the two groups. (Poo et al., 1992) The effect of UDCA treatment on the development of GOV in 180 patients with PBC was prospectively studied over a 4 year period in one study. The risk of developing varices in patients treated with UDCA was 16% versus 58% in those treated with placebo. (K. D. Lindor et al., 1997a) In contrast, the results of another study by Combes et al suggested a slight increase (however non significant statistically) in the risk of development of GOV and bleeding in patients treated with UDCA over 2 years. (Combes et al., 2004) A more recent study by Huet et al using HVPG demonstrated that UDCA use over 6 years prevented the progression of portal hypertension when compared with placebo. The use of placebo was associated with an increase in HVPG over 2 years and this returned to baseline values after a crossover phase in which patients were treated with UDCA over 4 years. (Pierre-Michel Huet et al., 2008) An important observation in the Huet study which might explain the contradictory findings seen in the study by Combes et al is that not all patients respond to UDCA in terms of normalisation of AST and improvement in HVPG and that patients who responded had better liver function at the time of initiation of UDCA therapy thus reiterating the importance of the responder and non responder concept and of starting UDCA as early in the course of the disease as possible. The
management of well established GOV in PBC is similar to that in chronic liver disease of other aetiology.

1.1.21 Screening strategies for GOV in patients with PBC

Given the high prevalence of GOV in PBC, the fact that almost 1 in 10 patients with asymptomatic / early PBC have GOV, and that this impacts poorly on survival be it in early or late stage disease, screening for GOV has an important place in the risk stratification and prognostication of these patients and has to be considered at diagnosis. However, current EASL and AASLD guidelines recommend screening for GOV with OGD in advanced (Stage IV) disease. (European Association for the Study of the, 2009; K. D. Lindor et al., 2009) These recommendations acknowledge previous evidence that histology may underestimate disease severity. (Garrido and Hubscher, 1996) They also acknowledge that the evidence regarding the selection of patients and timing of screening is contradictory (European Association for the Study of the, 2009; K. D. Lindor et al., 2009) especially in patients with early stage disease. Furthermore, a liver biopsy is rarely requested to make the diagnosis of PBC. (Zein et al., 2003) The default of indiscriminate screening all PBC patients with an OGD is associated with increased risk and cost and is likely to be unacceptable to all patients and healthcare providers. Alternative triggers are therefore needed to decide when to screen for varices, independent of histology. Several non-invasive tools based on laboratory parameters alone, or in combination with imaging, have been suggested to help identify PBC patients with GOV and who would benefit from OGD screening. Angulo et. al. prospectively followed 180 patients with PBC enrolled into a randomised placebo controlled trial of UDCA and found that 93% of patients with varices had a Mayo Score >4. (P. Angulo et al., 1999b) Bressler et al included a diverse
Canadian cohort of patients with varying aetiologies for chronic liver disease and demonstrated that in 86 patients with PBC or Primary Sclerosing Cholangitis, on multiple logistic regression, a platelet count < 200000 / mm$^3$ (Odds Ratio 6), an albumin <40 g/L (Odds Ratio 6) and serum bilirubin > 20 μmol/L (Odds Ratio 4) were independently associated with the risk of finding varices. This study also suggested that non invasive GOV screening thresholds previously suggested for patients with cirrhosis, where the aetiology of cirrhosis was predominantly non cholestatic disease, were not applicable in PBC. (Bressler et al., 2005) In another study from Florida, Levy et al found a platelet count of <140 000 mm$^3$ and / or a Mayo risk score of ≥ 4.5 to be independently associated with the risk of GOV in PBC. (Cynthia Levy et al., 2007) More recently male gender, albumin <3.5 g/dL, bilirubin ≥1.2mg/dL or 20 mmol/L, and/or PT ≥12.9 seconds has been suggested as a model (MABPT model) by the Mayo group to predict the presence of GOV in patients with early PBC. (Ali et al., 2011) Splenomegaly, on clinical examination has previously been shown to be an independent risk factor associate with the presence of large varices. (Naga Chalasani et al., 1999) However in a study including 9% of patients with cholestatic chronic liver disease, the presence of splenomegaly as defined on ultrasound criteria was not shown to be associated with the presence of GOV. (Zaman et al., 1999) Splenomegaly was found to significantly associated with the presence of GOV on univariate but not on multivariate analysis in another study including 143 patients. (Schepis et al., 2001) However the ideal criteria for non invasive prediction of GOV in PBC remain elusive. Furthermore, none of these criteria have been validated in the UK, it remains unclear if these non invasive predictive strategies are cost effective and finally, none help with
defining a percentage probability of detecting GOV in PBC patients to allow risk stratification.
1.2 Aims

Primary Aim
To develop and externally validate a cost effective and non-invasive clinical tool to predict the probability of finding GOV in patients with PBC – the Newcastle Varices in PBC (NVP) Score.

Secondary Aims
1. To identify risk factors associated with the development of GOV and bleeding from GOV in patients with PBC.

2. To establish the clinical impact of GOV on the natural history of PBC with particular reference to variceal bleeding episodes.

3. To establish the effect of the development of GOV and variceal bleeding on survival in patients with PBC.
1.3 Methods

1.3.1 Study setting, design and population

Newcastle Cohort

A cross sectional retrospective study was designed to identify all patients with a diagnosis of PBC (EASL and AASLD guidelines) who are under follow up at the Freeman Hospital, Newcastle upon Tyne, and who have undergone OGD for various clinical indications including screening for varices. The specialist liver service at the Freeman maintains a comprehensive database of all PBC patients under follow up. All patients on this database have been comprehensively characterised making the cohort fully representative. (Metcalf et al., 1997; James et al., 1999; M.I. Prince et al., 2001) Data from this cohort was used to develop the predictive tool and for internal validation as described below.

Cambridge Cohort

Similar to the Newcastle cohort, a cohort of PBC patients who have undergone an OGD for any clinical indication and under follow up at the Addenbrooke’s Hospital in Cambridge were identified. Clinical details for this cohort were collected. This cohort was used to externally validate the model.

Toronto Cohort

A further external validation cohort was set up using clinical details of PBC patients who attend the Toronto Western Hospital in Canada who have undergone an OGD for various reasons.
1.3.2 Inclusion and exclusion criteria

Inclusion criteria

1. All patients meeting diagnostic criteria for PBC according to EASL and AASLD guidelines (combination of cholestatic liver function tests and raised AMA (>1:40) and / or compatible histology) followed up at participating centres.
2. PBC patients who had OGD for any clinical reason including screening for varices

Exclusion criteria

1. PBC patients with HCC or portal vein thrombosis

1.3.3 Clinical data collection

Newcastle Cohort

Clinical records were reviewed for each patient on the PBC database and detailed clinical data collected. A previous study on the prevalence of portal hypertension in this cohort of PBC patients (D.E.J. Jones et al., 2002a) recorded biochemical, haematological, endoscopic and radiological / imaging data for each patient at various time points into this database. For the purposes of this study, data was extracted from this database at two time points – at first diagnosis of PBC and at the time of first OGD. In patients who were unstable at the time of the OGD, most recent steady state values were used. The Freeman Hospital Liver Transplant unit maintains a database of all patients who have had a liver transplant in the unit. This database was used to identify transplanted PBC patients. The national summary care records service, an online database that is constantly updated from primary care records, was used to gather mortality data. Collected data included the following:
• Demographic details including
  o Date of birth
  o Gender
  o Date first diagnosed with PBC as defined by the date at which the
diagnostic criteria for PBC were first met

• Liver biochemistry and blood haematological parameters including
  o Full blood count
  o Coagulation profile
  o Liver function tests including bilirubin, albumin, alanine amino-
  transferase (ALT) and alkaline phosphatase (ALP)

• Findings on OGD including
  o Number of OGDs
  o Date of each OGD
  o Presence or absence of GOV
  o Presence or absence of variceal bleeding as defined by the
  presence of bleeding seen from GOV or stigmata of recent
  haemorrhage present on GOV and associated blood in the upper
  GI tract or presence of GOV occupying more than 1/3rd of the
  oesophageal lumen and associated blood in the upper GI tract
  without other mucosal lesions

• Mayo Score at diagnosis

• Liver biopsy staging using the Scheuer score

• Abdominal ultrasound scan findings with particular attention to the
  presence of splenomegaly as defined by a bipolar measurement of >12
  cm

• Ursodeoxycholic Acid (UDCA) and beta blocker use, and finally,
• Outcome data in terms of date of death or liver transplantation in patients not transplanted and alive at the time of the study

Validation Cohorts (Cambridge and Toronto)
Clinical records were reviewed retrospectively for PBC patients from Cambridge and Toronto that met the inclusion and exclusion criteria requirements. Collected data included:

• Demographic details
• Liver biochemistry and blood haematological parameters similar to the Newcastle cohort
• Abdominal ultrasound findings with particular attention to the presence or absence of splenomegaly as defined above (available in the Cambridge cohort only), and
• Findings on OGD with reference to the presence or absence of GOV

1.3.4 Data Analyses and Statistical Methods
Data were collected using Microsoft Excel® and Microsoft Access® and analysed using Minitab® 15 and SPSS® 17 statistical packages. Health economics modelling was carried out using the Markov model which was constructed using java-script.

Univariate and Multivariate analyses
Each cohort of PBC patients from Newcastle, Cambridge and Toronto who underwent an OGD were divided into 2 groups based on the presence or absence of GOV. The group with GOV from the Newcastle cohort was further subdivided based on whether they had bled at index endoscopy or during the follow up period. Each group was characterised using basic descriptive statistics. Univariate analysis was used to identify statistically significant clinical
parameters from results of blood tests, imaging and liver biopsy to distinguish
the 2 groups. Non-parametric continuous data were analysed using the Mann
Whitney U test and categorical data using 2-tailed Fishers Exact test. In keeping
with the aim of creating a tool that is inexpensive, non invasive and universally
applicable, results from blood analyses that are measured routinely or available
in clinic during follow up of PBC patients and were significantly (p<0.05)
associated with the presence of GOV and bleeding on univariate analysis were
entered into binary logistic regression analyses. The presence or absence of
splenomegaly on ultrasound and PBC staging on liver biopsy were excluded
from this analysis. The former is an additional cost investigation in the UK which
would limit the use and cost effectiveness of the tool and the latter is an
invasive procedure which is not routinely performed in patients with PBC.
However, to cater to clinical environments where ultrasound is available
routinely in clinic without additional cost, a variant of the score was created to
include the presence or absence of splenomegaly on ultrasound. The Mayo
Score, a composite score created using the same variables as those entered
into the analyses individually, was also excluded from this analysis to avoid
multi-co-linearity. This is a statistical phenomenon that occurs when parameters
used for regression analysis are highly correlated, as would occur when a
composite score and the individual variables used to create this score were
entered into the same logistic regression analysis.

In the Newcastle cohort, where outcome data were collected, Kaplan-Meier
survival curves, censored for transplantation, were generated and the log-rank
test was used to compare survival amongst PBC patients with and without GOV
and amongst those who bled versus those that did not.
Predictive tool creation

Results of binary logistic regression of data from the entire Newcastle cohort were used to define the Odds Ratio (CI set at 95%) of finding GOV in these patients. Using a random number generator in SPSS, data from 70% of the cohort was grouped into a tool creation cohort and the remainder 30% was grouped into an internal validation cohort. Using binary logistic regression analysis on data from the tool creation cohort $\beta$ coefficients for each statistically significant parameter were identified. $\beta$ coefficients are statistically standardized estimates that establish the magnitude of effect of different independent variables on a dependant variable, when variables are measured in different units. (Schroeder et al., 1986) These $\beta$ coefficients can be used to create a prediction tool using a logit equation. This equation is derived as follows:

\[
\text{Probability} = \frac{1}{1 + e^{-z}},
\]

where $e$ represents the exponential function and $z$ is a number obtained by adding the products of the $\beta$ coefficients associated with each parameter and its numerical value. In other words $z = \beta_0 + \beta_1C_1 + \beta_2C_2 + \beta_3C_3 + \ldots$ and so on. Using these coefficients a probability tool was created to predict the probability of finding GOV in patients with PBC. The modelled tool created was checked for goodness of fit using the Hosmer-Lemeshow test which tests how closely the observed and predicted probabilities match and has the null hypothesis that the “model fits”; therefore a p value of >0.05 being desired. This tool was then both internally and externally validated using data from the internal validation cohort, the Cambridge cohort and the Toronto cohort. Sensitivity, specificity, positive predictive value and negative predictive value were calculated for the modelling, internal and external validation cohorts and finally for the entire cohort. A flexible approach was used and the performance characteristics calculated using 2 different cut offs (0.5 and 0.3).
which represent predicted probabilities of GOV being present of 50% and 30% respectively; the latter cut-off benefits from increased sensitivity at the expense of specificity and the former from increased accuracy at the expense of sensitivity. Receiver Operating Characteristic (ROC) curves were generated for the predictive tool in the modelling set, internal, external validation cohorts and a combination cohort. Performance characteristics and area under the ROC curve (AUROC) curve of the tool was compared to previously proposed predictive tools aiming to identify patients with suspected GOV.

*Modelling of treatment implications of using the NVP score*

Health economics modelling to define the cost implications of using the NVP Score was carried out with the assistance of Peter Mcmeekin, Institute of Society and Health, Newcastle University. An analysis was undertaken to explore the two metrics we believe are those by which the utility of the NVP Score will be judged, namely the number of bleeds prevented and the number of endoscopies carried out to prevent those bleeds. A multi-state Markov model was used to create a unique Cost Consequences analysis where 7 screening strategies were compared in terms of the number of endoscopies required and the number of bleeds. These included a “do nothing” arm where no screening would be initiated, an “OGD all” arm where every patient would undergo OGD at the onset of the cycle and then every 2 or 3 years according to AASLD guidelines and finally a “NVP Score” arm where every patient would be risk stratified using the NVP score at the onset and then every 2 or 3 years and endoscopy performed in those at high risk of GOV. The Markov model was terminated after 4 yearly cycles. The results represent the trade off between additional endoscopies saved (the consequences) versus incremental bleeds incurred (the costs). The Risk Prediction tool and the Cost-Consequences
analyses with options to choose various input models of prevalence and risk was uploaded onto the UK-PBC website as a supplemental tool that can be accessed online free of cost at http://www.uk-pbc.com/resources/uk-pbc-varices-prediction-tool.html. A number of assumptions (see Appendix – decision tree) were made about state transition probabilities in the Markov model. Aspects of these can be individually factored into this online model. This allows the model to be tailored to different healthcare systems and populations and allows flexibility amongst users to choose cut offs that best suit their practice.

1.3.5 Ethical Approval
As this was a clinical evaluation exercise, formal ethical approval was not sought and written informed consent was not needed.
1.4 Results

1.4.1 Demographics and prevalence of GOV in the study cohorts

Newcastle Cohort

The Newcastle Cohort comprised 330 PBC patients who underwent OGD at any time-point for any indication. Median age at first endoscopy was 64 years; 91.5% were female. OGD took place a mean 5 years following the diagnosis of PBC for any clinical reason including screening for varices. 159 patients who underwent OGD had GOV at that point. Baseline demographics and descriptive statistics of the 2 groups are shown in Table 7. Both groups were similar in age, gender, median dose of UDCA prescribed and time from diagnosis to OGD.

Toronto Cohort

The Toronto cohort comprised 157 patients who underwent OGD; 90% were female, median age at endoscopy was 55.6 and 22% had GOV at OGD. Patient characteristics are shown in Table 8.

Cambridge Cohort

The Cambridge cohort comprised 52; 89% were female, median age at OGD was 62.5 years and 31% had GOV at OGD. Patient characteristics are shown in Table 9.

Clinical characteristics of all cohorts are shown in Table 10.
Table 7: Patient characteristics and univariate analyses of factors associated with presence of GOV in the Newcastle cohort

<table>
<thead>
<tr>
<th></th>
<th>Varices (N=159)</th>
<th>No Varices (N=171)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age at OGD (IQR)</td>
<td>64 yr. (13)</td>
<td>64 yr. (15)</td>
<td>0.394</td>
</tr>
<tr>
<td>Females (%)</td>
<td>90</td>
<td>93</td>
<td>0.173</td>
</tr>
<tr>
<td>Median time lag of OGD from PBC diagnosis (IQR)</td>
<td>4 yr. (5)</td>
<td>5 yr. (7)</td>
<td>0.952</td>
</tr>
<tr>
<td>Mayo Score at PBC diagnosis (IQR)</td>
<td>5.7 (2.4)</td>
<td>4.4 (1.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ursodeoxycholic Acid Use (%)</td>
<td>41.5</td>
<td>62.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ursodeoxycholic acid dose, median (IQR)</td>
<td>600 (250)</td>
<td>600 (150)</td>
<td>0.055</td>
</tr>
</tbody>
</table>

Histological Stage at Diagnosis (%)

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Missing</td>
<td>14</td>
<td>17</td>
<td>0.723</td>
</tr>
<tr>
<td>Stage I, I-II, II</td>
<td>21 (13)</td>
<td>74 (43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage II-III, III</td>
<td>25 (16)</td>
<td>45 (26)</td>
<td>0.017</td>
</tr>
<tr>
<td>Stage III-IV, IV</td>
<td>99 (62)</td>
<td>35 (20)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Blood analyses at OGD (Median and IQR)

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>31 (32)</td>
<td>10 (9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>486 (451)</td>
<td>300 (461)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35 (8)</td>
<td>42 (5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets (x 10^9)</td>
<td>135 (80)</td>
<td>252 (116)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prothrombin Time (sec)</td>
<td>14 (2)</td>
<td>12 (2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Splenomegaly on Ultrasound at OGD (> 12cm) | 91% | 30% | <0.001 |
Table 8: Patient characteristics and univariate analyses of factors associated with presence of GOV in the Toronto Cohort

<table>
<thead>
<tr>
<th></th>
<th>Varices (N=32)</th>
<th>No Varices (N=115)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age at OGD (IQR)</td>
<td>55 yr. (15.0)</td>
<td>56 yr. (15.5)</td>
<td>0.8</td>
</tr>
<tr>
<td>Females (%)</td>
<td>90</td>
<td>91</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Blood analyses at OGD (Median and IQR)

<table>
<thead>
<tr>
<th></th>
<th>Varices (N=32)</th>
<th>No Varices (N=115)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>19 (20)</td>
<td>11 (12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>212 (265)</td>
<td>172 (150)</td>
<td>0.06</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>37 (7)</td>
<td>41 (4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelets (x 10⁹)</td>
<td>130 (78)</td>
<td>218 (128)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>INR</td>
<td>1.06 (0.2)</td>
<td>0.99 (0.1)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Table 9: Patient characteristics and univariate analyses of factors associated with presence of GOV in the Cambridge Cohort

<table>
<thead>
<tr>
<th></th>
<th>Varices (N=16)</th>
<th>No Varices (N=36)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age at OGD (IQR)</td>
<td>61 yr. (21.5)</td>
<td>64 yr. (17.3)</td>
<td>0.6</td>
</tr>
<tr>
<td>Females (%)</td>
<td>100</td>
<td>83</td>
<td>0.160</td>
</tr>
</tbody>
</table>

Blood analyses at OGD (Median and IQR)

<table>
<thead>
<tr>
<th></th>
<th>Varices (N=16)</th>
<th>No Varices (N=36)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>26.5 (33)</td>
<td>9 (5.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>209 (81)</td>
<td>179 (155)</td>
<td>0.33</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>36 (8)</td>
<td>43 (7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets (x 10⁹)</td>
<td>71.5 (45.5)</td>
<td>217.5 (113)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prothrombin Time (sec)</td>
<td>12.8 (1.95)</td>
<td>11.7 (1.1)</td>
<td>0.0054</td>
</tr>
<tr>
<td>Splenomegaly on Ultrasound at OGD (&gt; 12cm)</td>
<td>81.25%</td>
<td>36%</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Table 10: Comparison between Newcastle, Toronto and Cambridge cohorts

<table>
<thead>
<tr>
<th></th>
<th>Newcastle Cohort (N=330)</th>
<th>Toronto Cohort (N=147)</th>
<th>Cambridge Cohort (N=52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age at OGD</td>
<td>64 yr.</td>
<td>55 yr.</td>
<td>62.5 yr.</td>
</tr>
<tr>
<td>Females (%)</td>
<td>91.5</td>
<td>90</td>
<td>89</td>
</tr>
</tbody>
</table>

**Blood analyses at OGD (Median)**

<table>
<thead>
<tr>
<th></th>
<th>Newcastle</th>
<th>Toronto</th>
<th>Cambridge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>18</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>384</td>
<td>186</td>
<td>200</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>39</td>
<td>41</td>
<td>37</td>
</tr>
<tr>
<td>Platelets (x 10⁹)</td>
<td>189</td>
<td>193</td>
<td>150</td>
</tr>
<tr>
<td>INR</td>
<td>1.11</td>
<td>1.02</td>
<td>1.02</td>
</tr>
</tbody>
</table>

1.4.2 Clinical impact of GOV

83 of 159 patients in the Newcastle cohort with GOV (52% 95 CI 44%-60%) suffered 245 episodes of variceal bleeding during a median 11 years follow up (IQR 8). Patients with GOV that bled and those that did not, were similar in sex, age at endoscopy, UDCA use and dose of UDCA (Table 11). 39 of 159 patients (25% 95CI 18%-32%) presented with a variceal bleed at index endoscopy. Of the 120 patients who did not present with haemorrhage, 44 (37% 95CI 28%-46%) bled a median of 1.5 years (IQR 3.75) after GOV were identified. The proportion of patients with GOV that bled was similar in those on non-selective beta blockers and those that did not receive or tolerate these agents (48% vs. 52% respectively, p=0.75 Fisher’s exact test). However, the study was neither designed nor powered to detect any impact of beta blockade on variceal bleeding; data on adequacy of dose or the pharmacological response were unavailable. Of note, 21 (13% 95CI 8%-19%) of the 159 PBC patients with GOV had early stage (Stage I, I-II, II) disease at most recent biopsy. Variceal
haemorrhage was the first presentation of GOV in 3 early stage PBC patients (14% 95CI 3%-36%) and a further 5 (24% 95CI 8%-47%) bled during follow up.

**Table 11**: Patient characteristics and univariate analyses of factors associated with bleeding in those with GOV in the Newcastle cohort. IQR – Inter Quartile Range

<table>
<thead>
<tr>
<th></th>
<th>Bleeder (N=83)</th>
<th>Non Bleeders (N=76)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age at OGD (IQR)</td>
<td>62 yr. (13)</td>
<td>62 yr. (15.8)</td>
<td>0.850</td>
</tr>
<tr>
<td>Females (%)</td>
<td>93</td>
<td>86</td>
<td>0.199</td>
</tr>
<tr>
<td>Mayo Score at PBC diagnosis (IQR)</td>
<td>5.9(2.7)</td>
<td>5.5(1.6)</td>
<td>0.909</td>
</tr>
<tr>
<td>Ursodeoxycholic Acid Use (%)</td>
<td>37</td>
<td>35</td>
<td>0.334</td>
</tr>
<tr>
<td>Ursodeoxycholic acid dose, median (IQR)</td>
<td>600(100)</td>
<td>600(250)</td>
<td>0.777</td>
</tr>
</tbody>
</table>

**Histological Stage at Diagnosis (%)**

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<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Missing</td>
<td>8 (10)</td>
<td>6 (8)</td>
<td>0.706</td>
</tr>
<tr>
<td>Stage I, I-II, II</td>
<td>8 (10)</td>
<td>13 (17)</td>
<td>0.217</td>
</tr>
<tr>
<td>Stage II-III, III</td>
<td>12 (14)</td>
<td>13 (17)</td>
<td>1.000</td>
</tr>
<tr>
<td>Stage III-IV, IV</td>
<td>55 (66)</td>
<td>44 (58)</td>
<td>0.155</td>
</tr>
</tbody>
</table>

**Blood analyses at OGD (Median and IQR)**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>35.5 (41)</td>
<td>29 (23.75)</td>
<td>0.027</td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>469 (419.25)</td>
<td>514 (506)</td>
<td>0.522</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>35 (9.5)</td>
<td>36 (5.5)</td>
<td>0.133</td>
</tr>
<tr>
<td>Platelets (x 10⁶)</td>
<td>120 (60)</td>
<td>147 (88.5)</td>
<td>0.029</td>
</tr>
<tr>
<td>Prothrombin Time (sec)</td>
<td>14 (2)</td>
<td>13 (2)</td>
<td>0.135</td>
</tr>
<tr>
<td>Splenomegaly on Ultrasound at OGD (&gt; 12cm)</td>
<td>98%</td>
<td>83%</td>
<td>0.002</td>
</tr>
</tbody>
</table>
1.4.3 Factors associated with the presence of GOV

Univariate Analyses

On univariate analysis, in the Newcastle cohort, high bilirubin, high alkaline phosphatase, low albumin, low platelet count, higher prothrombin time, advanced histological stage at diagnosis (Scheuer stage >II), Mayo Score at diagnosis, not being on UDCA and splenomegaly on ultrasound were associated with the presence of GOV (all p<0.01) (Table 7).

Multivariate Analyses

In multivariate analyses using non invasively obtained variables only albumin, platelet count, alkaline phosphatase and the presence of splenomegaly were associated significantly with the presence or absence of GOV. The odds-ratios defining these associations are shown in Table 12 and can be used to quantify the incremental risk of having varices depending on every unit change in each clinical parameter. Therefore, a platelet count reduced by a value of 1x10⁹, albumin that is lower by 1 g/L and the presence of splenomegaly are associated with a 1.15% (95% CI 1.1% to 1.2%), 18% (95% CI 10% to 25%) and a 67% (95% CI 31% to 145%) increase in the odds of finding GOV, respectively.

1.4.4 Factors associated with bleeding in those with GOV

Univariate analyses

On univariate analysis, high bilirubin, low platelet count and splenomegaly were associated significantly with bleeding from GOV (Table 11).
**Table 12:** Multivariate analyses of parameters significantly associated with the presence of varices on univariate analyses

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Odds ratio (Exp B)</th>
<th>95% CI for odds ratios</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets (x10⁹)</td>
<td>&lt;0.001*</td>
<td>0.985</td>
<td>0.980</td>
<td>0.990</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>&lt; 0.001*</td>
<td>0.822</td>
<td>0.747</td>
<td>0.905</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase (IU)</td>
<td>0.032*</td>
<td>1.001</td>
<td>1.000</td>
<td>1.002</td>
<td></td>
</tr>
<tr>
<td>Presence of Splenomegaly</td>
<td>&lt;0.001*</td>
<td>6.742</td>
<td>3.139</td>
<td>14.480</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.272</td>
<td>0.981</td>
<td>0.949</td>
<td>1.015</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>0.496</td>
<td>0.608</td>
<td>0.145</td>
<td>2.551</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (µmol/L)</td>
<td>0.418</td>
<td>0.996</td>
<td>0.986</td>
<td>1.006</td>
<td></td>
</tr>
<tr>
<td>Prothrombin Time (s)</td>
<td>0.628</td>
<td>0.973</td>
<td>0.768</td>
<td>1.233</td>
<td></td>
</tr>
<tr>
<td>UDCA use</td>
<td>0.480</td>
<td>0.739</td>
<td>0.319</td>
<td>1.710</td>
<td></td>
</tr>
</tbody>
</table>

The Odds ratios signify the percentage odds of finding GOV for every 1 point change in that variable. * - statistically significant variables used to create the predictive equation.

**Multivariate analyses**

After adjusting for other variables significantly associated with bleeding in univariate analysis only the presence of splenomegaly was associated with bleeding (p=0.046) on multivariate analyses (**Table 13**). As lag time post diagnosis to presentation with bleeding may be a confounder and clinically significant, this was also entered into the multivariate analysis.
**Table 13:** Multivariate analyses of parameters significantly associated with bleeding from GOV on univariate analyses.

<table>
<thead>
<tr>
<th></th>
<th>P value</th>
<th>Odds ratio (Exp B)</th>
<th>95% CI for odds ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td><strong>Presence of Splenomegaly</strong></td>
<td>&lt;0.046*</td>
<td>0.111</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>Platelets (x10⁹)</strong></td>
<td>0.435</td>
<td>0.998</td>
<td>0.991</td>
</tr>
<tr>
<td><strong>Bilirubin (µmol/L)</strong></td>
<td>0.167</td>
<td>1.010</td>
<td>0.996</td>
</tr>
<tr>
<td><strong>Time lag post diagnosis (yrs)</strong></td>
<td>0.621</td>
<td>0.974</td>
<td>0.877</td>
</tr>
</tbody>
</table>

The Odds ratios signify the percentage odds of bleeding for every 1 point change in that variable. * - statistically significant variables

**1.4.5 Impact of GOV on survival**

Transplant free survival after diagnosis of PBC was better without GOV than with GOV (p<0.001) (Figure 8).

**Figure 8:** Kaplan Meier curves comparing survival following endoscopy in PBC patients with GOV versus those without GOV
1.4.6 Impact of GOV bleeding on survival

There was no significant difference in survival in patients with GOV that bled on follow up and those that did not (p=0.1) (Figure 9).

Figure 9: Kaplan Meier curves comparing survival in patients with PBC with GOV that bled vs. those that did not.

1.4.7 The Newcastle Varices in PBC Score

Using statistical methods described above, the probability of finding GOV in PBC was given either by the equation

\[
\text{The Newcastle Varices in PBC Score (NVP)} = \frac{1}{1 + e^{-(9.186 + 0.001*ALP - 0.178 *Alb - 0.015*PLT)}}
\]

Or \(\text{The Newcastle Varices in PBC Score – Splenomegaly variant (NVP-S)} = \)

\[
\frac{1}{1 + e^{-(6.385 - 0.138*Alb - 0.012*PLT + 2.013*X)}}
\]

where: Alb = Albumin in g/L, ALP = Alkaline Phosphatase in IU, PLT = Platelet x \(10^9\) and \(X=1\) if splenomegaly was present on USS and \(X=0\) otherwise.
An online calculator for the NVP is available at http://www.uk-pbc.com/resources/uk-pbc-varices-prediction-tool.html. The online tool includes a correction factor to standardise ALP results measured in laboratories with different reference upper and lower limit of normal to those of the reference range used for this study (30-130 IU). This correction factor (Chuang-Stein, 1992) is given as follows: standardised ALP to use in score = 30 + [(ALP - LLN) * (100 / (ULN - LLN))] where ALP = ALP result as obtained in external lab, LLN = lower limit of normal in external lab and ULN = Upper limit of normal in external lab. The p values of Hosmer-Lemeshow goodness of fit test for these models were 0.256 and 0.073 respectively indicating the models were adequate for the data set. Both models remained valid in both the internal (30% of the Newcastle cohort) and external Cambridge validation cohort. In addition the NVP score remained valid in the international Toronto external validation cohort.

1.4.8 Performance characteristics of the Score in all PBC patients

Both scores demonstrated discrimination exceeding 0.85 (AUROC) in the internal and external validation cohorts (Table 14 and Table 15) out-performing current criteria in both discrimination and accuracy. AUROC curves comparing the NVP with previously suggested criteria are shown in Figure 10 and Figure 11.
Table 14: Performance characteristics of the NVP score in modelling and internal validation cohorts and in the combined cohort at different probability cut-offs and comparison with other criteria.

<table>
<thead>
<tr>
<th>Test (probability cut off)</th>
<th>Sen.</th>
<th>Spe.</th>
<th>PPV</th>
<th>NPV</th>
<th>Acc.</th>
<th>AUROC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP Score – Modelling Set (0.5)</td>
<td>81.4</td>
<td>82.9</td>
<td>82.1</td>
<td>82.2</td>
<td>82</td>
<td>0.897 (0.856-0.938)</td>
</tr>
<tr>
<td>NVP Score – Internal Validation Set (0.5)</td>
<td>83.8</td>
<td>80</td>
<td>79.5</td>
<td>82.4</td>
<td>82</td>
<td>0.861 (0.777-0.944)</td>
</tr>
<tr>
<td>NVP Score - Combined cohort (0.5)</td>
<td>81.2</td>
<td>80.8</td>
<td>73</td>
<td>87</td>
<td>80.9</td>
<td>0.863 (0.831-0.896)</td>
</tr>
<tr>
<td>NVP Score - Combined cohort (0.3)</td>
<td>92.8</td>
<td>60.6</td>
<td>60.2</td>
<td>92.9</td>
<td>73.2</td>
<td>0.863 (0.831-0.896)</td>
</tr>
<tr>
<td>Cirrhotic (Stage IV) PBC</td>
<td>64.2</td>
<td>73.1</td>
<td>68.9</td>
<td>68.7</td>
<td>68.7</td>
<td>0.686 (0.628-0.744)</td>
</tr>
<tr>
<td>MRS &gt; 4.1</td>
<td>90.3</td>
<td>33</td>
<td>46.4</td>
<td>84.1</td>
<td>61.7</td>
<td>0.617 (0.536-0.697)</td>
</tr>
<tr>
<td>Platelets &lt;200</td>
<td>82.2</td>
<td>74.3</td>
<td>74.6</td>
<td>81.9</td>
<td>78.3</td>
<td>0.782 (0.731-0.834)</td>
</tr>
<tr>
<td>Platelets &lt;140</td>
<td>51.6</td>
<td>90.1</td>
<td>82.7</td>
<td>67</td>
<td>70.9</td>
<td>0.708 (0.651-0.766)</td>
</tr>
<tr>
<td>Platelets&lt;100</td>
<td>22.3</td>
<td>95.9</td>
<td>83.3</td>
<td>57.3</td>
<td>59.1</td>
<td>0.591 (0.529-0.653)</td>
</tr>
<tr>
<td>Total Bil ≥ 1.1 mg/dl &amp;/or alb &lt; 3.5 g/dL</td>
<td>80.9</td>
<td>73.1</td>
<td>73.4</td>
<td>80.6</td>
<td>77</td>
<td>0.768 (0.715-0.821)</td>
</tr>
<tr>
<td>MABPT</td>
<td>96.8</td>
<td>25.3</td>
<td>54.2</td>
<td>89.6</td>
<td>61</td>
<td>0.610 (0.549-0.671)</td>
</tr>
</tbody>
</table>

MRS - Mayo Risk Score, (P. Angulo et al., 1999b) MABPT – Males, Low Alb (<3.5 g/dL), High bil (≥ 1.2 mg/dL) and / or High PT (> 12.9), (Ali et al., 2011) Platelet cut off values as suggested by Bressler (Bressler et al., 2005) and Levy (Cynthia Levy et al., 2007)
### Table 15: Performance characteristics of the variant NVP-S (Newcastle Varices in PBC-splenomegaly) score in various cohorts

<table>
<thead>
<tr>
<th>Test (probability cut off)</th>
<th>Sen. %</th>
<th>Spe. %</th>
<th>PPV %</th>
<th>NPV %</th>
<th>Acc. %</th>
<th>AUROC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-S Score – Modelling Set (0.5)</td>
<td>88</td>
<td>82.6</td>
<td>83</td>
<td>87.7</td>
<td>85</td>
<td>0.912 (0.873-0.851)</td>
</tr>
<tr>
<td>NVP-S Score- Internal Validation Set (0.5)</td>
<td>86.8</td>
<td>68.3</td>
<td>71.7</td>
<td>84.8</td>
<td>77</td>
<td>0.874 (0.797-0.951)</td>
</tr>
<tr>
<td>NVP-S Score – Cambridge Validation Set (0.5)</td>
<td>94</td>
<td>67</td>
<td>56</td>
<td>96</td>
<td>81</td>
<td>0.934</td>
</tr>
<tr>
<td>NVP-S Score – Modelling Set (0.3)</td>
<td>94</td>
<td>74.4</td>
<td>78</td>
<td>92.8</td>
<td>84</td>
<td>0.912 (0.873-0.851)</td>
</tr>
<tr>
<td>NVP-S Score – Internal Validation Set (0.3)</td>
<td>94.7</td>
<td>58.5</td>
<td>67.9</td>
<td>92.3</td>
<td>75.9</td>
<td>0.874 (0.797-0.951)</td>
</tr>
<tr>
<td>AASLD / EASL - Cirrhotic (Stage IV) PBC</td>
<td>64.2</td>
<td>73.1</td>
<td>68.9</td>
<td>68.7</td>
<td>68.7</td>
<td>0.686 (0.628-0.744)</td>
</tr>
<tr>
<td>MRS &gt; 4.1</td>
<td>90.3</td>
<td>33</td>
<td>46.4</td>
<td>84.1</td>
<td>61.7</td>
<td>0.617 (0.536-0.697)</td>
</tr>
<tr>
<td>Platelets &lt;200</td>
<td>82.2</td>
<td>74.3</td>
<td>74.6</td>
<td>81.9</td>
<td>78.3</td>
<td>0.782 (0.731-0.834)</td>
</tr>
<tr>
<td>Platelets &lt;140</td>
<td>51.6</td>
<td>90.1</td>
<td>82.7</td>
<td>67</td>
<td>70.9</td>
<td>0.708 (0.651-0.766)</td>
</tr>
<tr>
<td>Total Bil ≥ 1.1 mg/dl &amp;/or alb &lt; 3.5 g/dL</td>
<td>80.9</td>
<td>73.1</td>
<td>73.4</td>
<td>80.6</td>
<td>77</td>
<td>0.768 (0.715-0.821)</td>
</tr>
<tr>
<td>MABPT</td>
<td>96.8</td>
<td>25.3</td>
<td>54.2</td>
<td>89.6</td>
<td>61</td>
<td>0.610 (0.549-0.671)</td>
</tr>
</tbody>
</table>

Sen. = Sensitivity, Spe. = Specificity, PPV = Positive Predictive Value, NPV = Negative Predictive Value, Acc. = Accuracy, AUROC = Area under receiver operating characteristic curve, MRS - Mayo Risk Score, Bil – Bilirubin, alb – albumin, MABPT – Males, Low Alb (<3.5 g/dL), High bil (≥ 1.2 mg/dL) and / or High PT (> 12.9). Platelet cut off values as suggested by Bressler et al and Levy et al.
Figure 10 ROC curves of the Newcastle Varices in PBC score derived in A) Tool derivation cohort, B) Internal validation cohort and C) Combined external validation cohorts.

Figure 11 Performance and Comparison of ROC curves of NVP score with currently suggested criteria as used in A) the Newcastle cohort and in B) the subgroup of patients with early PBC from the Newcastle cohort.
1.4.9 **Performance characteristics of the NVP Score in early PBC patients**

When applied to the subgroup of patients with early PBC the AUROC of the NVP score exceeded 0.9 again out-performing current criteria in discrimination and accuracy (Table 16).

**Table 16:** Performance characteristics of NVP Score in Early Stage Patients (Stages I-III) from the Newcastle cohort in comparison with previously suggested tools

<table>
<thead>
<tr>
<th><strong>Test (probability cut off)</strong></th>
<th><strong>Sen. %</strong></th>
<th><strong>Spe. %</strong></th>
<th><strong>PPV %</strong></th>
<th><strong>NPV %</strong></th>
<th><strong>Acc. %</strong></th>
<th><strong>AUROC (95% CI)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP Score (0.3)</td>
<td>80</td>
<td>86</td>
<td>62</td>
<td>94</td>
<td>83</td>
<td>0.904 (0.827-0.980)</td>
</tr>
<tr>
<td>NVP Score (0.5)</td>
<td>65</td>
<td>96</td>
<td>81</td>
<td>91</td>
<td>81</td>
<td>0.904 (0.827-0.980)</td>
</tr>
<tr>
<td>Total Bil ≥ 1.1 mg/dl &amp;/or alb &lt; 3.5 g/dL</td>
<td>71.4</td>
<td>95.8</td>
<td>83.3</td>
<td>91.9</td>
<td>83.6</td>
<td>0.829 (0.703-0.954)</td>
</tr>
<tr>
<td>Platelets &lt;200</td>
<td>76.2</td>
<td>81.1</td>
<td>53.3</td>
<td>92.3</td>
<td>78.6</td>
<td>0.782 (0.660-0.905)</td>
</tr>
<tr>
<td>Platelets &lt;140</td>
<td>47.6</td>
<td>94.6</td>
<td>71.4</td>
<td>86.4</td>
<td>71.1</td>
<td>0.696 (0.548-0.845)</td>
</tr>
<tr>
<td>Platelets&lt;100</td>
<td>14.3</td>
<td>95.9</td>
<td>50</td>
<td>79.8</td>
<td>55.1</td>
<td>0.551 (0.405-0.697)</td>
</tr>
<tr>
<td>MABPT</td>
<td>95.2</td>
<td>31</td>
<td>29</td>
<td>95.7</td>
<td>63.1</td>
<td>0.632 (0.508-0.757)</td>
</tr>
</tbody>
</table>

Sen. – Sensitivity, Spe. – Specificity, PPV – Positive Predictive Value, NPV – Negative Predictive Value, Acc. – Accuracy, AUROC – Area under receiver operating characteristic curve, Bil – Bilirubin, alb – albumin, MABPT – Males, Low Alb (<3.5 g/dL), High bil (≥ 1.2 mg/dL) and / or High PT (> 12.9). Platelet cut off values as suggested by Bressler et al and Levy et al.
1.4.10 Modelling the cost implications of using the NVP Score

Results of the cost consequence analyses in a hypothetical cohort of 1000 PBC patients, assuming prevalence of varices in PBC at 30%, (Bressler et al., 2005) bleeding from varices at 33% at 1 year and 41% at 3 year, (G.J. Gores et al., 1989a) rate of development of varices 11% per year (Mayo data) and standard prevalence data of outcomes in those with varices treated with beta-blockers or endoscopic band ligation and the performance characteristics of the tool in the combined cohort at a cut off of 0.3 as input was carried out with the help of Peter Mcmeekin, Institute of Society and Health, Newcastle University and results are shown in Table 17. Use of “OGD in all” every 2 years was associated with a significant reduction in the number of variceal bleeds compared to “doing nothing” (191 per 1000 patients over 4 years compared with 629, p<0.0001). This was, however, at the cost of 1938 endoscopies per thousand patients over the 4 year period. Application of the NVP score to pre-screen for need for OGD would be associated with only 13 additional bleeds, but would save over 1000 endoscopies over the 4 year period. The other screening modalities were equally effective at reducing the numbers of bleeds but did so at the cost of a significantly greater number of endoscopies than would have been the case with use of the NVP using the modelling approach.
**Table 17:** Cost Consequences analysis representing potential screening strategies for varices in PBC in a notional group of 1000 patients over a 4 year periods.

<table>
<thead>
<tr>
<th>Screening Approach</th>
<th><strong>Variceal Bleeds Over 4 Years</strong></th>
<th><strong>OGDs Over 4 Years</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>p (vs. NVP)</td>
</tr>
<tr>
<td>Do Nothing</td>
<td>629</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OGD</td>
<td>191</td>
<td>ns</td>
</tr>
<tr>
<td>Newcastle Varices in PBC Score (NVP)</td>
<td>204</td>
<td>ns</td>
</tr>
<tr>
<td>MRS&gt;4.5 and/or plat&lt;140</td>
<td>200</td>
<td>ns</td>
</tr>
<tr>
<td>MRS &gt;4.1</td>
<td>199</td>
<td>ns</td>
</tr>
<tr>
<td>Total Bil&gt; 1.1mg/dl and/or alb&lt;3.5g/dl</td>
<td>203</td>
<td>ns</td>
</tr>
<tr>
<td>MABPT</td>
<td>198</td>
<td>ns</td>
</tr>
</tbody>
</table>

Extra endoscopy values represent the additional endoscopies which would take place per 1000 patients per year using the relevant screening modality compared to the NVP to achieve the same level of varices prevention. Bil – Bilirubin, alb – Albumin, Plat – Platelets, MRS - Mayo Risk Score, MABPT – Males, Low Alb (<3.5 g/dL), High bil (≥ 1.2 mg/dL) and / or High PT (> 12.9)
1.5 Discussion

1.5.1 The Newcastle Varices in PBC Score

The Newcastle Varices in PBC (NVP) Score, and its variant model for use where ultrasound assessment of splenomegaly is readily available the Newcastle Varices in PBC Score – Splenomegaly (NVP-S), are non-invasive tools that predict the presence of GOV in patients with PBC, developed in a large well characterised cohort and validated internally and externally. Using a cut-off at 0.3 the NVP score has high sensitivity (93%), negative predictive value (93%) and discriminating value (AUROC of 0.86). The tool uses objective laboratory parameters that are measured routinely, readily and at low cost at follow up; it outperforms recent guidelines for screening in terms of accuracy and discrimination including previously suggested threshold cut offs values of platelet counts. In particular, it retains high sensitivity, negative predictive value and discriminating power (AUROC of 0.9) in patients with early PBC, a group in whom the use of such a tool would be of greatest value. A cost consequences evaluation of the NVP score to screen patients with PBC demonstrated significant savings in the number of endoscopies performed at the cost of a small increment in the number of variceal bleeds when compared to endoscopy as the screening strategy. The variant NVP-S score also demonstrated similar performance characteristics and discriminating power.

1.5.2 Prevalence and impact of GOV in patients with PBC

This study provides further evidence that GOV is common in PBC, found in early disease and associated with significant morbidity and mortality. More than 10% of patients with GOV had early stage PBC. The 5- and 10-year survival in PBC patients with GOV was 63% and 26% respectively, in contrast to 91% and
83% in patients without GOV. This reiterates the importance of using effective strategies, such as the NVP score, to identify PBC patients with GOV at an early stage to allow appropriate intervention.

1.5.3 Impact of bleeding from GOV in PBC patients

In this cohort, survival was similar in those with varices whether they bled or not. Patients with PBC tolerate variceal haemorrhage well. (M. R. Biagini et al., 1990a) It is also known that PBC patients have decreased blood loss when transplanted, (Popov et al., 1972) possibly due to an underlying hypercoagulable state. (Ritter et al., 1989; Ben-Ari et al., 1997; Segal et al., 1997; Pihusch et al., 2002) It is likely that severity of underlying liver disease predicts survival in PBC rather than decompensated portal hypertension.

1.5.4 Lack of Bilirubin and PT in the predictive score

Both bilirubin and alkaline phosphatase are independent predictors of outcome in PBC and are components of UDCA response criteria (Parés et al., 2006; Corpechot et al., 2008; Edith M. M. Kuiper et al., 2009) that predict survival in PBC patients who are treated with UDCA. Prothrombin time is an important measure of hepatic synthetic dysfunction in advanced cirrhosis and end stage liver disease. The absence of bilirubin and prothrombin time in the NVP Score and their statistical insignificance in multivariate analyses is noteworthy. A similar lack of significance of these parameters in predicting GOV in patients with PBC has been noted in previous studies. (Bressler et al., 2005; Cynthia Levy et al., 2007; Ali et al., 2011) In particular Ali et. al. showed that in early and mid histological stage PBC, PT and bilirubin were not significantly different between patients with and without GOV suggesting the role of presinusoidal portal hypertension in PBC. (Ali et al., 2011)
1.5.5 The role of pre sinusoidal portal hypertension in patients with PBC

Results from this and previous studies reiterate evidence that pre-sinusoidal portal hypertension secondary to nodular regenerative hyperplasia or the presence of portal phlebosclerosis (Colina et al., 1992; Nakanuma, 2003) might play a role in the development of GOV in PBC. These changes antecede abnormal lab parameters that accompany synthetic dysfunction accompanying cirrhotic late stage PBC or significant cholestasis which are features of advanced PBC, again emphasizing the value of the NVP tool in screening early stage patients with normal laboratory indices.

1.5.6 The role of splenomegaly in predicting GOV in patients with PBC

The presence or absence of splenomegaly on USS was shown in our study to be the single most important predictor of the presence of GOV. The presence of splenomegaly increases the odds of finding GOV six fold. However it is important to note that splenomegaly, or indeed, other biochemical and haematological parameters of portal hypertension may not be evident in early PBC and the greatest utility of the NVP score is to allow prediction of GOV in this group of patients where the results of this score may be the only discriminator available suggesting the need for OGD. Furthermore, the NVP Score outperformed the presence of splenomegaly, used on its own, in discriminating PBC patients with GOV (AUROC 0.88 vs. 0.79 respectively in the entire Newcastle Cohort). In fact, the NVP score itself can be used to accurately predict patients with splenomegaly with a high discriminating power (AUROC 0.85).
1.5.7 Exploring the role of UDCA in the development of GOV in PBC

The role of UDCA in preventing the development of GOV was highlighted recently. (Pierre-Michel Huet et al., 2008) The proportion of patients with GOV treated with UDCA was significantly lower than those without GOV, but not being treated with UDCA was not associated with GOV on multivariate analysis. Including UDCA in the predictive tool, for reasons of clinical significance, did not improve the model performance characteristics (accuracy 83% and 79% in modelling and validation cohorts respectively). The use of UDCA in PBC (and response) is associated with improvement in liver biochemistry including bilirubin, ALP and ALT. (Paul Angulo et al., 1999a; Parés et al., 2006; Pierre-Michel Huet et al., 2008; Edith M. M. Kuiper et al., 2009) ALP is included in the predictive score and improvement in this parameter after treatment with UDCA may outweigh the effect of UDCA on the NVP score. Appropriate weight-based dosing and adherence to UDCA treatment is difficult to ascertain during such clinical follow-up studies and this could influence results, limiting the interpretation of lack of significance of UDCA in multivariate analysis and exclusion from the score. The predictive value of alkaline phosphatase levels for GOV risk across all populations (UDCA treated and non-treated), together with the non-independence of UDCA therapy and alkaline phosphatase levels in predicting GOV on multivariate analysis and the well established reduction in alkaline phosphatase levels with UDCA would all be supportive, within the constraints of the data available, of the beneficial effect of UDCA therapy on GOV risk being linked to its anti-cholestatic effects further supporting its universal use in PBC.
1.5.8 Limitations of this study

This study was retrospective and therefore it was not possible to draw conclusions relating to size of GOV. However, the NVP tool is an initial screening strategy to help decide on the need for endoscopic screening of high risk patients and to seek those that would benefit from additional investigations. The absence of a qualifier describing the size of GOV did not reduce performance of the score. The study does suffer from selection bias as patients were included because an endoscopy had been requested, for various reasons including screening. However, the cohort was large and diverse and the score performed well in external validation. The retrospective nature of the study also precluded us from comparing NVP with transient elastography (“FibroScan®”) as a further potential modality for non-invasive assessment of portal hypertension risk in PBC.

1.5.9 Usability of this score in different health economic climates

An important attribute of the NVP tool is that it offers an objective percentage probability assessment of the risk of finding GOV in PBC patients and allows tailoring the cut off at which the need for further endoscopic evaluation is needed. An extension of this is reflected in choosing cost-consequences analysis to evaluate the health economics of the tool. A cost consequences analyses best reflects the outcomes that a decision maker would consider most important when deciding between alternative screening strategies (is minimising the risk of bleed from undiagnosed GOV or minimising the number of unnecessary OGD the local priority). Furthermore, extending the analysis by including financial costs or by associating quality of life measures with health outcomes would focus any results to distinct locales.
1.6 Conclusion

In conclusion, GOV in PBC are common, even in early PBC and have substantial effects on survival irrespective of whether they bleed or not. The Newcastle Varices in PBC Score is a simple, universally available, externally validated, highly discriminating and accurate predictive tool for clinical practice. Over a 1000 endoscopies would be saved if the score was used to screen 1000 PBC patients for varices over 4 years without any statistically significant increase in the number of variceal bleeds when compared to screening all PBC patients with an OGD over the same period.
CHAPTER 2: ROLE OF UDCA RESPONSE CRITERIA IN PREDICTING OUTCOMES IN PBC
2.1 Introduction

About 20% of patients with PBC do not respond to UDCA. It has been shown in a number of studies that the prognosis of patients who respond to UDCA is significantly better than that predicted by their Mayo Scores and that patients who normalise liver biochemistry on UDCA have no risk of progression to cirrhosis. (R. Poupon, 2010) Improvement in liver biochemistry during an initial period after commencement of UDCA treatment has therefore been evaluated in a number of cohort studies as a surrogate marker to predict outcome and impact on survival. These studies aim to identify “responders” to treatment who will benefit from UDCA versus non responders who will not and in doing so allow risk stratification to rationalise follow up and to enable early consideration of alternative treatment options including liver transplantation.

2.1.1 UDCA Response criteria

Various UDCA response criteria have been proposed previously and these are summarised in Table 18. The most commonly used criteria to gauge response to UDCA is the Paris I criteria which defines response as a bilirubin ≤ 1 mg/dL and ALP ≤ 3 ULN and AST ≤ 2 ULN 12 months after treatment with UDCA at a dose of 13-15 mg/Kg/d. (Corpechot et al., 2008) Patients who achieve response as per this criteria have a 10 year transplant free survival rate of 90% compared to 51% in those who do not (p <0.001). The Paris I criteria were recently validated as the most discriminatory predictor of response and outcome in a large well characterised PBC cohort in the UK. This study also suggested that female sex, age at diagnosis, low ALP, low bilirubin, high creatinine and lack of splenomegaly predicted response. (M. Carbone et al., 2013) The merits of this study are the inclusion of a large unselected cohort from the UK with detailed phenotypic data available at the time of analysis. This study cohort for the first
time included a significant proportion of younger age group and male patients making the analysis and interpretation of data meaningful. A recent study by the Dutch PBC group has shown that UDCA response was associated with lower risk of developing hepatocellular carcinoma. In this study, non response was associated with 10% and 20% risk of developing cancer at 9 and 15 years respectively (Figure 12) highlighting the need for screening in these patients. (E. M. Kuiper et al., 2010) In a recent international study, non-treatment with UDCA itself was not associated with cancer development; however, stratification by biochemical non-response at 12 months was associated significantly with future risk of HCC (HR 4.52, p<0.0001; IR 6.6 vs. 1.4, p<0.0001). Non-response predicted future risk in patients with early stage disease (IR 4.7 vs. 1.2, p=0.005), advanced disease (HR 2.79, p=0.02; IR 11.2 vs. 4.4, p=0.033), and when restricting the analysis to only male patients (HR 4.44, p<0.001; IR 18.2 vs. 5.4, p<0.001). On multivariate analysis biochemical non-response remained the most significant factor predictive of future HCC risk (adjusted HR 3.44, p<0.0001). (Trivedi et al., 2015)
**Figure 12:** Long term HCC risk in PBC patients in a Dutch cohort based on response and Non response to UDCA.

The log rank test suggests a significantly increased risk of developing HCC in patients who do not respond, equating to 10% and 20% risk of developing cancer at 9 and 15 years, when compared to patients with biochemical response to UDCA. *Figure adapted from Kuiper et al Eur J Gastroenterol Hepatol, 2010: 22(12), 1495-502.*
<table>
<thead>
<tr>
<th>Name of criteria</th>
<th>Yr.</th>
<th>No. of pts.</th>
<th>Dose of UDCA mg/Kg/d</th>
<th>Criteria</th>
<th>Time point (m)</th>
<th>Outcome (Responders)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mayo (Paul Angulo et al., 1999a)</td>
<td>1999</td>
<td>180</td>
<td>13-15</td>
<td>ALP &lt; 2 fold ULN OR Mayo Risk Score &lt;4.5</td>
<td>6</td>
<td>Favourable prognosis</td>
</tr>
<tr>
<td>Barcelona (Parés et al., 2006)</td>
<td>2006</td>
<td>192</td>
<td>15</td>
<td>ALP reduction 40% of pretreatment or ALP normalisation</td>
<td>12</td>
<td>Improved survival</td>
</tr>
<tr>
<td>Paris I (Corpechot et al., 2008)</td>
<td>2008</td>
<td>292</td>
<td>13-15</td>
<td>Bilirubin ≤ 1 mg/dL and ALP ≤ 3 ULN and AST ≤ 2 ULN</td>
<td>12</td>
<td>Survival similar to controls</td>
</tr>
<tr>
<td>Rotterdam (Edith M. M. Kuiper et al., 2009)</td>
<td>2009</td>
<td>375</td>
<td>13-15</td>
<td>Normalisation of bilirubin and albumin</td>
<td>12</td>
<td>Survival comparable to normal population</td>
</tr>
<tr>
<td>Paris II (Corpechot et al., 2011)</td>
<td>2011</td>
<td>165</td>
<td>13-15</td>
<td>Normal bilirubin ALP and AST ≤ 1.5 ULN</td>
<td>12</td>
<td>Survival without AE*</td>
</tr>
<tr>
<td>Sex and Age Criteria (M. Carbone et al., 2013)</td>
<td>2013</td>
<td>1090</td>
<td>13-15</td>
<td>Female gender and Age &lt;50 at presentation</td>
<td>-</td>
<td>Likelihood of response based on Paris I criteria</td>
</tr>
</tbody>
</table>

* AE = Adverse events over 7 yrs of liver-related death, OLT or referral to transplant unit, complication of cirrhosis, or histological evidence of cirrhosis.
ULN = Upper Limit of Normal
2.1.2 Do “Response Criteria” identify patients who have a good prognosis per se irrespective of treatment with UDCA?

An interesting question when considering response criteria is whether the attainment of these response criteria during follow up indicate a specific response to UDCA treatment or whether they reflect an inherent difference in the behaviour between two groups of PBC patients with a different course of disease progression whether it is treated or not? Components of response criteria are independent markers of prognosis in PBC. Bilirubin is an independent marker of severity in PBC and is the most powerful component of the Mayo risk score. ALP is an independent marker of outcome and is also a marker of cholestasis. Raised ALT and AST are markers of Autoimmune Hepatitis and the overlap syndrome which is associated with a poorer outcome.

It may therefore be suggested that in those patients who have biochemistry consistent with “response” one year after diagnosis whether this is a result of treatment or not, have a good prognosis.

A review of our database of patients with PBC to develop the Newcastle Varices in PBC score allowed a further opportunity to study the hypothesis that UDCA response criteria identify a subgroup of patients with PBC who have an inherently good prognosis irrespective of whether they have been treated with UDCA or not.
2.2 Aim

To assess in patients with PBC if “UDCA response” as per the Paris I criteria identify a cohort of patients with an inherently good prognosis whether they receive UDCA or not.
2.3 Methods

2.3.1 Study setting, design and population

A 10 yr prospective longitudinal follow up study was designed. 136 patients with PBC, who were initially identified in 1999 from our centre to study the impact and severity of fatigue, (Goldblatt et al., 2002) were included. 136 age and sex matched controls were identified from primary physician registers. Ten years ago, UDCA was not universally given to all PBC patients at our center – this was an era effect and reflected clinical practice at that time.

2.3.2 Clinical data collection

Apart from baseline demographic data, for the purposes of this study, biochemical data was collected for the entire cohort including bilirubin, ALT, ALP levels and Prothrombin time. Histological data on staging of disease was collected for all patients. Based on biochemical parameters that would define “response” as per the Paris I criteria (Corpechot et al., 2008) - reduction in ALP ≤ 3 times ULN, reduction in AST ≤ twice the ULN and a Bilirubin of ≤ 1mg/dl at least one year after beginning UDCA therapy, UDCA- receivers and Non-UDCA receivers were assigned to “responders” and “non- responder” groups (defined as per Paris I criteria after a minimum of 1 year of UDCA therapy in 1999 for the UDCA-group and at baseline in the non-UDCA group). Outcome data in terms of transplant free survival was collected by accessing the summary care record database to identify those patients and controls who were alive and cross referenced against the Freeman Hospital liver unit transplant database to identify patients who had been transplanted at the time of this study.
2.3.3 Data Analyses

Each group was characterised using basic descriptive statistics. Univariate analyses were used to identify statistically significant clinical parameters from results of blood tests and liver biopsy to assess if there were any differences between the groups. The log Rank test was used to compare survival between the PBC patient groups and controls.

2.3.4 Ethical Approval

As this was a clinical evaluation exercise, formal ethical approval was not sought and written informed consent was not needed.
2.4 Results

2.4.1 Demographic data

Of the 136 PBC patients in this study, 94 (69%) did not receive UDCA whilst 42 (31%) patients received UDCA. 124 (91%) of the 136 patients were female. Median age was 65.5yr (range 21-88yrs). There were no differences between the 2 groups at baseline with reference to biochemical parameters or histological stage. Demographic data is presented in Table 19.

Table 19: Baseline demographic data and comparison of UDCA receiving and Non UDCA Receiving PBC patients

<table>
<thead>
<tr>
<th></th>
<th>All patients N=136 Median (range)</th>
<th>UDCA Receiving patients N=42 Median (range)</th>
<th>Non UDCA receiving patients N=94 Median (range)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>124 (91%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>65.5 (21-88)</td>
<td>64 (45-86)</td>
<td>68 (21-87)</td>
<td>0.723</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.5 (0.2-7.7)</td>
<td>0.5 (0.2-7.7)</td>
<td>0.5 (0.2-6.1)</td>
<td>0.680</td>
</tr>
<tr>
<td>Alkaline Phosphatase (U/L)</td>
<td>131 (35-844)</td>
<td>159 (60-673)</td>
<td>120 (35-844)</td>
<td>0.697</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.2 (2.8-5.1)</td>
<td>4.1 (3.0-4.9)</td>
<td>4.2 (2.8-5.1)</td>
<td>0.227</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>13 (11-24)</td>
<td>13 (11-24)</td>
<td>13 (11-16)</td>
<td>0.441</td>
</tr>
<tr>
<td>Histological Stage</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>N.S</td>
</tr>
</tbody>
</table>
2.4.2 Responders and Non Responders

Of the 42 patients treated with UDCA 34 (81% 95% CI 66%-90%) of PBC patients treated with UDCA responded. Of the 94 that did not receive UDCA, 80 (85% 95% CI 76%-91%) had biochemical features that would be compatible with “response”. There was no significant difference in the proportion that met response criteria after UDCA treatment when this was compared with those that did not receive UDCA (p=0.559). However this study was not powered nor designed to detect this difference.

2.4.3 Comparison of survival between PBC patients and controls

The survival of patients with PBC as a whole was worse than that of age and sex matched controls. (Figure 13)

Figure 13: Kaplan Meier curves comparing survival between patients with PBC and age and sex matched controls.
2.4.4 Comparison of survival between UDCA receiving and non receiving PBC patients versus controls

Transplant free survival in UDCA receiving responders in our study mirrored that of controls, as expected. This was also significantly better than the transplant-free survival of PBC patients that do not receive UDCA at all (p<0.05). Importantly, transplant-free survival in non-UDCA receiving “responders” in our cohort was similar to that of UDCA receiving responders and indeed, to age and sex matched population controls (p=ns, Figure 14).

Non-UDCA receiving Non-Responders had a significantly worse outcome compared to responders and controls.

**Figure 14:** Transplant free survival in controls compared with PBC patients that “responded” to UDCA, those that did not receive UDCA but met the biochemical response criteria and with those that did not receive UDCA and did not meet the “response” criteria on follow up.
2.5 Discussion

The question we initially posed is one of prognostic risk stratification based on lab parameters that have been used in the context of measuring response to UDCA. Whether the same laboratory criteria predict prognosis in PBC patients who have not been treated with UDCA remains unclear and in an era where UDCA is universally prescribed to all patients with PBC, this question is difficult to answer. This study allows an insight into this area of ambiguity and provides some idea of the usefulness of these parameters in prognostic stratification in PBC irrespective of UDCA use. The findings in this study confirm that a lack of improvement in biochemical parameters after follow up for one year identifies a group of PBC patients that have a poor prognosis and for whom additional therapeutic options should be sought. Over a 10 year follow up period, patients who did not receive UDCA as a result of clinical practice at that time and who did not reveal any improvement in biochemistry had a prognosis that was significantly worse than those who had criteria consistent with a responder state. Most importantly, these patients had a survival that mirrored the Mayo predicted survival. The results seen in this study are consistent with those of other studies which aim to assess response in PBC patients treated with UDCA and to study the effect of response on survival.

There are some limitations of this study. The actual dose of UDCA in those that received UDCA was suboptimal (Mean dose 655mg). However, this does not affect the interpretation of the results as the key aim was to define if response criteria are effective as prognostic indicators if UDCA was not to be used. Furthermore, it would have been useful to compare various response criteria in terms of selection of criteria best predicting response in our cohort. However only the Paris I criteria were used. Recent evidence from the UK-PBC cohort
suggests that the Paris I criteria is the most discriminatory predictor of outcome in patients with PBC in the UK. (M. Carbone et al., 2013) Furthermore, the consistency of findings in larger cohorts of the general applicability of the different individual criteria implies that using the Paris I criteria should not disadvantage the interpretation of the results of this study.
2.6 Conclusion

It does not matter whether the attainment of parameters of biochemical response is a natural feature of untreated disease or a result of treatment. The usability of UDCA response criteria extend beyond their usefulness in predicting treatment outcomes in PBC patients treated with UDCA.
CHAPTER 3: EXACT SITE OF BIOTRANSFORMATION OF FOLATES IN HUMANS USING PORTAL VENOUS SAMPLING AT TIPS VENOGRAPHY
3.1 Introduction

The gastrointestinal tract and the liver are responsible for the absorption and biotransformation of a number of nutrients and drugs - the former by providing a metabolically active absorptive surface which allows active and passive movement of nutrients and solutes across it, possessing enzymes that allow biotransformation of various nutrients and the latter during first pass metabolism. Experimental assessment of bioavailability and the exact site of biotransformation of orally ingested essential nutrients and drugs can definitively be estimated in humans by means of portal venous blood sampling. However, accessing the human portal venous system to sample portal venous blood is challenging. (Sherlock, 1978) A number of methods have evolved to access portal venous blood. Direct cannulation of the portal vein and its tributaries via percutaneous transhepatic approach from a right mid-axillary line or subxiphoid approach can be performed. This is invasive and in one study was associated with an overall 20% complication rate, predominantly abdominal pain and a 6% rate of serious life threatening complications. (Miller et al., 1992) Trans-umbilical catheterisation of the portal vein may be undertaken for portal venous sampling. This however is again associated with significant adverse events including portal vein thrombosis and bleeding. (GÖthlin et al., 1975) None of these techniques can be used to sample portal venous blood in healthy human volunteers to study the biotransformation and metabolism of nutrients or drugs and allow pharmacokinetic profiling. To circumvent the need for human portal venous access, a number of studies requiring portal venous sampling have been performed in animals. However, a recent systematic review concluded that animal studies do not translate well into the clinical human domain with discordance seen between animal experiments and human trials.
(Perel et al., 2007) Animals used in experimental studies, are young and not
exposed to the same environmental influences or competing interactions and
interventions as humans. (Hackam, 2007) These influences and interactions
may modify phenotypic expression of genes and metabolic processes and
therefore metabolic studies in animals may not be representative of human
metabolic pathways. The biotransformation of folates is a typical example where
currently accepted wisdom on the exact site and bioavailability of folic acid
based on rat studies is contradictory to emerging evidence from preliminary in
vitro human cell culture studies.

### 3.1.1 Portal venous sampling in humans using TIPS

Some patients with early chronic liver disease and portal hypertension with
previous decompensation and TIPS have stable disease and almost normal
liver function especially when the inciting aetiology is modifiable or removed - as
seen most commonly after abstinence from alcohol. (Powell and Klatskin, 1968;
Brunt et al., 1974; Borowsky et al., 1981) Portal venography at the time of
insertion of TIPS or at surveillance venography offers a unique opportunity to
sample portal venous blood and in doing so, an opportunity to determine the
metabolic pathways of orally ingested nutrients and vitamins and the
pharmacokinetics of synthetic substitutes in vivo in humans. Portal venous
sampling at TIPS, as described above, has already been used to study the
occurrence of portal venous bacterial translocation in patients with cirrhosis
(Cohnen et al., 2003) and to measure the levels of endotoxaemia and Tumour
Necrosis Factor alpha in patients with alcoholic cirrhosis. (Treblicka et al., 2011)
More recently, portal venous sampling through TIPS has been used to
accurately measure pulsatile insulin release directly into the portal circulation of
human subjects (Song et al., 2000; Pørksen et al., 2002) and to directly study
hormonal changes in the portal circulation during an oral metabolic tolerance test. (Raddatz et al., 2008) These studies show that portal venous sampling through TIPS is feasible and safe.

3.1.2 The exact site of biotransformation of folates in humans

There exists significant controversy about the exact site of biotransformation of folates and in particular folic acid in humans. This has been comprehensively reviewed by Wright et al. (Wright et al., 2007) On the basis of studies predominantly in rat models, it was previously suggested that the predominant site of reduction of folic acid to dihydrofolate and then tetrahydrofolate forms was at the apical brush border cells of the small intestinal epithelium. Thus, currently accepted belief is that all folates including folic acid are converted to 5MTHF by the human small intestinal mucosa. These studies also suggested that when larger doses of folic acid (which may be considered non physiological) are administered, excess folic acid is passively transported into the systemic circulation. This currently accepted model is summarised in Figure 15. These assumptions have resulted in the validation of all short term experimental models that assess the relative absorption of various dietary and other folates with folic acid being considered as the standard reference folate. These views are challenged on 3 grounds - the complete absence of a 5MTHF response in the portal veins of human subjects given oral dose of folic acid when directly sampled, the low mucosal dihydrofolate reductase activity seen in man (exposing the pitfalls of extrapolating data from rat models to humans) and implications of the results of mathematical modelling of plasma response of 5MTHF to oral physiological doses of isotope $^{13}$C$_6$ labelled folic acid. (Wright et al., 2007) Measuring folates in the human portal vein is particularly challenging as accessing portal venous blood in healthy human volunteers is invasive,
associated with significant risk and neither feasible nor justifiable for the purposes of studying metabolic pathways. To date only 2 studies have been undertaken in human volunteers to assess the exact role of the small intestinal mucosa in the biotransformation of folic acid using portal venous sampling after an oral dose of folic acid was administered. Whitehead and Cooper undertook portal, hepatic vein and systemic venous sampling for 2 hours after oral dosing with 1000ug of folic acid in 3 adult volunteer patients undergoing umbilical vein catheterisation. It was shown that the intestinal mucosa changes 5-FTHF to 5 MTHF and transports FA unchanged. (Whitehead and Cooper, 1967) In another study carried out in Birmingham by Melikian and colleagues, portal and hepatic vein sampling was carried out in 11 patients who either had colorectal cancer with liver metastasis undergoing liver resection with umbilical vein and portal vein cannulation to deliver targeted chemotherapy or were undergoing hepatic vein catheterisation during investigation for portal hypertension. 0.5 mg of pure folic acid was administered and portal and hepatic vein sampling was carried out for a total of 2 hours post dose. Interestingly, no 5 MTHF was seen in the portal vein during this period whilst the folic acid levels were found to be transported unchanged into the portal vein. (Melikian et al., 1971) These studies suggest that the small intestinal mucosa was not involved in the biotransformation of folic acid but were criticised for the use of volunteers with cancer or those who had decompensated cirrhosis. They also used a differential microbiological assay to differentiate non methyl folate from total folate rather than mass spectrometric analysis now available in laboratories which can accurately measure individual folates. A novel method of accessing portal venous blood in humans at the time of a routine TIPS patency check by means of venography as mentioned above might allow an opportunity to study the role
of the small intestinal mucosa in the biotransformation of folic acid in humans. Whereas there is good evidence to suggest that a low folate status is associated with significant health problems for some individuals, dietary supplementation with folic acid may also be associated with significant patient and population-wide risk. This is a paradox given that both dietary reduced tetrahydrofolates and folic acid are taken up with similar affinity by the ‘proton-coupled folate transporter’ and the accepted wisdom mainly derived from rodent studies that physiological doses of folic acid are fully reduced and methylated in the intestinal absorptive mucosa and transferred to the hepatic portal vein as 5-MTHF. The latter being the main circulating plasma folate form as occurs after ingestion of all dietary folates. Mathematical modelling of systemic plasma response to single oral physiological doses of stable-isotope-labelled vitamin folates and folic acid has, however, challenged this consensus.
Figure 15: Schematic representation of folate absorption and biotransformation across the small intestinal mucosa.

Folate absorption from the gut lumen, metabolism in the mucosal cells and transport out into the hepatic portal vein as per currently accepted based on rat studies. All folates, according to current concepts, including folic acid are converted to 5MTHF by the human small intestinal mucosa prior to being transported into the portal vein. When larger doses of folic acid (which may be considered non physiological) are administered, excess folic acid is passively transported into the systemic circulation. PCFT – proton coupled folate transporter; DHFR – dihydrofolate reductase; MRP3 - multidrug resistance protein, THF – tetrahydrofolate.

3.1.3 Folates

In 1931, Lucy Wills, a chemical pathologist at the Royal Free Hospital in London, first described a nutritional factor in yeast extract (marmite) that when added to the diet could prevent and cure megaloblastic anaemia in pregnant
textile workers in Mumbai. (Wills, 1931) A number of different names were given to this factor by different pathologists including Vitamin M, Vitamin B₉, Vitamin Bₓ (folacin). The factor was finally named folic acid by Mitchell, Snell and Williams in 1941 in reference to its isolation from spinach leaves (folium = Latin for leaf). (Mitchell et al., 1941) The term “Folate” is a generic term that includes all pteroylglutamates that possess vitamin activity. They are one of eight different compounds which constitute the B vitamin family. Yeast extract (marmite), liver (turkey liver has the highest content), dried herbs especially dried spearmint, dark leafy greens like spinach, turnip greens and collards, beans such as mung and pinto, legumes, bean sprouts, asparagus, citrus fruits and peanuts are foods with a high folate content. Since their discovery, folates have been extensively investigated both with regards to their metabolic pathways and their role in health and disease.

3.1.4 Chemistry of Folates

Folates are a family of pteroylglutamates with different levels of reduction of the pteridine ring, different one carbon unit [methyl (CH₃-), formyl (CHO-), methylene (CH₂=), methenyl (CH₄-)] substitution and a varying number of glutamate residues. (Chango, 2012) Folic acid, pteroylmonoglutamic acid (PteGlu), is a synthetic form of the vitamin that does not occur naturally in significant amounts. (Wright et al., 2003) The chemical structure of folic acid is shown in Figure 16 and is made up of a pteridine ring, p-aminobenzoic acid and glutamic acid. Naturally occurring folates on the other hand are tetrahydrofolates with a hydrogen moiety at the 5, 6, 7 and 8 positions or dihydrofolates with a hydrogen moiety at the 7 and 8 positions and have one carbon units at the N5 and / or N10 positions. The chemical structures of tetrahydrofolate, 5 Methyl Tetrahydrofolate (5MTHF) and 5 Formyl
Tetrahydrofolate (5FTHF) have been shown in Figure 16. Folate polyglutamates (H₄PteGluₙ, where n= number of glutamate residues) are preferred by folate dependant enzymes as they are more metabolically active and better retained in cells when compared with folate monoglutamates (H₄PteGlu) and therefore natural folates are conjugated to a polyglutamyl chain containing different numbers of glutamic acids depending on the type of food. Natural folates lose their activity quite quickly over days to weeks when stored and a significant proportion of folate activity is lost during food harvesting, processing and preparation. (World Health Organisation, 2002) Folic acid on the other hand is a stable folate and therefore is most widely used in food supplementation. The molecular weight of PteGlu is 441.4 g/mol and the molecule is slightly soluble in water, quite soluble in salt form and insoluble in alcohol. It is sensitive to ultraviolet and visible light. (Chango, 2012) A number of stable isotope labelled folates have recently become available allowing the scientific community to make greater inroads into the study of folate metabolism than was ever possible before. (Powers, 2007)
Figure 16: Chemical structures of A) Folic Acid (Pteroyl monoglutamic acid)  B) 5-Methyl Tetra Hydrofolate and C) 5-Formyl Tetra Hydrofolate.
Folates are a family of pteroylglutamates with different levels of reduction of the pteridine ring, different one carbon unit [methyl (CH$_3$), formyl (CHO$^-$), methylene (CH$_2$=), methenyl (CH$_4^-$)] substitution and a varying number of glutamate residues. All folates have a pteridine ring, Paraminobezoic acid and glutamic acid.

3.1.5 Absorption of Folates

Naturally occurring folates are available to humans from 2 sources – that which is available in the diet and that which is synthetised by colonic bacteria. Dietary folates are polyglutamates which are associated with dietary proteins and from which they are released by digestive proteases. (Chango et al., 2012) Folate polyglutamates are then deconjugated by hydrolysis at the mucosal epithelium brush border cells by folyl-poly-$\gamma$-glutamate carboxypeptidase to the corresponding monoglutamates. Prior to 2007, a large number of studies had focused on 2 predominant mechanisms of folate transport – a neutral pH optimized reduced folate carrier, RFC, which is an anion exchanger expressed ubiquitously in all human cells and the folate receptors (FR), FR$\alpha$ and FR$\beta$, which are glycosylphosphoinositol (GPI)-linked high-affinity folate binding proteins that deliver their substrates into cells via an endocytic process. However, these transport mechanisms did not adequately explain the large quantities of folate transport across the intestinal epithelium. Furthermore, intestinal folate transport characteristically occurs at low pH. In 2007, Zhao et al characterised a saturable carrier mediated pH and energy dependant transport mechanism in the duodenum and proximal jejunum which unlike other epithelial tissues, is unique in its non selectivity - having similar affinity for both oxidised (folic acid) and reduced folate forms and for antifolates such as methotrexate. (Rongbao Zhao and Goldman, 2007) This is in accordance with a number of
studies that have shown that untransformed folic acid can appear in the systemic circulation when large presumably un-physiological doses of folic acid were administered. (Whitehead and Cooper, 1967; Melikian et al., 1971) In one study, when oral doses of folic acid in excess of 589nmol were administered orally, untransformed folic acid was shown to appear in the systemic circulation of man. (Kelly et al., 1997) This suggests that the there is a saturation point and evidence that intestinal conversion to 5-MTHF is not a prerequisite for transport therefore indicating that non physiological overdosing with folic acid is possible. This is particularly relevant in light of emerging evidence that suggests that in comparison to animals, human livers have low dihydrofolate reductase activity which can convert folic acid to dihydrofolate and which can then be converted to tetrahydrofolate. (Kamen et al., 1985; Whitehead et al., 1987) This would lead to significantly increased amounts of untransformed folic acid in the systemic circulation should un-physiological high doses be administered. This is important because it is believed that Folic acid has significantly higher bioavailability when compared with natural folates and given the fact that it is one of the most stable folates, it is used extensively in food supplementation and fortification. However, a recent study showed that the bioavailability of dietary folates is about 80% that of the amount present in natural dietary folates obtained from vegetables, fruit and liver (Winkels et al., 2007) and therefore a diet rich in natural folates may be all that is necessary to improve folate status in deficiency states. (Powers, 2007) Folates transported across the intestinal epithelium enter the mesenteric veins and then via the portal vein reach the liver where they undergo extensive first pass metabolism. The liver rapidly removes about 10-20% of the dietary folates (Chango et al., 2012) preferentially removing folic acid and allowing a significant proportion of 5-MTHF to pass
through into the systemic circulation making it the major plasma folate available for tissues. (Rogers et al., 1997) Folates in the body are mainly stored in the liver and undergo entero – hepatic recirculation which allows the plasma folate levels to be maintained. (Wright et al., 2007) With regards to excretion, folates are filtered by the glomerulus and re absorbed in the proximal tubule with daily excretion of folates being in the range of 1 to 12 μg. The proximal tubular capacity for re absorption of folates can be saturated when plasma folate levels are high, resulting in net renal folate excretion. (Chango et al., 2012)

3.1.6 Metabolism and Biochemical Function of folates

Folates are the major one carbon donors within cells. Normal cellular folate status is essential for cell division and homeostasis. Folates are involved in the synthesis of purine bases adenosine and guanosine, the pyrimidine nucleoside base thymidine and in many methylation reactions. Folates are of utmost importance in the methionine cycle wherein 5-MTHF re-methylates homocysteine to methionine which in turn is metabolized to S-adenosylmethionine. The latter is the major methyl donor in cellular reactions and may control gene transcription by cytosine methylation in the DNA which in turn affects gene expression and protein synthesis. A number of ill effects of folate deficiency are due to increased levels of circulating homocysteine and these have been extensively investigated in literature. Likewise, a high folate intake may be associated with low homocysteine levels which have been associated with cardiovascular risk, cerebrovascular disease and cognitive decline. (Reviewed by Dierkes and Nygard, 2012) The metabolism of folic acid and a summary of its interactions are shown in Figure 17 (as adapted from Fowler, 2001). After transferring the methyl group to homocysteine, 5MTHF is converted to tetrahydrofolate (THF) which enters the folate cycle to enable the
synthesis of purine nucleosides and thymidine and allow for further single carbon needing pathways to function. Folate metabolism occurs both in the cytoplasm and the mitochondria both containing a parallel array of enzymes with the former preferring incorporation of one carbon units from formate with purine and thymidine synthesis and homocysteine remethylation whilst the latter preferring one carbon incorporation from serine and resulting in the release of formate. (Chango, 2012) Lack of thymidine in folate deficiency may result in the mis-incorporation of uracil into DNA leading to DNA breakage and chromosomal breakage (Blount et al., 1997) and DNA instability which is associated with carcinogenesis especially in cases where the repair interferes with normal apoptotic mechanisms. (Blount et al., 1997; Duthie et al., 2010)
Figure 17: Schematic representation of the metabolism of folic acid and its interactions (adapted from Fowler et al., 2001)

Intracellularly, 5-methylTHF functions as a methyl donor for homocysteine remethylation. The resulting THF can directly be converted into 5,10-methyleneTHF by the action of serine hydroxymethyltransferase (SHMT). Conversion of THF into 5,10-methyleneTHF, via 10-formylTHF and 5,10-methenylTHF, is catalyzed by the trifunctional enzyme methylenetetrahydrofolate dehydrogenase (MTHFD1) that has
formyltetrahydrofolate synthetase, methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase activities. The 10-formylTHF can donate one-carbon groups for purines biosynthesis, whereas 5,10-methylenetetrahydrofolate can be used as a cofactor for the conversion of Uridine 5’ Phosphate (dUMP) into Thymidine 5’ Phosphate (dTMP). The latter reaction is catalyzed by the thymidine synthase (TYMS) enzyme and produces dihydrofolate (DHF), which requires subsequent reduction back to THF by the action of dihydrofolate reductase (DHFR). In addition to being a cosubstrate for dTMP synthesis, 5,10-methyleneTHF can also be reduced to 5-methylTHF by the riboflavin (vitamin B2)-dependent enzyme methylenetetrahydrofolate reductase (MTHFR), which competes for 5,10-methyleneTHF with TYMS.

### 3.1.7 Folates in health and disease

The primary manifestation of a low folate status in humans is macrocytic or megaloblastic anaemia. Peri-conceptual supplementation of folic acid in women has been shown to lead to a significant reduction in the incidence and recurrence of neural tube defects such as spina bifida in new borns. (MRC Vitamin Study Research Group, 1991; Czeizel and Dudás, 1992) A low folate status as explained above results in high levels of homocysteine which in turn has been shown to be a risk factor for cardiovascular disease, (David et al., 2002) stroke (Casas et al., 2005) and has been associated with dementia and Alzheimer’s disease. (Seshadri et al., 2002)

### 3.1.8 Folic acid supplementation – too much of a good thing?

Studies in the early nineties showing a reduction in neural tube defects in newborns with peri-conceptual folic acid supplementation led to the mandatory fortification of flour in the USA (1.4mg/Kg, 1998), Canada (1.5mg/Kg, 1998) and
Chile (2.2mg/Kg, 2000). (Wright et al., 2007) This has been shown to double the plasma folate levels in these countries after fortification was introduced. (Jacques et al., 1999) Given the identification of a low folate status or high homocysteine levels as risk factors, a number of observational cohort studies, randomised controlled trials of FA supplementation and meta-analysis have been carried out to establish if FA supplementation prevents or reduces the burden of cardiovascular, cerebrovascular, cognitive decline and malignant disease (especially colorectal cancer) in communities. The results of these randomised controlled trials and meta-analysis have been contradictory, in that though observational cohort studies have shown a reduced risk for participants ingesting high amounts of folate or taking supplemental folic acid, or those with high blood folate levels, randomised controlled trials of folic acid supplementation and other meta-analysis have failed to demonstrate these benefits. (Dierkes and Nygard, 2012) The authors of this article comment that though this discrepancy might reflect bias in observational studies and the insufficient length of time supplementation is needed to demonstrate clinically relevant endpoints, differences in the chemical structures and metabolic pathways of natural folates and synthetically derived folic acid might be one of the most important reasons. A recent large and well cited meta analysis involving data from 8 large randomised trials and including almost 37500 patients confirmed that dietary supplementation with folic acid to lower homocysteine levels had no significant effects within 5 years on cardiovascular events or on overall cancer or mortality in the populations studied. (Clarke et al., 2010) Worryingly however, a number of concerns have been raised about the possibility of causing harm by folate fortification programs and folic acid supplementation. (Kim, 2006) In 2006, an NIH study of just over 25000 women
participants with a baseline age of 55 to 74 years with complete information on dietary and multivitamin intake information, the risk of post menopausal breast cancer was increased in women taking supplemental folic acid. Similarly, a trial designed to assess the effect of folic acid supplementation on lowering colorectal adenoma incidence, reported quite unexpectedly, an increased risk of multiple and advanced colorectal adenomas and prostate cancer in the supplemented cohort. (Cole et al., 2007) The temporal relationship between colorectal cancer incidence and folic acid fortification programs was subjected to mathematical modelling. Fortification, in this model, was wholly or partially responsible for the increase in colorectal cancer incidence in the mid 1990s. (J. B. Mason et al., 2007) One explanation for this unexpected increase risk of developing advanced neoplasia is the possibility, as is seen in animals, that high folate levels might suppress tumourigenesis in normal tissue but might enhance the growth of established early tumours. (Kim, 2004b; Kim, 2004a) Other concerns about mandatory fortification of folic acid include accelerating the risk of cognitive decline in older patients with low vitamin B12 status (which might be masked), inducing a suboptimal response to methotrexate based therapy in with autoimmune conditions such as rheumatoid arthritis and increasing the risk of multiple births and therefore maternal and neonatal mortality. (Wright et al., 2007) Thus an understanding of the exact site of biotransformation of folates and in particular folic acid in humans is essential before any further folic acid programs, such as the one being considered by the Department of Health in the UK, are undertaken.
3.2 Aims

**Primary Aim**

To define, in-vivo, the exact site of biotransformation of folic acid in humans by portal venous sampling from well compensated chronic liver disease patients with in-situ TIPS exposed to orally ingested labelled folic acid or a labelled dietary folate (formyltetrahydrofolic acid).

**Secondary Aims**

1. Create a descriptive database of all patients who have had TIPS at the Freeman Hospital, Newcastle upon Tyne

2. To compare transplant free survival between patients with covered and uncovered TIPS
3.3 Methods

3.3.1 Study setting, design and population

A randomised, prospective, open-labelled, cross-over study was designed to ascertain the exact site of biotransformation of folic acid in humans. The study was carried out in collaboration with the Institute of Food Research (IFR), Norwich and Nottingham University. The Freeman Hospital, Newcastle upon Tyne, is a tertiary referral centre that has been offering a regional TIPS service to patients with complications of portal hypertension since 1992. All patients who had TIPS from the year 1992 to the end of the year 2000 were identified through an extensive search of all paper based interventional radiology requests and all interventional radiology activity registers. The latter record all interventional radiology attendances, for any indication, at the point of entry into the intervention room and are updated with summary results after the procedure. Patients who had TIPS after the year 2000 and until 2009 were identified from paper requests and activity registers and in addition cross referenced with an online radiology database that logs all radiology activity which has been active since 2000. All patient attendances at the Freeman Hospital are clinically coded and logged into a central database. For completeness, clinical coding was used to find all patients with in-situ TIPS under active or previous follow up at the Freeman Hospital. These were then cross checked with the existing database to ensure that the TIPS database is accurate, comprehensive and up-to-date. Around 44000 requests were reviewed to identify patients that have had TIPS for any clinical indication. A detailed search using the national summary care records database was carried out to identify patients alive at the time of this study. The Freeman Hospital Liver Transplant database was used to identify patients that had been
transplanted. This master database was used to identify participants for this study and to obtain preliminary data about outcomes in these patients. Clinical records were reviewed to collect the following data:

- **Demographic details including**
  - Date of birth
  - Gender
  - Aetiology of liver disease
  - Date of Liver Transplantation, if transplanted
  - Survival data and date of death

- **Data at the time of TIPS including**
  - Date of TIPS placement
  - Type of Stent used (covered / uncovered)
  - HVPG measurements before and after TIPS

Patients with a patent TIPS offer a unique opportunity to sample portal venous blood at the time of their TIPS patency check when carried out using venography. Such patients followed up at the Freeman Hospital, Newcastle upon Tyne, were invited to take part in the folate study subject to the inclusion and exclusion criteria listed below. Pharmacokinetic analysis of portal plasma samples obtained from study participants for folate metabolites was carried out at the IFR Norwich using processes derived at the University of Nottingham.

### 3.3.2 Inclusion and exclusion criteria

**Inclusion criteria**

1. Compensated Child Pugh A cirrhosis with TIPS in situ with patency demonstrated on US doppler or TIPS venogram at the last annual check

2. No evidence of decompensation of liver disease in the last 6 months
3. Abstinent from alcohol use
4. Intact normally functioning gut

Exclusion criteria

1. Ongoing oral folate supplementation
2. Coexisting malignancy or recent treatment for malignant disease
3. Unable to provide informed consent

3.3.3 Patient consent and allocation

Eligible patients identified from amongst those in the TIPS database were sent information about the study and those who expressed an interest in taking part were invited to discuss the study in further detail in a preconsent clinic and to undergo further screening using a screening questionnaire. (Appendix – FOLTIPS screening questionnaire v1) Interested patients were given detailed written information (Appendix - Patient information leaflet 5) about the study and a consent form to take home, read, sign and return if they were willing to participate. Participants were then allocated randomly and sequentially to receiving either a physiological 500nmole dose of $^{13}$C$_{5}$-folic acid or $^{13}$C$_{5}$-6S-5-FTHF (Merck Eprova, Schauffhausen, Switzerland) at the time of their annual TIPS venography check and $^{13}$C$_{5}$-6S-FTHF or $^{13}$C$_{5}$-folic acid, respectively, in the cross-over phase at their next annual patency check.

3.3.4 Preparation of labelled folate doses

Oral doses of labelled folic acid (L-Folic Acid-$^{13}$C$_{5}$; Pte$[^{13}$C$_{5}$]Glu) and labelled formyl folate (5-formyltetrahydrofolate; [6S]-5-CHO-H$_{4}$Pte$[^{13}$C$_{5}$]Glu, Ca salt) were prepared for use in the study in the designated diet kitchen in the Human Nutrition Unit (HNU) at the Institute of Food Research by our collaborators. The diet kitchen is registered for food preparation with the local Environmental
Health Organisation and complies with their guidelines. Each dose was prepared using the following standard operating protocol.

*Labelled Folic Acid*

Folic Acid (L-Folic Acid-\(^{13}\)C\(_5\); Pte\(^{13}\)C\(_5\)Glu) was purchased from Merck Eprova AG, Schaffhauseen supplied as a sealed vial containing 20mg powder stored at -20ºC in the HNU freezer until further use. In the HNU kitchen, wearing appropriate coat, hat and gloves, a 7.5% solution of sodium bicarbonate (Sodium Bicarbonate BP; pharmaceutical grade, purchased from a pharmacy, e.g. Boots the Chemist) was prepared using sterile water. 1 ml of this solution was added to a vial containing 20 mg of Pte\(^{13}\)C\(_5\)Glu to bring the pH to slightly alkaline, in order for the Folic Acid, which was provided as a free acid, to dissolve fully in water. This mixture was gently swirled till the yellow powder was completely dissolved and transferred to a sterile bottle with at least 100 ml capacity using a sterile plastic pastette. The vial containing the Folic Acid was then rinsed out with sterile water and the rinsing solution was transferred to the sterile bottle to collect any residue remaining in the vial. The solution in the sterile bottle was made up to a total volume of approximately 100ml using sterile water and mixed gently. 1ml of this solution was then transferred to a sterile 50ml falcon tube and to it was added 19ml of sodium phosphate buffer (0.1M sodium phosphate (PO\(_4\)-Na) buffer, pH 7.0) to achieve a 20x dilution. Using the spectrophotometer (UV/Vis) and a matched pair of cuvettes, absorbance of the 20x dilution was read against buffer i.e. the sodium phosphate buffer used to dilute the folic acid solution as the blank at 282nm wavelength. The amount of stock solution required for 500nmole folic acid was calculated using the following equation: \( \frac{0.690}{A_{282}} \) of the stock solution = x volume required (mls) for 500nmole. This volume was dispensed to 2 ml amber
tubes (amber 2ml sterile screw cap tubes from Fisher, cat.no. TUL-918-076H). The spectrophotometer was wavelength-checked using calibration standards prior to use. A “test sheet” was created at each instance of dose preparation and a copy kept with the paperwork for the dose preparation. The phosphate buffer-diluted solution of folic acid was discarded and the amber tubes stored upright (racked) at -20ºC in a designated HNU food-grade freezer (temperature monitored twice a day, and linked to a monitored warning system should temperature fluctuate above or below the set temp). The tubes containing 500nmol doses were transported in person to the Freeman Hospital (Newcastle upon Tyne) over dry ice (temperature recorded at the beginning and end of transportation) and transferred to -20ºC storage at the central pharmacy at the Freeman Hospital (24 hour temperature monitoring). All pipetting was carried out using a Gilson P1000 pipette purchased solely for use in the HNU kitchen and sterile tips (1000ml) purchased from stores and taken directly to the HNU kitchen.

Labelled Formyl Folate

5-formyltetrahydrofolate - [6S]-5-CHO-H₄Pte[¹³C₅]Glu, Ca salt was purchased from Merck Eprova AG, Schaffhausen supplied as a sealed vial containing 20mg powder stored at -20ºC in the HNU freezer until further use. 1 ml of sterile water was added to a vial containing 20 mg of [6S]-5-CHO-H₄Pte[¹³C₅]Glu and swirled gently to dissolve fully and transferred to a sterile bottle with at least 100 ml capacity using a sterile plastic pastette. The vial containing the formyl folate was then rinsed out with sterile water and the rinsing solution was transferred to the sterile bottle to collect any residue remaining in the vial. The solution in the sterile bottle was made up to a total volume of approximately 100ml using sterile water and mixed gently. 1ml of this solution was then transferred to a
sterile 50ml falcon tube and to it was added 19ml of sodium phosphate buffer
(0.1M sodium phosphate (PO$_4$-Na) buffer, pH 7.0) to achieve a 20x dilution.
Using the spectrophotometer (UV/Vis) and a matched pair of cuvettes,
absorbance of the 20x dilution was read against buffer i.e. the sodium
phosphate buffer used to dilute the formyl folate solution as the blank at 282nm
wavelength. The amount of stock solution required for 500nmole formyl folate
was calculated using the following equation: 0.930/A$_{282}$ of the stock solution =
x volume required (mls) for 500nmole. This volume was dispensed to 2 ml
amber tubes (amber 2ml sterile screw cap tubes from Fisher, cat.no. TUL-918-
076H). The spectrophotometer was wavelength-checked using calibration
standards prior to use. A “test sheet” was created at each instance of dose
preparation and a copy kept with the paperwork for the dose preparation. The
phosphate buffer-diluted solution of formyl folate was discarded and the amber
tubes stored upright (racked) at -20°C in a designated HNU food-grade freezer
(temperature monitored twice a day, and linked to a monitored warning
system should temperature fluctuate above or below the set temp). The tubes
containing 500nmol doses were transported in person to the Freeman Hospital
(Newcastle upon Tyne) over dry ice (temperature recorded at the beginning and
end of transportation) and transferred to -20°C storage at the central pharmacy
at the Freeman Hospital (24 hour temperature monitoring). All pipetting was
carried out using a Gilson P1000 pipette purchased solely for use in the HNU
kitchen and sterile tips (1000ml) purchased from stores and taken directly to the
HNU kitchen.
3.3.5 Study protocol

Pre sampling preparation

Eligible consented patients scheduled to come in for their routine annual TIPS surveillance were requested to have blood tests, which included liver function tests, renal functions, full blood count, coagulation profile, CRP and B12 and folate levels, prior to the procedure. A Pre TIPS venography information leaflet was sent to the patient no later than 1 month prior to the test day and receipt / understanding of preparation required confirmed by a telephone call. To avoid increased gut permeability, participants were asked to avoid alcohol and non steroidal anti inflammatory drugs for 2 weeks prior to the procedure. Similarly, to avoid spurious results due to supplementation, they were asked to avoid vitamin B containing supplements for at least 2 weeks prior to the procedure. Patients were fasted for at least 8 hours or overnight prior to the test but were encouraged to keep themselves well hydrated with water. On the day of the procedure, patient consent was re-confirmed and preliminary brief interview was carried out to confirm abstinence from alcohol, the absence of non steroidal anti-inflammatory drug use within the last few weeks and the absence of recent illness or new medications being started prior to the test day. Participants were then escorted to interventional radiology where a routine TIPS venogram was carried out by an experienced radiologist (JR) to assess and confirm TIPS patency. A portal venous catheter (Beacon ® Tip Royal Flush® Plus High Flow Catheter 65 cm 5Fr Cook Medical Europe Limited) was left in situ in the portal vein and the position of the tip confirmed radiologically using fluoroscopy. The non inserted catheter length was marked and the catheter was then flushed with heparin sodium 10 l.U./ml (Hepsal® Wockhardt UK Ltd.) solution. The catheter was secured using Tegaderm™ (3M United Kingdom PLC) transparent
adhesive plaster and the participant transferred in supine position to the programmed investigation unit ward for the study.

**Portal and Systemic Venous Sampling**

The study was carried out in a side room on the programmed investigation unit in the Freeman Hospital, Newcastle upon Tyne. After an uncomplicated TIPS venography, the participant was nursed in a supine position and allowed to recover for 30 minutes with routine post venography vital observations including temperature, heart rate, blood pressure and oxygen saturations being measured at transfer and every 15 minutes thereafter for 1 hour and hourly until the completion of the study. Depending on sequential randomization a vial containing either labelled folic acid or labelled formyl folate was retrieved from -20ºC storage and allowed to reach room temperature whilst being shielded from light in an opaque bag during participant recovery. Participant consent to continue with the study was re confirmed and a large bore 18g cannula (BD Venflon™ Becton, Dickinson U.K. Limited) was sited in the left median cubital fossa and flushed with 0.9% sodium chloride solution. The site of the portal venous catheter was checked to confirm that it was not displaced. The vial contents were transferred to a new clean plastic beaker. The vial was then rinsed with 10 ml of sterile water and the rinsate transferred to the beaker and gently swirled to ensure mixing. Using a plastic straw the patient was asked to ingest the 500nmol dose of either labelled folic acid or formyl folate dissolved in sterile water from the plastic beaker. The beaker was rinsed twice with 10 ml of sterile water and the participant asked to ingest this rinsate using the straw. The time at point 0 was noted on the study sampling sheet and a clock timer was started. The study sampling sheet including the participant study code number, the date of the test, the details of the sampling points and a provision for
entering the exact time of portal and systemic venous sampling and comments
(Appendix – Time Sheet). Timed portal (15, 25, 35, 45, 55, 65 and 85 minutes) and systemic (30, 60, 90, 120, 150, 180, 210 and 240 min) venous blood samples were collected in green topped 13 mm x 75 mm 4ml Lithium Heparin BD vacutainers® and held at 4ºC. At each portal sampling time point, using strict aseptic precautions and after confirming the marked non inserted position of the catheter, a 2.5 ml disposable syringe was used to aspirate 2 ml of serosanguinous fluid (0.9 ml heparin saline flush with 1.1 ml of portal venous blood). The syringe containing this mix of flush and portal venous blood was discarded. A new 2.5 ml syringe was then used to aspirate 2 ml of portal venous blood and transferred into a previously labelled vacutainer which was gently inverted 5 times. This vacutainer was held at 4ºC prior to processing. The portal catheter was flushed with 0.9 ml of Heparin Sodium 10 IU/ml solution and position confirmed again. At each systemic venous sampling, using strict aseptic precautions, a 2.5 ml syringe was used to aspirate 2 ml of systemic blood from the peripheral 18g cannula previously sited and the entire syringe with contents was discarded. 2 ml of systemic blood was then aspirated from the peripheral cannula and transferred to a pre labelled lithium heparin vacutainer®, inverted 5 times and held at 4ºC prior to processing. The peripheral cannula was flushed with 0.9% sodium chloride. The portal cannula was removed at 100 minutes and the site of insertion was dressed appropriately. The patient was then allowed to sit up. Throughout the procedure regular vital observations were measured. Care was taken to maintain strict asepsis throughout sampling and to avoid displacing the portal venous catheter from its original insertion site. After study sampling was completed, the peripheral cannula was removed and the site dressed. The site of insertion of
the portal venous catheter was checked and the patient observed for 30 minutes and given a light meal. A post discharge information leaflet was given to each participant including my contact details and out of hours contact details should they feel unwell in any way. Each participant was contacted by telephone the same day in the evening to check well being and to report any adverse events experienced and then in 1 week and 1 months time.

**Plasma preparation and analysis**

Portal and systemic venous samples were held at 4ºC prior to plasma preparation which was carried out by chilled centrifugation (3300G for 10 minutes at 4ºC) using a Heraeus Primo R Thermo Centrifuge (Thermo Scientific, UK). Post preparation, 250μl aliquots of plasma were transferred to two 2 ml amber light protected micro tubes (Eppendorf Safe-Lock tubes™) using a 1000μl pipette and snap frozen in dry ice prior to being transported to catalogued storage at -80ºC at Newcastle University. These were finally transferred in dry ice to long term storage at -80ºC at the IFR in Norwich for analyses using a high sensitivity LC-MS/MS assay developed to enable this study and described in detail previously. (King et al., 2012) In brief, each sample (5 µL) was analysed using an Agilent 1200 binary HPLC coupled to an AB Sciex 4000 Qtrap triple quadrupole mass spectrometer. HPLC was achieved using a binary gradient of solvent A (MilliQ Water + 0.1% formic acid) and solvent B (HPLC grade acetonitrile + 0.1% formic acid) at a constant flow rate of 250 μl/min. Separation was made using a Phenomenex Kinetex 2.6u C18 100 x 2.1 mm column maintained at 50°C. Injection was made at 2% B and held for 2.5 min, ramped to 10% B to 6 min and then to 43% B by 15 min. A 98% B column wash was then applied until 23 min and then the column equilibrated to initial conditions for 10 minutes. The mass spectrometer was operated in
electrospray positive mode to monitor specific parent / fragment transitions for the folate target compounds as folic acid (FA): 442/295, 13C-FA : 447/295, 5MeTHF : 460/313, 13C-5MeTHF : 465/313, 5F0THF: 474/327, 13C-5F0THF : 479/327, MTX: 455/308. Optimised ionisation and collision energies were tuned and applied to each transition (not reported). Quantification was applied using AB Sciex Analyst 1.5 software to integrate detected peak areas relative to the MTX internal standard.

3.3.6 Pharmacokinetic Modelling and Statistical Analysis
Pharmacokinetic modelling and statistical analysis for this part of the study was carried out with the help of Dr. Jack Dainty at the Institute of Food Research, Norwich. It has been shown previously that it is possible to estimate the apparent absorption of labelled folate doses by modelling their appearance in plasma. (Kok et al., 2004; de Meer et al., 2005; Wright et al., 2005) The analysis for this is based on a technique called Compartmental Modelling. (Dainty, 2001) A compartment is a theoretical construct that may combine material from several different physical spaces and defined as an amount of material that acts as though it is well mixed and kinetically homogeneous and a compartmental model consists of a finite number of compartments with specified interconnections between them. The interconnections are a representation of the flux of material that is transported from one location to another. By defining a compartmental model in this way, researchers can reduce a complex metabolic system into a small number of pathways and compartments. The outputs from such a model include the quantity of mineral or vitamin stored in certain body pools, the rate and extent to which the nutrient moves from one pool to another and how long it will remain in the body before being excreted. This can inform the approach of the nutritionist about status, daily requirements
and retention and, only through modelling, can this type of information be obtained from humans in a non-invasive way.

**Figure 18:** A 2 compartment model (used with permission from Dr. Jack Daint)

As an example, consider the 2-compartment model shown in **Figure 18.** The circles represent compartments and the arrows are pathways showing the direction of transfer of material. Associated with each pathway is a rate constant, which is conventionally written as $k_{(i,j)}$ that denotes transfer of material, from compartment “$j$”, to compartment “$i$”, per unit time. The types of compartment models which are usually found in nutrition are described by constant coefficient, ordinary, first-order differential equations. The basis of these equations is that the rate constants do not alter over the course of the experiment and, by applying The Conservation Of Mass to the system under study, it is possible to solve the resultant series of equations. If the mass of material in compartment 1 is denoted as $P$ (for plasma) and that in compartment 2 as $E$ (the “rest” of the body), the system can be described by:

$$\frac{dP}{dt} = k_{1,2} \cdot E - (k_{2,1} + k_{0,1}) \cdot P$$

$$\frac{dE}{dt} = k_{2,1} \cdot P - k_{1,2} \cdot E$$
To solve these equations, one of the two compartments needs to be sampled (measured) so that the parameters ($k_{0,1}$, $k_{1,2}$ and $k_{2,1}$) can be fitted to the resultant, measurement data. It is often the case that the only compartment that can be sampled is the plasma. This limits the number of “other” compartments that can be used in the model, if the parameters are to be identified, uniquely. This is called \textit{a priori} identifiability and is used to identify, given noise-free data, the number of compartments a model may have in order that all its parameters will have a single solution. Often investigators have an in-depth knowledge of the metabolism of a particular nutrient, and want to include many compartments, but this can lead to uncertainty in the value of the subsequent parameters. Any model that is not \textit{a priori} identifiable must be carefully evaluated and the results from it used cautiously. The fitted parameters from a model should always be quoted with their calculated precision so that an assessment of the quality of the model can be made. This is called \textit{a posteriori} identifiability and plays an important part in drawing nutritional conclusions from the modelling of real data. The \textit{a posteriori} identifiability is dependent on good quality data and the correct choice of model structure for the system under investigation. The 2-compartment model is \textit{a priori} identifiable. If the data from the plasma samples are of sufficient quality and they support a 2-compartment rather than, say, a one or three compartment structure then the model should be \textit{a posteriori} identifiable. A compartment model is, therefore, constrained by the type of data collected, the location it is collected from and the statistical considerations involved in fitting experimental data to models. It is known from pharmacokinetic studies, that the bioavailability of certain drugs can be estimated from their appearance in the plasma, over the first few hours post-ingestion. The underlying mathematics (differential equations) of compartmental
modelling depend on the principle of mass balance – the rate of change in the plasma folate concentration is equal to the rate of folate absorption minus the rate of removal of folate from the plasma for utilisation and storage. So, the estimation of rate of folate removal is very important if the absorption is to be estimated correctly. Intensive blood sampling was carried out to ensure that the peak in plasma concentration was not missed and the changes in folate concentration were fully characterised. Simulation of ten sampling times, equally spaced over 360 minutes, indicated that there would be enough degrees of freedom to estimate all the model parameters “a priori” from a one or two compartment model, given noise free data. This is an important part of the modelling process and must be performed to ensure that a model is theoretically possible, given the experimental design. The number of parameters that need to be estimated in the one compartment model are the quantity of folate absorbed, the rate of folate loss from the plasma and the time period during which absorption is taking place. The same parameters would be estimated from a two compartment model, as well as the rate of re-appearance of folate from the second compartment (rest of body). In developing a model whose only function is the estimation of folate absorption, sampling for days is unnecessary because the absorptive process takes place over a few hours. It became possible to decide which of the two competing models (one or two compartment) was the most appropriate after the human study was performed and the data were collected. This was carried out in SAAMII (SAAM Institute Inc, Seattle, WA) by looking at the residual plots, examining the uncertainty on the parameters and using the model comparison parameters called Akaike’s information criterion (AIC) and Bayesian information criterion (BIC). When comparing two or more model structures, the one with the lowest AIC (or BIC) is
preferred, because these parameters measure the goodness of fit of the model and also take into account the number of parameters that are used in fitting it. If two models have a comparable goodness of fit to the data, the simplest model (one with the least parameters) is preferred.

**Figure 19:** Schematic overview of compartmental model for folate absorption (used with permission from Dr. Jack Dainty)

*Figure 19* illustrates the final model which is a single compartment. It is assumed that mass, $M$, of the folate dose, is absorbed from the small intestine, into the plasma, at a constant rate, $R$, for a time, $T$. This is known as "zero-order" absorption and equates to a constant infusion of folate from the gut into the plasma, which is, of course, a simplification, as the true process is more likely to be first-order (time-varying) in nature. A first-order absorptive process had been examined as part of the model development but it did not improve the absorption predictions and also required the estimation of an additional parameter. Therefore, by applying the Principle of Parsimony, the simpler process of zero-order absorption was preferred. By fitting equations to the
plasma concentration data (C) for either labelled or unlabelled folate, it is possible to estimate the mass of folate absorbed (M), the period of time when absorption has taken place (T) and the rate constant of elimination from the plasma (k). All fitting can be performed in Excel (Microsoft Corporation, 2002) or SAAMII but the latter is preferable as it gives statistics that indicate how good the fit is. The fractional folate absorption can then be calculated by knowledge of the mass of folate (M) appearing in plasma, divided by the mass of the dose of folate. In the present work, the estimate for folic acid absorption is based on the appearance of labelled folic acid in the portal and systemic blood after the administration of the labelled folic acid dose. The estimate for formyl absorption is based on the appearance of labelled 5MTHF in the portal and systemic blood after the administration of the labelled formyl dose.

The proportion of folate in un-modified and modified form in the portal vein and systemic circulation for the different oral dosing formulations at the initial 15m post-dosing time-point was compared using a paired t-test.

3.3.7 Ethical Approval

The study was formally approved by the Newcastle and North Tyneside 1 Research Ethics Committee (08/H0906/82) and via a substantial amendment to accommodate crossover sampling (08/H0906/82/ ver 2.0 dated 09/02/11) (Appendix – Ethics)

3.3.8 Study Funding

This work was funded by responsive mode grants BBF0144571, BBF0141041 & BBF0125941 from the UK Biotechnology and Biological Sciences Research Council (BBSRC).
3.4 Results

3.4.1 Study participants

Using the comprehensive search strategy as outlined in the methods section, 336 patients were identified who have had a TIPS at the Freeman Hospital, Newcastle upon Tyne since this service has been available from 1992 upto 2009. 13 (4% 95% CI 2% - 6%) of the 336 patients were either non UK residents who had been treated whilst on their visit to the UK and returned to their country or had relocated in the UK without any further contact details available either through their last known primary care provider or the hospital records. Their demographic records were unavailable. 323 (96%; 95% CI 94%-98%) of the 336 patients had demographic records available. Median age at TIPS was 53yrs (Range 20-82). 60% (95%CI 54%-65%) of the entire cohort was male. Alcoholic liver cirrhosis was the commonest cause of portal hypertension requiring TIPS (Figure 20). Data about the type of stent used was available in all but 17 patients (almost all were in the pre 2000 era). Almost all patients had an uncovered Wallstent prior to the year 2000 (N=176). After this period, the covered Viatorr® stent was the main stent used (N=130). 191 patients had documentation of HVPG measurements before and after TIPS in either the radiology report or clinical records. Mean HVPG pre TIPS was 18.2 (±5.7) Hg mm and mean HVPG post TIPS was 7.3 (± 2.5) Hg mm.
For the folate study, using the strategy described in the methods, 32 patients with an in-situ TIPS were identified as potential participants and their records reviewed to identify those patients who would meet inclusion criteria for this study. A CONSORT flow chart is shown in Figure 21. Six subjects meeting all study criteria participated in the study. 2 of 6 participants could not take part in a cross over study. As a consequence, data from 4 cross over studies (4 labelled folic acid and 4 labelled formyl folate) and 2 labelled folic acid studies were included in the final analyses.
3.4.2 Baseline characteristics and Demographics

Demographic details for patients in the folate study are shown in Table 20. All participants had well compensated Child Pugh A liver disease, normal synthetic liver function and normal renal functions. All participants underwent routine TIPS venography that confirmed a patent TIPS prior to each study.

3.4.3 Portal and systemic folate concentrations

When the portal venous concentrations of provitamin folic acid and its reduced and methylated vitamin form 5-methyltetrahydrofolic acid (5-MTHF), the product of physiological reduction and methylation by the gut wall) were compared after dosing with labelled folic acid, un-metabolised labelled folic acid levels were seen to rise significantly and more rapidly than labelled 5-MTHF concentrations (Figure 22). This suggests a greater proportion of absorbed folic acid crossed the gut wall unmodified than is reduced and methylated within the cells of the gut wall. The finding of portal labelled folic acid early after dosing was mirrored by its appearance in the peripheral circulation (Figure 23). In contrast, the unmodified form was extremely low in the portal circulation when patients were dosed with labelled formyltetrahydrofolic acid ($^{13}$C$_5$-6S-5-FTHF), a reduced form of “natural folate”, in the cross-over arm of the study (Figure 24).
Table 20: Demographics and clinical characteristics of participants

<table>
<thead>
<tr>
<th>Sex &amp; Age (y)</th>
<th>Aetiology of chronic liver disease</th>
<th>BMI (Kg/m²)</th>
<th>CP</th>
<th>CPG</th>
<th>MELD</th>
<th>eGFR (ml/min/1.73m²)</th>
<th>B12 (pmol/L)</th>
<th>RCF (nmol/L)</th>
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<tbody>
<tr>
<td>M 57</td>
<td>NASH</td>
<td>33</td>
<td>6</td>
<td>A</td>
<td>10</td>
<td>115</td>
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<td>741</td>
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<tr>
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<td>5</td>
<td>A</td>
<td>6</td>
<td>119</td>
<td>576</td>
<td>392</td>
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<tr>
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<td>A</td>
<td>6</td>
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<td>151</td>
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<td>6</td>
<td>A</td>
<td>10</td>
<td>82</td>
<td>349</td>
<td>185</td>
</tr>
<tr>
<td>F 53</td>
<td>Budd Chiari (on warfarin)</td>
<td>23</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>96</td>
<td>692</td>
<td>236</td>
</tr>
</tbody>
</table>

* This patient was on warfarin therefore coagulation parameters were not interpretable. M- male, F-Female, BMI – Body Mass Index, CP – Child Pugh Score, CPG – Child Pugh Grade, MELD – Model for End Stage Liver disease, eGFR – E Glomerular Filtration rate, B12 – Vitamin B12 reference range 170-700 ng/L, RCF – Red cell folate reference range 160-600 Ug/L
**Figure 22:** Measured concentrations of labelled folic acid and labelled 5-MTHF in portal venous blood after dosing with labelled folic acid.

![Portal](image)

**Figure 23:** Measured concentrations of labelled folic acid and labelled 5-MTHF in systemic venous blood after dosing with labelled folic acid.

![Systemic](image)
**Figure 24:** Measured concentrations of labelled 5-formyltetrahydrofolic acid (5-FTHF or “natural folate”) and labelled 5-MTHF in portal venous blood after dosing with labelled 5-FTHF

![Portal](image)

**Figure 25:** Measured concentrations of labelled 5-FTHF and labelled 5-MTHF in systemic venous blood after dosing with labelled 5-FTHF

![Systemic](image)
At the initial 15 minute sampling time point, a consistent pattern was seen across all participants with a median 86% [range 60-88%] of labelled folate in the hepatic portal vein following a dose of folic acid being unmodified folic acid which was significantly higher (p<0.01) than the median 14% for labelled 5-MTHF. In contrast, following a dose of 5-FTHF, only a median 3% [range 2-6%] of labelled folate in the hepatic portal vein was unmodified 5-FTHF which was significantly lower (p<0.001) than the median 97% for labelled 5-MTHF. Between oral treatments, the proportion of unmodified folic acid in the hepatic portal vein was significantly greater (p<0.0001) than unmodified 5-FTHF (Figure 26). Additionally, at the 15 minute time point the median concentration of labelled 5-MTHF (0.965 nmol/L) derived from folic acid was only 3.7%, and significantly lower (p<0.01), than the median concentration of labelled 5-MTHF (25.776 nmol/L) derived from 5-FTHF.
Figure 26: Percentage of the dose of folic acid and 5-FTHF crossing into the portal circulation in an unmodified form after dosing with each in the cross-over study. Data are presented for 15 minutes following folate dosing; the first sampling time point. Plots delineate the median, max and min and inter-quartile range value.

3.4.4 Adverse events
No adverse events were reported in participants taking part in the study. All patients found the study acceptable and indeed two thirds consented to the cross over study.

3.4.5 Comparison of survival in patients with uncovered and covered TIPS
45 of the 323 (11% 95%CI 8%-15%) who had a TIPS procedure at our centre were transplanted after a median of 13 months (Range 1-129 mths). Over a median follow up period of 6 years (IQR 2-9 yrs), 205 of the 278 (74% 95%CI 68%-79%) patients who did not receive a transplant died and 73 (26% 95%CI 21%-32%) were alive. (Figure 27)
Figure 27: Flowchart representing the outcome of patients treated with TIPS at the Freeman Hospital Newcastle upon Tyne

Overall transplant free survival in patients who had TIPS at our centre was 21 months (95%CI 14-28 months). This is shown in Figure 28. There was no significant difference in the transplant free survival between patients with uncovered TIPS or covered TIPS (p=0.647, log rank test; Figure 29).
Figure 28: Transplant free survival in all patients who had a TIPS between 1992 and 2009

Figure 29: Kaplan Meier curves comparing transplant free survival between patients with covered and uncovered TIPS
3.5 Discussion

3.5.1 The site of biotransformation of folates in humans
This study elegantly and unambiguously demonstrates that the majority of a physiological oral dose of folic acid passes into the portal venous circulation in an unmodified form. Almost 90% of labelled folic acid administered orally passes into the portal vein unmodified in humans and definitively proves that folic acid is not metabolised by the small intestinal epithelium in humans. In contrast to folic acid, the physiological dietary folate 5-formyltetrahydrofolic acid is very nearly completely methylated, appearing in the portal venous circulation almost entirely as 5-methyltetrahydrofolic acid; an observation which confirms normal gut wall function in terms of both gut permeability and folate methylation capacity and that the small intestinal mucosa simply re arranges the one carbon moiety in dietary folates to the active 5 methyl tetrahydrofolate form. These findings fundamentally challenge the accepted model of folic acid uptake, in which folic acid is proposed to be fully reduced and methylated in the gut wall as previously suggested by high quality rodent studies (Tani and Iwai, 1983) and instead suggests that, whilst human methylation capacity is adequate, reduction capacity in the form of dihydrofolate reductase activity is strictly limited.

3.5.2 The effect of portosystemic shunting on the study results
The appearance of unmodified folic acid after dosing in the peripheral circulation reflects the shunt-based experimental model which causes, by its nature, significant by-passing of first-pass liver metabolism and therefore limited any conclusions that could be drawn about the actual capacity of the liver to bio-transform folic acid.
3.5.3 Different folates have different rates of entry into the portal vein

Folate excretion out of gut absorptive cells into the hepatic portal vein is undertaken by ‘multidrug resistance protein MRP-3’. (R. Zhao et al., 2009) Since MRP-3 excretes folic acid with much lower efficiency than the reduced tetrahydrofolate form, (Zeng et al., 2001) it is not surprising that after an oral dose of folic acid the rate of total labelled folate (folic acid + 5-MTHF) entering the human portal vein is significantly lower than the total labelled folate (5-FTHF + 5-MTHF) after an oral dose of 5-FTHF.

3.5.4 In vivo studies are superior to animal model or in vitro studies

Our findings disagree with currently held beliefs and assumptions about the exact site of biotransformation of folic acid. The most obvious explanation for the discrepancy between our findings and those of studies, on which the consensus of full gut reduction and methylation of folic acid metabolism was based, is their use of rodent models (most typically rat) and human cell lines because of the difficulty of doing in vivo human studies. Both of these approaches have inherent weaknesses. Rats have significantly higher levels of DHFR than humans, (S. W. Bailey and Ayling, 2009) making them a poor model for human biology. Human cell lines also exhibit elevated levels of DHFR activity, potentially as a consequence of the traditional use of high levels of folic acid in tissue culture medium. (Kamen et al., 1985) Although this study is complex and resource intensive, it demonstrates that directly studying gut uptake of folic acid (and potentially other nutrients in the future) is feasible in human subjects, safe (no adverse events were reported) and highly acceptable (all approached patients volunteered for re-dosing and all found the procedure fully acceptable). In vivo human studies are, of course, the most relevant for the understanding of human biology. Interestingly, our findings confirm and extend
those of even earlier human studies published in the 1960s and 1970s. (Whitehead and Cooper, 1967; Melikian et al., 1971) These studies were, at the time, criticised for administering large, non-physiological, doses of folic acid and were subsequently largely disregarded. In the light of our findings, however, the fact that no dose-derived 5-MTHF was initially detected in hepatic portal vein plasma was a pertinent finding, albeit one overlooked by later researchers.

3.5.5 Limitations of the study

Due consideration must be made of the fact that although the study subjects were as normal as was feasible, no healthy person has a shunt into their portal circulation allowing access to the portal circulation for sampling. The characteristics of the study participants, which allowed them to be participants, also, therefore, represented a potential limitation of the study. Care was taken in selecting study subjects, however, to minimise confounding biological processes. All subjects had inactive liver disease and normal liver synthetic function and all were abstinent from alcohol. The major confounding factor, that of increased gut permeability, was eliminated by determining – as part of the study – complete methylation of natural folate implying the passage of all labelled folate through enterocytes. Given the principal measurements were gut transport into the hepatic portal vein (i.e. “upstream” of the liver) and gut permeability was normal, we believe our observations are robust. More caution needs to be applied to systemic circulation data given the capacity for periportal shunting effects. When single physiological doses of stable-isotope-labelled folic acid have been given to normal human volunteers who were neither exposed to mandatory fortification nor self-supplementation, no unmetabolised labelled folic acid was seen in the systemic circulation, only labelled 5-MTHF. (Wright et al., 2005) If folic acid is mainly transferred to the
hepatic portal vein un-metabolised, one may now conclude that under those experimental conditions all folic acid entering the hepatic portal vein must have been removed on ‘first-pass’ metabolism by the liver (by the ‘proton-coupled folate transporter’), (R. Zhao et al., 2009) which exhibits a slightly greater affinity for folic acid than for reduced folates, and biotransformed to 5-MTHF prior to enterohepatic recirculation. As human liver has recently been shown to have not only low but highly variable DHFR activity, (S. W. Bailey and Ayling, 2009) then arguably, chronic exposure to folic acid even in physiological doses (as would be the case with mandatory fortification) may induce saturation and explain the observed systemic circulation of unmetabolised folic acid. (Regan L. Bailey et al., 2010)

3.5.6 Biological patterns of systemic response to FA and dietary folates

The key finding of this study is that the biological patterns following supplementation with folic acid (as would be the case with supplements and/or food fortification containing folic acid) and folate (as would happen with increased dietary intake) are very different. One potential explanation for the apparent paradox of risk associated with folic acid supplementation, despite the obvious benefits associated by reversing low body folate levels, would that the adverse effects are a direct consequence of the presence of unnatural circulating concentrations of unmetabolised folic acid following prolonged exposure to this artificial entity. That the risk is not associated with elevated folate exposure in general is suggested by the Danish population study (Roswall et al., 2012) which showed that, in contrast to patients taking folic acid supplements in whom increased mortality rates were seen, patients consuming a naturally high folate diet had no increased risk.
3.5.7 TIPS can be used safely for portal venous sampling in humans

There were no adverse effects noted in the study both at the time of venous sampling and on follow up. Portal venous sampling at the time of venography was acceptable to patients and almost all screening clinic invitees agreed to take part, with further consent to take part in the cross over phase.
3.6 Conclusion

In conclusion, low enzyme activity of dihydrofolate reductase (DHFR) may compromise both mucosal and liver biotransformation of folic acid in humans. For dietary supplement capsules, it is suggested that folic acid could be partially, or completely, replaced with 6S-5-MTHF (the normal systemically-circulating folate form), the multiple advantages of which have been noted previously. (Wright et al., 2010) It is also suggested that effort is made to microencapsulate 5-MTHF so that losses from manufacture and use in voluntary fortified foods (e.g. breakfast cereals) and subsequent processing (e.g. heat) of products following UK proposals for mandatorily-fortified flour (if implemented), are minimised.
The work carried out during this project aimed to rise up to the clinical challenge of predicting the presence of portal hypertension in patients with PBC and to utilise the opportunity for portal venous sampling presented at routine TIPS venography to answer important questions about the actual role of the human small intestine and liver in the metabolism of folic acid.

We have shown that in PBC, GOV are common even in early disease and that the presence of varices is a poor prognostic marker in these patients. The NVP and NVP-S scores are inexpensive bed side tools which can be used to predict the presence of varices in patients with PBC, including those with early disease, with high accuracy and can be used at different probability cut offs to suit differing health economic locales. We have also shown that patients with PBC who develop GOV have a poorer prognosis than those who do not. Therefore, the NVP and the NVP-S scores are also prognostic scores which can be used to identify PBC patients with a poorer prognosis. Unlike the Mayo Score, they do not contain subjective variables such as the presence of peripheral oedema. Further studies to evaluate their use in this setting should be the focus of further research in this setting.

Our work has demonstrated that portal venous sampling through TIPS is feasible and safe and can be used to study metabolic pathways in humans. Though the effect of shunting on the interpretations poses a limitation, this can be adjusted for in future studies by studying absorptive pharmacokinetics in patients with TIPS and healthy matched controls. This factor however did not limit the interpretation of our study which specifically aimed to find the exact site of biotransformation of folates. We have found that synthetic folic acid, the form taken in folic acid supplements bought over the counter, is not processed by the
body in the same way as natural folates, the form found in nature. This can lead to unprocessed folic acid circulating in the blood stream, with unknown potential health effects. With the UK government considering adding folic acid to all bread flour (to reduce the 900 neural tube defect births thought to be related to low maternal folate levels in the UK every year) our study suggests a need to think about using a different folate form for the fortification, or better understand the implications of excess folic acid. Fortification can also be done with natural forms of folate. These are already licensed for use, under the names Metafolin® (Merck KGaA, Darmstadt, Germany) and Quatrefolic ® (Gnosis S.P.A, Milan). These have been approved for use as a food supplement and are metabolised in the same way as natural folates, and so avoid the problem identified in this study. If these can be adapted for use in food fortification, they present a way of improving the folate status of the population, reducing the risk from unmetabolised folic acid in the blood and any possible risks to health.

“A pessimist sees the difficulty in every opportunity; an optimist sees the opportunity in every difficulty” said Winston Churchill.

We have risen to the challenge of non invasive prediction of portal hypertension in patients with PBC and optimised the opportunity presented in the management of portal hypertension to study the exact site of biotransformation of folic acid in humans.
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17 September 2008

Professor David EJ Jones
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Dear Professor Jones

Full title of study: Where is the Initial Site of Folic Acid Biotransformation in Humans?

REC reference number: 08/H0906/82

Thank you for your letter of responding to the Committee’s request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA). The favourable opinion for the study applies to all sites involved in the research. There is no requirement for other Local Research Ethics Committees to be informed or SSA to be carried out at each site.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

This Research Ethics Committee is an advisory committee to North East Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England
Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<tr>
<td>Application</td>
<td>Parts A+B</td>
<td>04 July 2008</td>
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<tr>
<td>Investigator CV</td>
<td>D E J Jones</td>
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<tr>
<td>Protocol</td>
<td>REF G521304</td>
<td>01 July 2008</td>
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Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document “After ethical review – guidance for researchers” gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRDS website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencesgroup@nres.npsa.nhs.uk.

The Research Ethics Committee is an advisory committee to North East Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England
With the Committee's best wishes for the success of this project

Yours sincerely

Mr. Chris Turnock
Chair

Email: vicky.eggleston@sunpct.nhs.uk

Enclosures:

"After ethical review – guidance for researchers" SL- AR2 for other studies

Copy to:

Joint Research Office
(Research & Development)
Newcastle upon Tyne Hospitals NHS Foundation Trust
Research & Development
4th Floor, Leazes Wing
Royal Victoria Infirmary
Newcastle upon Tyne
Newcastle & North Tyneside 1 Research Ethics Committee
TEDCO Business Centre
Room 002
Rolling Mill Road
Jarrow
NE32 3DT
Tel: 0191 428 3584
Fax: 0191 428 5432

09 March 2011

Professor David Jones
ICM
Level 4 William Leech Building
Newcastle University
Framlington Place
Newcastle upon Tyne
NE2 4HH

Dear Professor Jones

Study title: Where is the Initial Site of Folic Acid Biotransformation in Humans?
REC reference: 08/H0906/02
Amendment number: Ver 2.0 dated 09/02/11
Amendment date: 25 February 2011

The above amendment was reviewed at the meeting of the Sub-Committee held on 08 March 2011.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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<th>Document</th>
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<tr>
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<td>Dr Imran Patanwala</td>
<td>01 March 2011</td>
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<tr>
<td>Notice of Substantial Amendment (non-CTIMPs)</td>
<td>Ver 2.0 dated 09/02/11</td>
<td>25 February 2011</td>
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<tr>
<td>Participant Information Sheet</td>
<td>Version 3</td>
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Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval
All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| 08/H0606/02: | Please quote this number on all correspondence |

Yours sincerely

Miss Laura Kirkbride  
Committee Co-ordinator

E-mail: laura.kirkbride@sotw.nhs.uk

**Enclosures:**  
List of names and professions of members who took part in the review

**Copy to:**  
Newcastle upon Tyne Hospitals NHS Foundation Trust
## Newcastle & North Tyneside 1 Research Ethics Committee

### Attendance at Sub-Committee of the REC meeting on 08 March 2011

<table>
<thead>
<tr>
<th>Name</th>
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<th>Capacity</th>
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<tbody>
<tr>
<td>Dr Thomas J Chadwick</td>
<td>Clinical Trials Statistician</td>
<td>Expert</td>
</tr>
<tr>
<td>Mr Gary Player</td>
<td>Biomedical Scientist</td>
<td>Expert</td>
</tr>
<tr>
<td>Mr Christopher Roy-Toole</td>
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<td>Lay Plus</td>
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<tr>
<td>Mr Chris Turnock</td>
<td>Learning &amp; Teaching Advisor</td>
<td>Expert</td>
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### Written comments received from:

<table>
<thead>
<tr>
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<tr>
<td>Dr Simon Woods</td>
<td>Senior Lecturer</td>
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</tbody>
</table>

### Also in Attendance:

<table>
<thead>
<tr>
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<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miss Laura Kirkbride</td>
<td>Committee Coordinator</td>
</tr>
</tbody>
</table>
How is Folic Acid Metabolised in People?

Information for Participants

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully and discuss it with others if you wish. The summary section briefly outlines the studies and the implications that taking part in it would have for you. Part 1 tells you the purpose of the study and what will happen to you if you take part in more detail. Part 2 gives you more detailed information about the conduct of the study. If you have any questions or would like more explanation, please ask. You can choose not to take part in the study. If you do decide to take part, you can change your mind at any time. Your decision will not affect the standard of care you receive in any way.

SUMMARY
This study will answer important questions about how the vitamin folic acid is taken up by the body which are very relevant given government plans to add folic acid to food in the UK. The study is possible because in TIPSS patients we have the unique opportunity to take blood samples through the TIPSS from the portal vein. This will be done immediately after a normal TIPSS check venogram. If you are willing to participate in the study, and our tests suggest you are suitable, 3 things will happen to you over and above what would normally happen at a TIPSS check

1) The tube used to check the TIPSS will not be removed immediately, instead it will be left in place for a maximum of 4 hours (after which it will be removed as normal)

2) We will ask you to take a single dose of one of two types of folic acid (similar in dose to a normal supplement tablet but modified to have a safe “label” contained within it to allow us to measure it)

3) Once you have taken the folic acid tablet we will take a series of small blood samples both from the tube into the TIPSS and from a vein to measure the levels of folic acid

4) We will ask you if you wish to take part in a “cross over” study, with your next annual TIPSS check-up venogram. This would involve the same procedure as above but on this occasion you will be given another type of folic acid supplement again similar in dose to a normal supplement and modified to have a safe “label” contained within it to allow us to measure it.

PART 1
What is this study about?

There is a lot of information available now which suggests that increasing the amount of folic acid in our diet may have health benefits. These include reducing serious conditions in babies developing in the womb such as spina bifida. It has also been suggested that folic acid may be helpful in reducing the risk of heart disease and stroke. Based on this information many people elect to increase the amount of folic acid in their diet through the use of vitamin supplements which are available in all UK pharmacies and supermarkets. Folic acid supplements are also recommended for all women trying to become pregnant and who are already pregnant in the UK.

Given its health benefits the government is considering adding folic acid to flour in the UK meaning that anyone who eats bread (or any other type of food made from flour) will automatically receive an increased dose. A number of countries (including the USA and Canada) have already introduced folic acid “fortification” of flour and have seen significant reductions in conditions such as spina bifida.

The plans to fortify flour with folic acid in the UK are, however, currently on hold. The reason for this is that concerns have emerged about some potential health risks associated with long term high level exposure to folic acid (as might happen in someone eating fortified flour in addition to an otherwise healthy diet and who was also taking folic acid containing vitamin supplements). Amongst the concerns is the possibility of an increased risk of bowel cancer. One of the potential reasons for this may be that, unlike animals, humans appear to have only a limited capacity to metabolise folic acid meaning that too much in the diet may result in too much getting into the body. It is thought that in animals this risk of harm is prevented by folic acid being changed, partly by the bowel wall
and partly by the liver, into a modified form, with both components of this metabolism system having significant spare capacity. We already know that humans have much lower capacity to modify folic acid within the liver. Up until now, however, it has not been possible to study folic acid breakdown in the bowel wall. Were humans to have a high capacity to modify folic acid in the bowel wall then this would provide significant reassurance about the benefits of folic acid fortification of food. In contrast, were humans to lack this capacity (as they lack it in the liver) it would raise concerns about the proposed policy because of its potential risk.

**Why have I been asked to take part?**

The reason why we have, until now, not been able to answer the question as to whether folic acid is modified by the bowel wall is that it is normally not possible to take blood samples from the portal vein (the blood vessel which takes blood from the bowel to the liver and which carries all the nutrients (including folic acid) to the liver). You are being asked to participate in this study because you are one of a small but unique group of people in whom, because you have a TIPSS in place, it is possible to take blood samples from the portal vein via the TIPSS at the time of a TIPSS-check procedure.

**Do I have to take part?**

It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.
What would it involve?

First, the doctor will ask you some basic questions about your health, and make a note of your recent hospital blood tests. If your answers suggest that you would be a suitable participant for the study, the first stage will involve a test to make sure that you absorb dietary contents normally. This will involve taking a urine (water) sample following an oral dose of two agents which are safe and widely used in medical practice (Lactulose and Mannitol) which shouldn’t be absorbed from the diet. If, based on this screening test, you are thought to be suitable then the test itself would involve taking some blood samples from the portal vein through the tube which is used to carry out a normal TIPSS check procedure (an x-ray procedure used to check whether the TIPSS remains open and which is one of the tests used to answer this question on an annual basis within the Freeman hospital in all TIPSS patients). We will also take blood samples from an arm vain trough a normal drip. Prior to the blood sampling you will be asked to take a single dose of either folic acid or a modified form of folate. You will then be asked if you would like to take part in a repeat study with your next annual TIPSS check up. If you are happy to take part, then the procedure will be repeated using modified folate (if you were given folic acid in the first study) or folic acid (if you were given modified folate in the first study).

Why will I be asked to have to have it repeated?

We want to make sure that the differences we hope to see in the way the body changes folic acid is because of the inability of the bowel wall to do so rather than our own individual differences to do so. This can best be answered if were able to repeat the test with both folic acid and the modified folate and compare the results.
Do I have to take part in the repeat study?

Again, it is up to you to decide. We will describe the repeat study and go through this information sheet again when we request your willingness to take part in the repeat study. We will then ask you to sign another consent form to show you have agreed to take part in the repeat study. You are free to withdraw at any time, without giving any reasons. This would not affect the standard of care you receive.

Are there any risks associated with taking part?

The study has been designed to make it as low risk as possible but there are 3 things which you should consider.

We will ask you to take a dose of folic acid. The folic acid dose we will be asking you to take is the same as would be present in a single folic acid supplement tablet available in all chemists and supermarkets in the UK and the folate dose would be the equivalent to single days green vegetables in somebody following the UK’s “five a day” recommendation for fruit and vegetables intake in the diet. The only difference between this folic acid and the type which you would buy in a supermarket is that it has been modified to include a harmless “label” which allows the tablet form to be identified in your blood.

We will be taking blood samples. The total volume of blood which will be taken over the course of the 4 hour study will be approximately 30mls (this is equivalent to about 3 times the volume taken in a normal clinic visit for a TIPSS patient and about one tenth the volume that would be taken from a blood donor.
in a single donation). Taking this amount of blood of blood over a period of 4
hours is thought to be completely safe.

In order to carry out the study we will need to leave the tube used to carry out
the TIPSS check procedure in place for longer than would normally be the case
(to allow us to take the blood samples) giving rise to theoretical risks of infection
and clotting. The investigators will take the utmost care to keep this risk to a
minimum. The approach of leaving the tube in place in the portal vein after a
TIPSS check to allow blood sampling has been used safely before in the UK. At
the end of the study the tube will be removed in the exactly the same way as
would happen normally in the X-ray department.

If you agree to repeat this study with your next annual TIPSS check up, you will
be taking an extra dose of folic acid or folate. Taking an extra dose is again
thought to be completely safe. The repeat study will be carried out in an entirely
similar way to the first study as explained above.

What will happen to the blood samples?

The blood samples will be used to measure the level of folic acid and its
modified forms. The measurements will be performed at the University of
Nottingham. The samples will all be destroyed during the course of the study.
If you decide to withdraw from the study following the taking of the blood
samples but before the analysis the samples cannot be returned to you but can
be destroyed at your request

PART 2

What will happen if I don’t want to carry on with the study?
You are free to withdraw from the study at any time and the blood samples obtained to that point destroyed.

**What will happen if I don’t want to carry on with the repeat study?**

You are free not to take part in the repeat study, or withdraw from the whole study at any time it and the blood samples obtained to that point will be destroyed.

**What if there is a problem?**

If you have a concern about any aspect of the this study you should ask to speak to the researchers (Professor David Jones and Dr Mark Hudson who can be contacted via 0191 233 6161) who will do their best to answer your questions. If you remain unhappy and wish to complain formally you can do this through the normal NHS complaints procedure. Details can be obtained form the hospital. In the event that something does go wrong and you are harmed during the research, and this is due to someone’s negligence, then you may have grounds for legal action for compensation against Newcastle-upon-Tyne NHS Foundation Trust who are sponsoring the study.

**Will my taking part in the study be kept confidential?**

If you join the study some parts of your medical records and the data collected for the study will be looked at by authorised persons from Newcastle University. They may also be looked at by representatives from the regulatory authorities and by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. Any information about you which leaves the
hospital will have your name and address removed so that you cannot be recognised.

**Who is organising and funding the research?**

The research will be sponsored by Newcastle-upon-Tyne NHS Foundation Trust and carried out by members of staff from this Trust and Newcastle University. The research is being funded by the Biotechnology and Biological Sciences Research Council (BBSRC), a UK government funded research council established to support scientific research.

**Who has reviewed the study?**

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your safety, rights wellbeing and dignity. This study has been reviewed and given a favourable opinion by Newcastle and North Tyneside 1 Research Ethics Committee. The research has also been independently reviewed by 4 outside experts on behalf of the BBSRC as part of its normal assessment process before deciding to pay for it.

**What if I have questions later?**

Please contact:

Dr Mark Hudson or Professor David EJ Jones

via the hospital switchboard (Tel 0191 2336161).
## APPENDIX – SCREENING QUESTIONNAIRE

### FOLTIPSS STUDY

**CONFIDENTIAL**

Volunteer Screening Questionnaire

**Study title:** “Where is the site of biotransformation of Folates in Humans”

(FOLTIPSS study)

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<td>Age:</td>
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<tr>
<td>Height:</td>
<td>Weight:</td>
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<tr>
<td>Blood Pressure:</td>
<td>Pulse:</td>
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**Cause of Chronic Liver Disease:**

- **Past Medical history:**
  - Ethanol: Y N
  - NASH: Y N
  - Hepatitis C: Y N
  - Hepatitis B: Y N
  - PBC: Y N
  - PSC: Y N
  - Autoimmune: Y N
  - Other: Elaborate / Others
  - Date of TIPSS: 
  - Type of surveillance: 
  - Date of last surveillance: 
  - Surgical history: 

**Prescribed medication:**

- Dietary Supplements: Y N

- Herbal remedies: Y N

---

FOLTIPSS screening questionnaire v1 10/12/2009

Volunteer code no.: 21/01/2010
FOLTIPSS STUDY

Are you currently suffering from any illness/injury?  Y  N
If yes give details below:

Do you take pain killers regularly?  Y  N (Specify: ....................)
Have you recently passed black stools  Y  N
Are you / could you be pregnant:  Y  N
Have you been pregnant within the last 12 months:  Y  N
Do you /or have you ever smoked:  Y  N
If yes how many per day:.................. When did you stop smoking:..................
Do you drink alcohol:  Y  N
If yes how many units per week: .................................................................

Have you any known allergies:  Y  N
Food:........................................... Drugs:.............................................
Other:..............................................................................................................

Do you agree to us informing your GP of your participation in the study or of any results found:  Y  N

Name and Address of your General Practitioner:
..............................................................................................................
..............................................................................................................
..............................................................................................................

Telephone number:.........................
Form completed by (print):.....................
Signature:........................................
Date:...............................................
URINE DIPSTICK TEST RESULTS

Date of sample:..........................  Time of sample:..................

RESULTS:

Bilirubin:.............  Blood:.............  Leucocytes:.............  Nitrates:.............

Test performed by:..........................  Signature:..........................
Date:...........................................  Time:...........................................

N.B. If positive for blood and volunteer is female please ask if they are menstruating, if answer is yes, repeat the test once volunteer has ceased menstruating.

Menstruating:  Y  N

Date due to finish menstruating:.....................  Repeat test:  Y  N


Date of repeat test:.............................  Time:.............................
Repeat test performed by:..........................  Signature:..........................

Comments:.................................................................................................
.................................................................................................
.................................................................................................
.................................................................................................

FOLTIPSS screening questionnaire v1 10/12/2009
Volunteer code no.: 21/01/2010
**Clinical Notes / Comments:**

*Text continues with no entries highlighted.*

**Investigations:** (Date: ..................)

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**Ultrasound:**

**Dated:**

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FOLTIPSS screening questionnaire v1 10/12/2009

Volunteer code no.: 21/01/2010
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