SINGLE NUCLEOTIDE POLYMORPHISMS OF MANNAN BINDING LECTIN AND COMPLICATIONS OF CHRONIC LIVER DISEASE

Thesis submitted for the degree of

Doctor of Medicine (MD)

at the University of Leicester

Dr Robert Su Chun Lo
B.Med.Sc, MB BCh, MRCP (UK)
Research Fellow

Digestive Diseases Centre
Royal Derby Hospital
Derby DE22 3NE
DEDICATION

This thesis is especially dedicated to my dearest wife Irene for her love, moral support, and encouragement, and to our precious daughter Jasmine for her love and inspiration.
ACKNOWLEDGEMENTS

I wish to express my utmost gratitude to my supervisors Dr Jan Freeman and Dr Andrew Austin for patiently guiding me through my research, for their stimulating discussions and for the financial support. I wish to thank my Leicester Mentor Dr John de Caestecker for his continual encouragement. I also wish to thank Mr Andy Lee from the University of Nottingham Derby Medical School, for teaching me the laboratory techniques (ELISA and real time PCR); and Mr Apostolos Fakis, Statistician at the Royal Derby Hospital, for providing me with invaluable knowledge in statistical analysis. I am grateful to Dr Ka-Kit Li and Dr Vincent Lai for their contributions in drafting the initial study protocols. Last but not least, I am indebted to all my families and friends, to whom this thesis is dedicated to, for their support, patience and encouragement throughout.
DECLARATION

This thesis is a record of work conducted at the Digestive Diseases Centre, Royal Derby Hospital. I confirm that the work of this thesis and its preparation including patient recruitment, venipuncture, and blood analysis, is entirely my own work, which has not previously been submitted for a higher degree.

Dr Robert Su Chun Lo

June 2014
# TABLE OF CONTENTS

1. Literature reviews .................................................................11
   1.1 Complications of Chronic liver disease: Pathophysiology and clinical implication .........................................................12
   1.2 Mannan Binding Lectin and the liver .................................30
   1.3 Gut microflora and probiotics in chronic liver disease ..........50

2. Aim ...........................................................................................64

3. Methods ....................................................................................66

4. Results ....................................................................................72
   4.1 The relationships between single nucleotide polymorphisms of Mannan Binding Lectin and complications of chronic liver disease.73
   4.2 Prognostic value of single nucleotide polymorphisms of Mannan Binding Lectin in the development of liver related death ........83
   4.3 The effect of probiotics treatment on systemic inflammation and bacterial translocation in acute decompensated chronic liver disease ....93

5. Discussions ..............................................................................98

6. Appendix ..................................................................................107
   6.1 Papers and presentation ......................................................108
   6.2 References .........................................................................109
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLD</td>
<td>Chronic liver disease</td>
</tr>
<tr>
<td>CP</td>
<td>Child Pugh</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HE</td>
<td>Hepatic encephalopathy</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LBP</td>
<td>Liposaccharide binding protein</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>Mannan-associated serine proteases</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan Binding Lectin</td>
</tr>
<tr>
<td>MBP</td>
<td>Mannan Binding Protein</td>
</tr>
<tr>
<td>MELD</td>
<td>Model for End-Stage Liver Disease</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non alcoholic fatty liver disease</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SBP</td>
<td>Spontaneous bacterial peritonitis</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structural MBL-2 genotype and allele frequencies in different population</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>Association between MBL deficiency and diseases</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>Common preparations of probiotics</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>Frequency of MBL SNPs</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Comparison of clinical characteristics between MBL SNPs and wild types</td>
<td>77</td>
</tr>
<tr>
<td>6</td>
<td>Association of MBL SNPs and LBP &amp; TNF-α</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>Association of different stages of CLD and LBP &amp; TNF-α</td>
<td>79</td>
</tr>
<tr>
<td>8</td>
<td>The prevalence of previous variceal bleed and/or spontaneous bacterial peritonitis</td>
<td>81</td>
</tr>
<tr>
<td>9</td>
<td>Univariate analysis of risk factor between patients with previous CLD and patients without</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>Causes of liver related death on follow up</td>
<td>85</td>
</tr>
<tr>
<td>11</td>
<td>Risk factors independently associated with liver related death</td>
<td>92</td>
</tr>
<tr>
<td>12</td>
<td>Baseline clinical characteristics</td>
<td>95</td>
</tr>
<tr>
<td>13</td>
<td>Effect of probiotic therapy on TNF-α, LBP and Child Pugh score</td>
<td>96</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pathways of complement activation</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>Schematic representation of MBL gene structure, MBL polypeptide structure</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>and MBL oligomeric structure</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Association between Child Pugh class and liver related death</td>
<td>87</td>
</tr>
<tr>
<td>4</td>
<td>Association between MBL SNPs and liver related death</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>Association between MBL SNPs and liver related death in Child Pugh class A.</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>Association between MBL SNPs and liver related death in Child Pugh class B.</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>Association between MBL SNPs and liver related death in Child Pugh class C.</td>
<td>91</td>
</tr>
<tr>
<td>8</td>
<td>Changes in LBP and TNF-α from study baseline to study end</td>
<td>97</td>
</tr>
</tbody>
</table>
ABSTRACT

Background

Mannan-binding lectin (MBL) is an innate immune system pattern recognition protein that kills a wide range of pathogenic microbes through complement activation. Infection is a major complication in patients with chronic liver disease (CLD). Infection is also thought to lead to variceal bleeds. These complications cause significant mortality and morbidity. MBL single nucleotide polymorphisms (SNPs) may result in an inability to protect against pathogens which may contribute to the development of infection including spontaneous bacterial peritonitis (SBP). Association between MBL SNPs and SBP/variceal bleed is unclear. The aim of this thesis is to comprehensively assess the association of MBL SNPs and SBP/variceal bleed, and its role as a prognostic marker in liver related death.

Methods

A case control study was performed. CLD patients with a prior history of complications (SBP/variceal bleed) were compared to those without. All patients were followed up subsequently in a longitudinal study to assess MBL SNPs as a risk factor for liver related death. Lastly, an open labelled proof of concept study was performed to determine the effect of 4 weeks probiotics therapy on bacterial translocation, systemic inflammation and the severity of liver disease.
Results

No significant relationships were found between MBL SNPs and baseline inflammation or bacterial translocation. MBL SNPs was not a risk factor for the occurrence of SBP/variceal bleed in both the univariate and multivariate analysis. In a Kaplan-Meier survival analysis, MBL SNPs was not associated with increased liver related death. MBL SNPs was also not an independent predictor in a multivariate Cox-regression analysis. The introduction of 4 weeks of probiotics therapy did not alter the baseline inflammation, bacterial translocation and severity of liver disease significantly.

Conclusion

The results of this thesis suggest that MBL SNPs do not associate with the occurrence of CLD complications (SBP/variceal bleed), and that MBL SNPs is not a prognostic marker for liver related death.
CHAPTER 1

LITERATURE REVIEWS
CHAPTER 1.1

COMPLICATIONS OF CHRONIC LIVER DISEASE: PATHOPHYSIOLOGY AND CLINICAL IMPLICATIONS
LIVER CIRRHOSIS

Cirrhosis is considered to be the end product of progressive liver fibrosis. Fibrogenesis is widely accepted to be a consequence of liver injury and inflammation. The process involves several key components in particular stellate cells, cytokines, proteinases and their inhibitors (Dooley et al., 2011). Injury to hepatocytes leads to activation of the immune system and recruitment of inflammatory mediators. The resulting inflammatory response stimulates and activates stellate cells in the perisinusoidal space of the liver. These include TGF-β1 from endothelial, Kupffer cells and platelets, lipid peroxides from hepatocytes. The stellate cell is the principle cell involved in fibrogenesis. Activated stellate cells are self-perpetuating through a number of autocrine systems. They result in a significant increase in extracellular matrix (ECM) complex deposition. ECM complexes are not uniform; they differ in age and chemical composition. Over a period of many months, the collagen fibrils of the complex undergo secondary processing, becoming cross-linked. The process confers resistance to degradative enzymes and ultimately irreversibility occurs (Rockey and Bissell, 2006). All over the world, the incidence of liver diseases is rising and carries an increasing economic burden for health providers (Bell et al., 2008). An epidemic of liver disease is expected over the next few years due to rising rates of obesity and increasing alcohol consumption (Lo et al., 2009).
LIVER CIRRHOSIS AND MORTALITY

Liver cirrhosis is associated with a substantially increased mortality risk compared with the general population. In the United Kingdom, patients with compensated liver cirrhosis have a nearly five-fold increased risk of death, while those with decompensated cirrhosis have a near 10-fold increased risk (Fleming et al, 2012). In the USA, cirrhosis accounted for more than 25,000 deaths and 373,000 hospital discharges in 1998 (Popovic and Kozak, 2000). In a 2006 USA National Statistics Report, cirrhosis was the twelfth leading cause of death (Miniño et al., 2006). Furthermore, liver cirrhosis mortality rates increased steeply during the 1990s. This is especially true in Scotland when between the periods 1987—1991, and 1997—2001, cirrhosis mortality in men in Scotland more than doubled (Leon and McCambridge, 2006).

STAGING OF LIVER CIRRHOSIS

Child Pugh score (Pugh et al, 1973) is commonly used to stage the severity of chronic liver disease.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 point</th>
<th>2 points</th>
<th>3 points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (μmol/L)</td>
<td>&lt;34</td>
<td>34-50</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>&gt;35</td>
<td>28-35</td>
<td>&lt;28</td>
</tr>
<tr>
<td>INR</td>
<td>&lt;1.7</td>
<td>1.71-2.3</td>
<td>&gt;2.3</td>
</tr>
<tr>
<td>Ascites</td>
<td>None</td>
<td>Mild</td>
<td>Moderate-severe</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>None</td>
<td>grade 1-2</td>
<td>grade 3-4</td>
</tr>
</tbody>
</table>
A total Child Pugh score of 5 to 6 is classified as A (well compensated disease), 7 to 9 is class B, and 10 to 15 is class C (decompensated disease). Child Pugh classes correlate with survival. The one-/two-year survival for class A, B, C are 100%/80%, 80%/60%, and 45%/35%, respectively (Infante-Rivard et al., 1987).

COMPLICATIONS OF CHRONIC LIVER DISEASE
The 3 major complications of cirrhosis of the liver are formation of ascites, hepatic encephalopathy and varices. They are features of liver decompensation and are consequences of portal hypertension, which is defined as an increase in hepatic sinusoidal pressure to 6mmHg or greater.

PORTAL HYPERTENSION
Portal hypertension is an increase in pressure in the portal vein and its tributaries. It is defined as a portal pressure gradient, which is the difference in pressure between the portal vein and the hepatic veins, of greater than 5mm Hg. However, portal pressure gradient is only clinically significant when it reaches 10 mmHg or greater. This is called clinically significant portal hypertension, and predicts the development of varices (Groszmann et al., 2005), and decompensation of cirrhosis (Ripoll et al., 2007).
Anatomically, portal vein is formed by the union of the superior mesenteric vein and the splenic vein. The mesenteric vein collects blood from the splanchnic circulation (Snell, 2003). Portal venous inflow therefore is determined by vasoconstriction or vasodilatation of the splanchnic arterioles.

The initial mechanism in the pathogenesis of portal hypertension is an increase in vascular resistance. This can occur at any level within the portal venous system. Portal hypertension is therefore classified as prehepatic (portal or splenic vein thrombosis); intrahepatic (cirrhosis), and posthepatic (Budd-Chiari syndrome). Cirrhosis remains the commonest cause of portal hypertension.

In cirrhosis, approximately two thirds of the increased resistance is caused by hepatic architectural distortion secondary to fibrosis and regenerative nodules, and the remaining resistance is caused by intrahepatic vasoconstriction (Miñano and Garcia-Tsao, 2010). This is caused by the activation of stellate cells with active contraction of myofibroblasts and vascular smooth muscle cells in portal venules (Pinzani and Gentilini, 1999), which in turn is caused by increased endogenous vasoconstrictors, such as endothelin, and reduced nitric oxide bioavailability (Wiest and Groszmann, 2002).

Formation of portosystemic collaterals occurs as a consequence of the high pressure in the portal vein and decreases the increased resistance. However, even when portal blood flow is entirely diverted through the collaterals, portal hypertension persists. This is due to a concomitant increase in portal venous inflow, which in turn is caused by splanchnic
vasodilatation (Sikuler and Groszmann, 1986), mostly mediated by an increase in nitric oxide (Wiest and Groszmann, 2002). Collaterals can be the result of dilatation of preexisting vascular channels, as well as neo-angiogenesis. Neo-angiogenesis contributes to both portosystemic collaterals as well as splanchnic blood flow causing portal hypertension (Fernandez et al., 2004).

**ASCITES**

Ascites is defined as pathological accumulation of fluid in the peritoneal cavity (Runyon, 2009). A portal pressure of at least 12 mmHg is required for the formation of ascites (Ginès et al., 1997). It is the commonest complication of cirrhosis, and is the commonest cause of ascites in the West. Approximately 50% of newly diagnosed cirrhotics will develop ascites within 10 years (Ginès et al., 1987). Development of ascites is an important landmark in the natural history of cirrhosis as approximately 15% of patients with ascites die in 1 year and 44% die in 5 years (Planas et al., 2006).

It is now widely accepted that formation of ascites is governed by the arterial vasodilatation theory (Schrier et al., 1988). Patients with cirrhosis and ascites usually have a marked reduction in systemic vascular resistance and in mean arterial pressure plus an increase in cardiac output (Groszmann, 1994). These abnormalities result in hyperdynamic circulation which is considered a pre-requisite for the development of ascites (Kowalski and Abelmann, 1953).
Increased levels of circulating vasodilators are thought to be the major contributor of vasodilatation in patients with cirrhosis. Although not precisely defined, potential vasodilators identified include nitric oxide (NO) (Vallance and Moncada, 1991), glucagon (Benoit and Granger, 1986), vasoactive intestinal peptide, prostaglandins and prostacyclin (Ros et al., 1995).

NO is thought to be the primary mediator of vasodilatation (Vallance and Moncada, 1991). There are a few observations to support this statement. First, the activity of endothelial NO synthase, which promotes the synthesis of NO from L-arginine, is increased in the arterial vessels of cirrhotic rats with ascites (Ros et al., 1995). Second, the serum levels of nitrite and nitrate, which are indexes of in vivo NO synthesis, are significantly higher in patients with cirrhosis (Guarner et al., 1993). Third, inhibition of the synthesis of NO in rats with cirrhosis significantly increases the arterial pressure and systemic vascular resistance, decreases the cardiac index (Clària et al., 1992) and reverses the impaired response to vasopressors (Sieber et al., 1993).

In cirrhotics, NO synthesis may be stimulated by endotoxin or other bacterial products, such as bacterial DNA from the gastrointestinal tract, which are not effectively cleared by the impaired reticuloendothelial cell function as well as secondary to portal-systemic shunting. This is evidenced by the findings that NO concentrations in portal venous blood are higher than those of peripheral veins (Battista et al., 1997). Also, endotoxin is significantly related to serum nitrite and nitrate levels (Guarner et al., 1993). Administration of antibiotics significantly reduces plasma endotoxin levels and the serum
concentration of nitrite and nitrate (Guarner et al., 1993), increases systemic vascular resistance hence mitigating the vasodilatation phenomenon in cirrhotics (Albillos et al., 2003).

As a consequence of vasodilatation, carotid and renal baroreceptors sense the reduction in pressure, and activate sodium- retaining neurohumoral mechanisms in an attempt to normalise perfusion pressure. These include the renin-angiotensin-aldosterone system, sympathetic nervous system, and antidiuretic hormone (Henriksen et al., 1992).

The net effect is avid sodium and water retention as cirrhotic patients are effectively volume depleted. The presence of sodium retention is indicative of at least 50% reduction in liver function (Wensing et al., 1997). The degree of sodium retention is inversely related to survival. Prognosis of patients with ascites and urinary sodium excretion below 10 mEq/day (230 mg/day) is as low as five to six months, in comparison to over two years in those with a higher rate of sodium excretion (Arroyo et al., 1981).

Water retention is largely related to increased release of antidiuretic hormone (ADH). This inability to excrete water regularly leads to the development of hyponatraemia and hypoosmolality (Arroyo et al., 1994).

The activation of vasoconstrictor systems reduces renal blood flow (Sacerdoti et al., 1993). However, renal perfusion may initially be maintained due to vasodilators which include prostaglandins and perhaps nitric oxide. Nevertheless, the natural progression of
liver disease overtakes these protective mechanisms, leading to progressive renal hypoperfusion, a gradual decline in the glomerular filtration rate, and eventually hepatorenal syndrome (Fernandez-Seara et al., 1989).

**SPONTANEOUS BACTERIAL PERITONITIS (SBP)**

SBP is defined as a spontaneous mono-infection of the ascitic fluid in the absence of an identifiable intra-abdominal source of infection or inflammation. The incidence of SBP in cirrhotic patients with ascites ranges between 10 to 30% (Rimola et al., 2000). At the time of hospital admission, 12% of patients were found to have SBP although nearly 50% of the patients were not symptomatic (Borzio et al., 2001). A diagnostic paracentesis is therefore recommended for all cirrhotic patients with ascites admitted to the hospital. The diagnosis of SBP is made when the ascitic fluid absolute polymorphonuclear leukocyte (PMN) is more than 250 cells/mm$^3$ (Hoefs et al., 1982).

SBP is associated with significant mortality and morbidity. Despite early recognition and prompt initiation of antibiotic treatment and volume expansion, at present 1 in 5 patients still die in the hospital with an episode of SBP (Garcia-Tsao, 2001).

It is thought that bacteria translocation, a process whereby enteric bacterial cross the intestinal barrier and gain access to the mesenteric lymph nodes and blood vessels, is the underlying mechanism responsible for SBP. This is supported by the fact that more than two thirds of cases of SBP are due to normal enteric flora, mainly gram-negative bacteria (Garcia-Tsao, 1992). A low ascitic protein in patients is known to be associated with the
development of SBP (Lo et al, 2010). This susceptibility is thought to be due to the lower concentration of ascitic complement and decreased opsonic activity (Runyon et al., 1985). However, there is currently no role for primary antibiotic prophylaxis due to the lack of evidence (Moore and Aithal, 2006), and concerns of the development of drug resistance (Ghassemi and Garcia-Tsao, 2007). The roles of probiotics in the prevention of SBP will be discussed later in review chapter 1.3.

HEPATIC ENCEPHALOPATHY (HE)

HE is a serious, potentially reversible, syndrome of neuropsychiatric dysfunction in patients with advanced liver failure. As defined by the Working Party in 1998, HE can be categorised into 3 broad groups: type A, which occurs in acute liver failure; type B, which occurs in patients with bypass shunts, for example radiological inserted transjugular intrahepatic portosystemic shunt; and the most commonly recognised, type C, which occurs in patients with chronic liver disease (Ferenci et al., 2002).

Within type C HE, different patterns exist. Many patients suffer from intermittent episodic HE. Episodes of HE may be isolated events, but more commonly they are recurrent. On the other hand, another presentation is chronic persistent HE which is marked by an ongoing deficit in neuropsychological functioning and failure to achieve complete symptom resolution.
Episodic HE is usually acute and may be precipitated by over-diuresis, large volume paracentesis, fluid and electrolyte depletion, gastrointestinal haemorrhage, surgery, alcoholism, sedative medications, infections, constipation, as well as a large protein meal (Dooley et al., 2011). Systemic inflammatory response syndrome (SIRS) has also been shown to cause HE (Shawcross et al., 2004).

Development of HE in the setting of liver cirrhosis signifies liver failure and carries a poor prognosis. The survival probability in cirrhotics after the first episode of acute hepatic encephalopathy is 42% at 1 year and 23% at 3 years (Bustamante et al., 1999).

West Haven criteria have been widely used to classify clinically detectable HE (Conn et al., 1977). They range from grade 1 for the presence of mild symptoms such as lack of awareness, through to grade 4 for comatose and unresponsive patients.

Outside of West Haven classification, minimal HE is HE without overt signs or symptoms recognisable in clinical settings, but nonetheless patients with minimal HE demonstrate deficiencies in several psychometric tests (Frederick, 2011). These patients experience decreased global functioning, increasing falls, impaired driving ability, and reduced quality of life (Groeneweg et al., 1998).

The exact mechanism causing HE is still unknown. The general consensus is that elevated levels of ammonia and a concurrent inflammatory response causing astrocytes to swell and fluid to accumulate in the brain, resulting in cerebral oedema.
Ammonia is the most well characterised neurotoxin that precipitates HE. It is toxic at elevated concentrations and must be removed from the body (Zieve, 1987). In humans, ammonia is mostly eliminated through the formation of urea in the liver. Direct evidence for the role of ammonia in the pathogenesis of HE was demonstrated in a study using radio-labelled nitrogen in Positron Emission Tomography (PET) imaging of patients with severe liver disease and HE (Lockwood et al., 1991).

The primary source of ammonia is the gastrointestinal tract, and it enters the circulation via the portal venous system. Ammonia is produced by enterocytes from glutamine and by colonic bacterial catabolism of nitrogenous sources, for example ingested protein and secreted urea. Helicobacter pylori which digest urea in the stomach is another potential source (Suto et al., 2001).

In the normal liver, the ammonia from the portal vein is cleared in the liver by converting it into glutamine and hence preventing it from entering into the systemic circulation (Häussinger et al., 1985). In the cirrhotic liver, the increase in blood ammonia in the systemic circulation is a consequence of impaired liver function and of shunting of blood around the liver (Riggio et al., 2005). Muscle wasting which is part of stigmata of cirrhosis may also contribute to hyperammonaemia since muscle is an important site for extrahepatic ammonia removal (Olde Damink et al., 2002).
Astrocytes are the only cells in the brain that can metabolise ammonia (Cooper and Plum, 1987). The enzyme glutamine synthetase is responsible for the conversion of glutamate and ammonia to glutamine (Olde Damink et al., 2009). Intracellular levels of glutamine, therefore, increase enormously as the ambient ammonia concentrations rise secondary to liver cirrhosis and failure (Thomas et al., 1988). Glutamine is an osmolyte causing water to move inside the astrocyte causing it to swell. This swelling leads to cerebral oedema and intracranial hypertension (Häussinger et al., 2000), culminating in HE in acute liver failure.

In addition to ammonia, many other molecules have been implicated in the pathogenesis of HE. Neurosteroids are neuroactive steroids that are synthesised in the central and peripheral nervous system. Their increased productions in HE are secondary to upregulation of the 18 kDa translocator protein (previously known as peripheral-type benzodiazepine receptor) (Butterworth, 2000). Ammonia and manganese are also thought to enhance neurosteroid synthesis by activating these translocator proteins (Ahboucha and Butterworth, 2007). Neurosteroids are positive allosteric modulators of GABA-A receptor, which increase influx of chloride ions and thereby enhance the inhibitory GABAergic tone. These effects are responsible for some clinical sequelae in patients with type C HE (Ahboucha et al., 2006).
VARICES

Development of gastro-oesophageal varices is the most direct consequence of portal hypertension. The portal vein-systemic collateral circulation develops and expands as a consequence of portal hypertension (Gupta et al., 1997). Blood flow in the low volumes that normally perfuse these collaterals and flow toward the portal circulation is reversed.

The sites of collateral formation are the rectum (where the inferior mesenteric vein connects with the pudendal vein and rectal varices develop); the umbilicus (where the vestigial umbilical vein communicates with the left portal vein and give rise to caput medusae); the retroperitoneum (where, in females, collaterals develops between ovarian vessels and iliac veins); and the most clinically significant distal oesophagus and proximal stomach (where gastroesophageal varices are the major collaterals between the portal venous system and systemic venous system) (Snell, 2003).

The development of gastroesophageal varices requires a portal pressure gradient of at least 10 mm Hg. A higher portal pressure gradient of 12 mm Hg is thought to be a pre-requisite for varices to bleed (Miñano and Garcia-Tsao, 2010).

Not all patients with portal pressure gradient of greater than 12 mm Hg bleed. Local factors that increase variceal wall tension are also a requirement. Law of Laplace (T=Pr/w) governs that patients with large varices (r) in sites of limited soft tissue support hence thin wall thickness (w), with elevated portal pressure (P), tend to have increased variceal
wall tension (T) and are at greatest risk of variceal bleed (Dooley et al., 2011). Soft tissue support is notably weak at the gastroesophageal junction.

Oesophageal varices are present in approximately 40% of patients with cirrhosis and as many as 60% of patients with cirrhosis and ascites (Bosch et al., 2003). New varices develop at a rate of 5% in cirrhotic patients without varices in index endoscopy, and small varices progress to large varices at a rate of 10-15% annually (de Franchis and Primignani, 2001). Up to 25% of patients with newly diagnosed varices will bleed within 2 years. Despite endoscopic and pharmacological intervention, the risk of death with acute variceal bleed is 5% to 8% at one week and about 20% at six-weeks (de Franchis and Primignani, 2001).

BACTERIAL INFECTION AS TRIGGER FOR VARICEAL HAEMORRHAGE

Bacterial infections occurred in up to two third of cirrhotic patients with variceal haemorrhage (Goulis et al., 1998). About two thirds of these infections are present at hospital admission while the remaining third develop during admission (Borzio et al., 2001). Also, bacterial infections are more common in cirrhotic patients with acute variceal bleeding than in those admitted to hospital with other forms of decompensation, such as encephalopathy (Borzio et al., 2001). Vivas et al (Vivas et al., 2001) studied 91 cirrhotic patients presenting with an upper gastrointestinal bleed. They found a strong association between infection and failure to control bleeding as well as mortality. A larger study by Goulis et al involving 163 admissions for variceal bleeding in 137
patients found a significant association between infection and early rebleeding as well as death.

Antibiotic prophylaxis in cirrhotic patients with gastrointestinal bleeding significantly increased the short term survival rate, further supporting the idea of bacterial infection as a trigger for variceal bleeding (Soares-Weiser et al., 2003).

It is possible that bacterial infection or endotoxaemia triggers variceal haemorrhage via the following mechanisms:

- Increase in portal pressure
- Impairment of coagulation
- Impairment of liver function

Bacterial infection increases the production of endothelin 1, the most potent mediator of stellate cell contraction. Acting synergistically with cyclo-oxygenase products, it increases the intrahepatic portal pressure and therefore the variceal pressure culminating in variceal bleed.

Impairment of coagulation happens because sepsis causes defects in platelet aggregation (Vincent et al., 2002). This may occur either via a decrease in platelet aggregation due to the increased nitric oxide production, or due to the fact that endotoxin and Endothelin-1 can impair platelet aggregation through release of prostacyclin. Moreover, production of cytokines in the presence of bacterial infection can lead to activation of clotting factors and fibrinolysis (Grignani and Maiolo, 2000).
Infections have been shown to aggravate liver dysfunction in patients with cirrhosis (de Mattos et al., 2003). Normal liver microcirculatory function is maintained by a balance of vasoconstrictors (Endothelin-1) and vasodilators (NO, carbon monoxide) (Baveja et al., 2002). Both endotoxaemia and cytokines such as TNF-α and Interleukin-1 (IL-1) cause hepatic necrosis by disruption of the microcirculation. Alteration in the microcirculation due to endotoxin is mediated by Kupffer cells (Han, 2002). Kupffer cells also cause infiltration of leukocytes which are a source of reactive oxygen and nitrogen species, leading to lipid peroxidation in the liver and secondary damage to hepatocytes (Hewett et al., 1992). Also, TNF-α is itself hepatotoxic (Thalheimer et al., 2005).

**INFLAMMATORY CYTOKINE TNF-α**

TNF-α is the marker for systemic inflammation studied in this thesis. It is an endotoxin-induced cytokine which is well established as a marker for systemic inflammation (Diez-Pina et al., 2009). It is a member of a group of cytokines that stimulate the acute phase reaction. It is produced mainly by activated macrophages and T cells as a transmembrane precursor protein. The cytoplasmic tail of this protein is then cleaved to release soluble TNF-alpha (Beutler and Cerami, 1989).

The biological activity of TNF-alpha requires the aggregation of three TNF-alpha monomers to form trimeric TNF-alpha, which then acts by binding to one of two types of receptors: TNFR1 or TNFR2 (Hehlgans and Männel, 2002). TNFR1 and TNFR2 are also
known as p55 and p75, respectively. The trimeric structure of the receptors mimics that of the active cytokine (Zhang, 2004).

TNFR1 and TNFR2 both exert multiple effects on the immune system, including the following (Roach et al., 2002):

1) Stimulation of the release of the inflammatory cytokines interleukin (IL)-1beta, IL-6, IL-8,

2) Upregulation of the expression of endothelial adhesion molecules and chemokines,

3) Coordination of the migration of leukocytes to targeted organs
CHAPTER 1.2

MANNAN- BINDING LECTIN AND THE LIVER
HISTORY OF MANNAN BINDING LECTIN

Back in 1968, a patient with a serum-dependent defect in phagocytosis of yeast particles was reported in the literature (Miller et al., 1968). This defect was linked to the complement system a decade later, as C3 was deposited in lower amounts on yeast surfaces incubated in sera from affected individuals (Turner et al., 1981). Subsequently in 1989, this defect was linked with low levels of mannan-binding protein (MBP), in a study which showed a highly significant correlation between the serum MBP level and the generation of C3b opsonins (Super et al., 1989).

Mannan-binding lectin (MBL), also known as mannan-binding protein, is an important component of the innate immune system. It was first discovered by Kawasaki et al. as early as 1978 when a protein was extracted from rabbit liver using mannan particles from Saccharomyces cerevisiae as a probe (Kawasaki et al., 1978). MBL is a protein that activates the complement system via the lectin pathway (Figure 1) (Ikeda et al., 1987). The complement system provides immediate defence against infection and has a pro-inflammatory response.
**MBL PROTEIN**

Lectins that are dependent upon the presence of calcium ions are named C-type lectins. MBL belongs to the class of collectins in the C-type lectin superfamily. Lectins are involved in complement activation via the lectin pathway. MBL is primarily produced by the liver (Ji et al., 2005). It circulates throughout the body and is able to recognise a wide variety of common pathogens through repeating carbohydrate sequences present on microbial surfaces. MBL binding of pathogens initiates complement activation.

MBL has an oligomeric structure (400-700 kDa), built of subunits that contain three identical polypeptide chains of 25 kDa each (Garred, 2008). Each polypeptide consists of a cysteine rich N terminal region, a collagen-like region, a hydrophobic neck region, and a carbohydrate recognition domain (Figure 2). The collagenous regions of three such chains interact to give a classical triple helix. MBL in serum primarily consists of trimers and tetramers of 9 and 12 polypeptides respectively, but the oligomers can range from dimers or hexamers (Garred, 2008). These higher-order oligomers are essential for the function of MBL (Wallis and Drickamer, 1999).

MBL binds carbohydrates in the presence of calcium through the c-terminal carbohydrate-recognition domain (CRD) (Sheriff et al., 1994). CRD is able to form bonds with hydroxyl groups on specific ligands, including mannan, N-acetylglucosamine and glucose (Turner, 2003). These carbohydrates are found on pathologic microorganisms, such as bacteria, fungi, parasitic protozoans and viruses. The CRD also recognises molecular structures of dying host cells, including nucleic acids and the metalloproteases
(Garred, 2008). On the other hand, carbohydrates that are found on mammalian glycoproteins, such as D-galactose and sialic acid, have no affinity for MBL protein. MBL protein is therefore able to distinguish self from non-self in its role in activating the innate immune pathway.

MBL interacts with mannan-associated serine proteases (MASP-1 (Matsushita and Fujita, 1992) and MASP-2 (Thiel et al., 1997), which activates both the classical and alternate pathways of the complement system and may also bind to novel phagocyte receptors, resulting in opsonisation, phagocytosis and cell lysis.

SERUM MBL LEVELS IN NORMAL POPULATION

Kilpatrick et al measured serum MBL concentrations in 566 blood donors and found that median serum MBL level was 1.3µg/ml (range 0-8.4µg/ml). The proportion of individuals with very low MBL level (<0.1 µg/ml) was 10% (Kilpatrick, 2003).

SERUM MBL IN LIVER CIRRHOSIS

A large Hungarian study found that among patients with liver cirrhosis of various aetiologies, the median MBL level was 1.118 µg/ml. This MBL level was not statistically different compared to the healthy control (1.027 µg/ml), hepatitis C patients without liver cirrhosis (1.139 µg/ml), and non-cirrhotic patients with autoimmune liver diseases (0.959 µg/ml) (Altorjav, 2010). Cirrhotic patients with very low MBL level (<0.1 µg/ml) was
10.7%. Again this prevalence was comparable to other groups of patients mentioned above.

**MBL2 GENE**

The relative efficiency of MBL function for an individual is largely determined by polymorphisms within the MBL2 gene, located on chromosome 10 (Sastry et al., 1989). The MBL2 gene is also known as collectin subfamily member 2 (Garred, 2008).

There are two human MBL genes but MBL-1 is a pseudogene (Guo et al., 1998) and only MBL-2 encodes a protein product (MBL). MBL-2 comprises four exons with exon 1 encoding a signal peptide, a cysteine-rich region and part of the glycine-rich collagenous region; exon 2 encodes the remainder of the collagenous region; exon 3 encodes an α-helical coiled-coil structure which is known as the ‘neck’ region; and the fourth exon encodes the carbohydrate-recognition domain which adopts a globular configuration (Taylor et al., 1989) (Figure 2). The promoter region of the MBL gene contains various elements which also enhance MBL transcription (Taylor et al., 1989).

The first pathogenic mutation in MBL2 was found in 1991 (Sumiya et al., 1991). Three point mutations or single nucleotide polymorphisms (SNPs) within the first exon of MBL2 significantly affect MBL functions (Sumiya et al., 1991). They are designated ‘B’, ‘C’ and ‘D’ in contrast to ‘A’, which is the normal or wild type.
Allele B represents a point mutation at codon 54 causing substitution of a glycine with an aspartic acid (CCC to GAC) (Sumiya et al., 1991). Allele C represents a point mutation in codon 57 causing a glycine to be substituted with a glutamic acid (GCA to GAA) (Lipscombe et al., 1992). Allele D represents a point mutation in codon 52 causing an arginine to be substituted with a cysteine (CGT to TGT) (Madsen et al., 1994).

Allele ‘B’, ‘C’ and ‘D’ are collectively referred to as ‘O’. Generally, wild type A/A is associated with the highest MBL levels, A/O with intermediate and O/O with low or absent MBL.

Within any given population there are different functional levels of circulating MBL, due to the differences in the frequency of MBL 2 genetic polymorphisms (Table 1). B allele is frequent among Caucasians (0.14), Chinese and Eskimos; C allele is very frequent among Africans (0.29) but rare in Caucasians and absent in Asians; and D allele has very low frequency among all populations (Garred et al., 2006).

In addition to the three mutations in exon 1, there are several other polymorphisms in promoter 1 and 5’ untranslated regions, which can partly account for variation in the MBL serum concentration. 3 common regulatory polymorphisms are H/L variant situated at position -550, X/Y variant at position -221, and P/Q variant at position +4 (Madsen et al., 1995). These 3 loci are closely linked and four promoter haplotypes (LXP, LYP, LYQ and HYP) are commonly found. These regulatory and coding polymorphisms are in strong linkage disequilibrium, with only seven haplotypes defined in human populations (HYPA, LYQA, LYPA, LXPA, HYPD, LYQC and LYPB). Among haplotypes carrying
the wild-type A allele, HYP A results in normal/high levels of MBL, whereas LXPA is associated with reduced level of MBL (Casanova and Abel, 2004).

**MBL VARIANT PROTEIN**

The MBL variant proteins are unstable and probably have a shorter half-life in the circulation (Naito et al., 1999). They have a lower molecular weight and do not bind mannann effectively nor activate complement. They interfere with the formation of higher order oligomers (Garred et al., 2003b). This genetically determined MBL deficiency status increases the risk of infections and other disease-specific complications especially when the immune system is already compromised. It is involved in a variety of diseases. Non hepatological manifestations are summarised in table 2. Associations between MBL polymorphisms and liver diseases will be discussed in detail.
MBL SNPs IN THE NORMAL POPULATION

Among normal British Caucasians, the prevalence of A/A was 60\%, A/B was 21\%, A/C was 5\%, A/D was 10\% and O/O was 4\% (Garred et al., 2006).

MBL SNPs IN LIVER CIRRHOTIC PATIENTS

To date, there is no published literature on the prevalence of MBL SNPs among patients with liver cirrhosis.

MBL AND LIVER FIBROSIS

Brown et al found that among HCV patients, those with severe liver fibrosis had significant higher mean serum MBL level (9.5 µg/ml) compared to those with mild fibrosis only (5.0 µg/ml) (Brown, 2008). This is due to the increased complement activation and increased activity of the MBL/MASP1 complex which are pro-inflammatory. The cytokines produced then enhance a rise in extracellular matrix that is important in the pathogenesis of liver fibrosis (Lata et al., 2011)
MBL POLYMORPHISMS AND THE LIVER

MBL polymorphisms have been studied in the field of hepatology in the following areas:

- Viral hepatitis B
- Viral hepatitis C
- Liver transplantation

MBL polymorphisms and hepatitis B

Yuen et al (Yuen et al., 1999) studied 190 hepatitis B and C Chinese patients with compensated cirrhosis and 117 normal controls. The codon 54 mutation of MBL was associated with progression of disease in chronic hepatitis B infection, and the development of SBP.

Chong et al (Chong et al., 2005) studied 320 hepatitis B surface antigen (HBsAg) inactive carriers; 199 HBsAg carriers with hepatocellular carcinoma or cirrhosis; 87 spontaneously recovered individuals who were HBsAg negative and anti-HBs and anti HBc positive; and 484 controls who were naïve to HBV. They found that MBL genotypes with low MBL levels were associated with the occurrence of cirrhosis and hepatocellular carcinoma.

Thio et al (Thio et al., 2005) studied 189 persons with HBV persistence, and 338 individuals who had naturally recovered from HBV infection. They found that those
homozygous for the combination of promoter and exon 1 genotypes which produced the lowest amount of functional MBL were more likely to have viral persistence. Conversely, those combinations associated with the highest amount of functional MBL had significantly increased odds of recovery from infection.

Filho et al (Filho et al., 2010) investigated 102 patients infected with HBV and 232 healthy controls. They found a significantly increased frequency of haplotypes associated with low serum MBL (LXA, HYO, LYO) in the HBV-infected group. This finding may indicate that MBL has a protective role against HBV infection.

Hohler et al (Höhler et al., 1998) however, did not find any association between MBL polymorphisms and susceptibility to chronic HBV infection. They studied 61 patients with chronic HBV infection, 28 patients with acute infection and in 60 controls. The frequency of MBL variants at codon 52 and 54 was not increased.

Lack of association was also confirmed by Bellamy et al (Bellamy et al., 1998). They retrospectively studied MBL genotypes in 990 patients tested for persistent HBV carriage. 337 were positive for anti-HBc, and of these, 180 had persistent infection with positive HBsAg serology. MBL genotype was found not to be associated with positive anti-HBc.
**MBL polymorphisms and hepatitis C**

Matsushita et al (Matsushita et al., 1998b) studied MBL polymorphisms in 93 patients with chronic hepatitis C (45 responders and 48 non-responders to interferon) and 218 healthy controls. They found that homozygous carriage of the variant allele of codon 54 of MBL may predict poor response to interferon in chronic hepatitis C patients. The same authors also found that LXPA or LYPB haplotypes were more prevalent in patients who were resistant to interferon therapy (Matsushita et al., 1998a).

Sasaki et al (Sasaki et al., 2000) in another Japanese study examined 52 HCV-infected patients (8 with chronic inactive hepatitis, 31 with chronic active hepatitis, and 13 with liver cirrhosis) and 50 normal controls. Although they did not find any significant relationship between MBL polymorphisms and the levels of HCV RNA, all patients with heterozygous or homozygous codon 54 mutations had chronic active hepatitis or liver cirrhosis, suggesting that MBL polymorphisms may influence the course of HCV infection.

Halla et al (Halla et al., 2010) investigated MBL polymorphisms in 186 HCV patients and 232 healthy controls. They found that frequency of genotypes related to low production of MBL was higher in patients with HCV than in controls, hence conferring susceptibility. They also showed that inheritance of HYO haplotype was associated with fibrosis severity.
Koutsounaki et al (Koutsounaki et al., 2008) investigated the variant alleles in MBL2 gene promoter and exon-1 regions in 80 Caucasian HCV-infected patients. They found that polymorphism homozygosity in exon-1 region was significantly related to progression of HCV infection to liver inflammation and liver fibrosis.

On the other hand, Kilpatrick et al (Kilpatrick et al., 2003) studied 180 hepatitis C patients and 566 blood donors and found no association between MBL deficiency and susceptibility to hepatitis C infection, course of the disease, and the response to antiviral therapy. However, no MBL polymorphisms were measured in this study.

The lack of association between MBL polymorphisms and the prevalence of chronic hepatitis C was confirmed in a Brazilian study involving 73 hepatitis C patients and 92 seronegative controls (Vallinoto et al, 2009).
MBL polymorphisms and liver transplantation

Bouwman et al (Bouwman et al., 2005) investigated 49 patients undergoing orthotopic liver transplantation, and genotyped 25 of the recipients. They found that the presence of MBL variant alleles in the MBL gene of the donor liver, but not in the recipient, was associated with a strongly increased incidence of clinically significant infections after transplantation.

Worthley et al (Worthley et al., 2009) studied 102 patients that underwent orthotopic liver transplantation. Both the donors and recipients were genotyped. The presence of MBL2 coding mutations in the donor was significantly associated with clinically significant infection in the recipient. Recipients of MBL-deficient livers had almost a 3-fold greater likelihood of developing clinically significant infection.

A much larger study was performed by de Rooij et al (de Rooij et al., 2011). They studied 295 liver transplant patients and all were genotyped for recipient and donor SNPs. They found that MBL2 SNPs in the donor liver showed an increased risk of cytomegalovirus infection. Another interesting finding is that genetic donor-recipient mismatch for MBL2 increased the cytomegalovirus infection risk.

On the other hand, a very large single-centre study involving 290 donor livers failed to find any association between the donor MBL genotypes and the risk of bacterial infection after liver transplantation (Curvelo et al, 2011).
POTENTIAL REASONS TO ACCOUNT FOR CONFLICTING MBL2 ASSOCIATION STUDIES

The role of MBL in the susceptibility to various diseases is sometimes controversial since for the same disease, association, or the lack of it, have been reported.

Potential reasons to explain the conflicting results in MBL2 association studies are the techniques used for MBL2 genotyping, the size of the samples studied, and the choice of controls (Segat and Crovella, 2011) (de Melo et al., 2011). With regards to MBL2 genotyping techniques, there are currently several genotyping methods, such as multiplex PCR (Skalníková et al, 2004); real time PCR with fluorogenic probes (Steffensen et al, 2003), and melting temperature assay (Arraes et al., 2006), which are not entirely similar. Therefore there is a suggestion that a blind double check on randomly chosen samples with direct sequencing should always be considered in this kind of study (Segat and Crovella, 2011). With regards to the choice of control, the selection of the control population as well as the possibility of replicating the results in other ethnic groups is a crucial point in MBL2 association studies, since the frequencies of MBL2 polymorphisms show some variability within different populations (Garred et al., 2006).
COMPLEMENT SYSTEM

The complement system is a major component of innate immunity. It consists of plasma and membrane proteins, which mediate 3 pathways, namely classical, alternative and lectin. The lectin pathway is the most recently described of these pathways (Ikeda et al., 1987). Activation of these pathways of cascading enzymatic reactions results in the deposition of fragments that promote inflammatory and immune response. The classical pathway is activated when IgM or IgG antibody bind to antigens such as viruses and bacteria. The activation of Lectin pathway is similar to the classical pathway except that antibody is replaced by a lectin, which includes MBL. Lectins bind to repetitive sugar patterns on the surface of a pathogen. These lectins are associated with MBL associated serine proteases (MASPs) that are structurally and functionally similar to the classical pathway component C1. The MASPs also cleaves C2 and C4.

The major effect of the complement system, regardless of the pathway, is the deposition of the complement fragment C3b on the target (Figure 1). The effects are membrane modification and promotion of the inflammatory response.

Complement fragments including C3b deposit in large numbers on microbes and unwanted materials such as apoptotic cells and necrotic tissue. This coating or opsonisation of bacteria allows specific receptors on peripheral blood cells, especially phagocytes, to bind these ligands. Additionally, activation of complement results in the formation of the membrane attack complex (MAC, C5b-9). MAC perturbs the bacterial cell membrane, causing lysis of the microbes.
Activation of the complement system leads to the release of peptides. These are potent mediators of inflammatory and immune responses. These fragments are known as anaphylatoxins (C3a and C5a), and bind to their respective receptors on cells to initiate inflammation and activate immune cells (Ward, 2004).

Additionally, complement is also bactericidal as C3a and C4a directly kill microorganisms (Pasupuleti et al., 2007).
Figure 1: Pathways of Complement Activation

MASPs: MBL associated serine proteases
Figure 2: Schematic representations of MBL gene structure, MBL polypeptide structure and MBL oligomeric structure

**MBL2 gene**

![MBL2 gene Schematic]

Promoter 0  Promoter 1

**MBL 25 Kd polypeptide**

![MBL 25 Kd polypeptide Schematic]

N terminal Cysteine  Collagen-like domain  C-type CRD domain  Coiled coil neck region

**MBL tetramer**

![MBL tetramer Schematic]
Table 1: Structural MBL2 genotype and allele frequencies in different population

<table>
<thead>
<tr>
<th>Ethnic group</th>
<th>genotype frequencies in %</th>
<th>allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
<td>A/B</td>
</tr>
<tr>
<td>Europeans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Danish</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td>British</td>
<td>60</td>
<td>23</td>
</tr>
<tr>
<td>Sub-Saharan Africans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kenyan</td>
<td>51</td>
<td>6</td>
</tr>
<tr>
<td>Ghanian</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>Nambian</td>
<td>79</td>
<td>6</td>
</tr>
<tr>
<td>South African</td>
<td>51</td>
<td>NF</td>
</tr>
<tr>
<td>Asians</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hong Kong Chinese</td>
<td>78</td>
<td>20</td>
</tr>
<tr>
<td>Japanese</td>
<td>59</td>
<td>36</td>
</tr>
<tr>
<td>Australasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papua New Guinean</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>Aboriginals</td>
<td>100</td>
<td>NF</td>
</tr>
<tr>
<td>Americans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eskimos</td>
<td>78</td>
<td>18</td>
</tr>
<tr>
<td>Argentinean</td>
<td>30</td>
<td>56</td>
</tr>
<tr>
<td>Patient groups</td>
<td>Diseases</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>sepsis (Israëls et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Infant</td>
<td>recurrent infections (Miller et al., 1968), failure to thrive (Richardson et al., 1983), chronic diarrhoea (Candy et al., 1980), atopic disease (Turner et al., 1978)</td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>acute respiratory tract infection (Koch et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Intensive care</td>
<td>sepsis, septic shock and fatal outcome (Garred et al., 2003a)</td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>disease severity (Chalmers et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Infections, prolonged febrile neutropaenia (Peterslund et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Immunocompromised</td>
<td>acute invasive aspergillosis (Lambourne et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal surgery</td>
<td>infections (Siassi et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>General population</td>
<td>recurrent or severe infections (Hoeflich et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>Patients with pneumonia</td>
<td>susceptibility and worse outcome (Garcia-Laorden et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>E Coli pyelonephritis (Smithson et al., 2007), Fallopian tube occlusion (Sziller et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>HIV infection</td>
<td>worse outcome (Garred et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>General population</td>
<td>meningococcal disease (Hibberd et al., 1999), Legionnaires’ disease (Eisen et al., 2007), recurrent tonsillitis (Grasso et al., 2007), poor immune response to influenza vaccination (Tang et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>severe disease (Saevarsdottir et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>disease severity (Garred et al., 2001)</td>
<td></td>
</tr>
<tr>
<td>Coeliac disease</td>
<td>disease development (Boniotto et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>disease development (Araujo et al., 2007)</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>disease development and disease severity (Wang et al., 2008)</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 1.3

GUT MICROFLORA AND PROBIOTICS IN CHRONIC LIVER DISEASE
INTESTINAL MICROFLORA

There is a strong relationship between liver and the gut. The gut supplies blood to the portal system and intestinal blood content activates liver functions. On the other hand, the liver secretes bile and influences intestinal function.

Intestinal microflora forms a complex ecological system. It contains a large amount of microbes that weigh more than 1 kg. This quantity exceeds the number of cells in the human body 10-fold. The microbial community of the intestine consists of more than 500 species, most of which have not been cultivated, and many have yet to be identified. The intestinal microflora contains both bacteria that are fixed in the intestine (autochthonous, resident) and bacteria that only pass through the intestine (transient allochthonous) (Saavedra and Tschernia, 2002). Most of the bacteria in the intestine form an anaerobic bioreactor that helps to digest difficult polysaccharides and synthesizes micronutrients including vitamins and short-chain fatty acids. The fermentation products of these bacteria can provide up to 10% of the daily energy needed by an individual (Macpherson and Harris, 2004).

The intestinal microflora has a variety of important physiological functions. It produces vitamins, degrades bile acids, digests nutrients, and forms important barrier against pathogens by producing local and general immunity (Abt and Artis, 2009).
INTESTINAL MICROFORA IN CHRONIC LIVER DISEASE

In patients with liver cirrhosis, abnormal colonisation of the small intestine with colonic bacteria is well established. At least 50% have intestinal bacterial overgrowth. This is in comparison with healthy individuals who have only small amount of these bacteria in the small intestine. The main causes are thought to be hypochlorhydrosis, a decrease in IgA secretion, a decrease in intestinal motility and malnutrition. In the clinical setting, increasing small intestinal motility pharmacologically with cisapride has been shown to reduce bacterial overgrowth in patients with cirrhosis (Pardo et al., 2000)

BACTERIAL TRANSLOCATION

Bacterial translocation is defined as the migration of bacteria from the intestinal lumen to mesenteric lymph nodes of other extra-intestinal sites (Berg and Garlington, 1979). The most effective bacteria to translocate to the mesenteric lymph nodes are Gram-negative members of the Enterobactereaceae family such as Escherichia coli and Klebsiella spp, enterococci and other streptococci species (Steffen et al., 1988). In an animal study, the prevalence of bacterial translocation to mesenteric lymph nodes is round 40% in cirrhotic rats with ascites (Guarner et al., 1997) and around 80% in such animals with spontaneous bacterial peritonitis (Llovet et al., 1994). Available evidence has suggested that bacteria isolated from the mesenteric lymph nodes are genetically identical to strains causing SBP in the same animal (Llovet et al., 1998). In patients who are undergoing liver transplantation or liver resection, positive mesenteric lymph node culture for enteric bacteria was found in nearly 31% of Child-Pugh class C cirrhosis, which was 5 times
higher than in child Pugh Class A or B (Cirera et al., 2001). In a study on cirrhotic patients undergoing partial hepatectomy, almost 20% were found to have positive mesenteric lymph node culture, and most of the post-operative infections were due to the same bacteria (Yeh et al., 2003).

Bacterial DNA was found in as many as one third of cirrhotic patients with portal hypertension and culture negative ascites, with Escherichia coli the most frequently identified bacterial species (Such et al., 2002a). The presence of bacterial DNA is associated with increased local levels of pro-inflammatory cytokines, which may be clinically significant (Francés et al., 2004). Endotoxaemia is also postulated to increase portal pressure and impair haemostasis (Thalheimer et al., 2005), and the onset of variceal bleeding (Lata et al., 2005).

**Marker of bacterial translocation: Liposaccharide binding protein (LBP)**

LBP is the marker for endotoxaemia or bacterial translocation studied in this thesis. It is a soluble acute-phase protein that binds to LPS of gram-negative bacteria to elicit immune responses, by presenting the LPS to important cell surface pattern recognition receptors called CD14 (Grunwald et al., 1996) and TLR4 (Muta and Takeshige, 2001). The end result is the production of proinflammatory cytokines (Schumann et al., 1990). Serum LBP is a biomarker for infection and sepsis. Elevated serum LBP in patients with severe sepsis is also strongly associated with increased mortality (Villar et al., 2009).
PATHOGENESIS OF BACTERIAL TRANSLOCATION IN CIRRHOSIS

Apart from intestinal bacterial overgrowth, cirrhotics also exhibit changes in intestinal mucosa as well as intestinal immunity. Morphological changes can be secondary to increased levels of nitric oxide as an animal study has shown that nitric oxide dilates tight junctions in cultured intestinal epithelial cells (Salzman et al., 1995). This represents the breaching of the first line of mucosal defence against paracellular absorption, and hence the increased potential for bacterial translocation. Dilatation of the intercellular space below tight junctions, which is the second line of defence against paracellular absorption, has also been found in cirrhotics (Such et al., 2002b). Thick-walled, dilated capillaries together with oedema of the lamina propria, fibromuscular proliferation, a reduced villous/crypt ratio and thickened muscularis mucosa in the small bowel are also found in cirrhotics (Misra et al., 1997), and have been proposed to play a role in bacterial translocation (Hashimoto and Ohyanagi, 2002). On the other hand, intestinal immunity has also been described as a contributing factor. This is because an animal study has been shown that there is an increased number of intraepithelial lymphocytes with markedly impaired proliferative activity and capacity for production of interferon-γ, correlating with increased bacterial translocation (Inamura et al., 2003). To summarise, the main mechanisms leading to bacterial translocation are bacterial overgrowth, a deficit in the local immune response of the mucous membrane, a decrease in phagocytic activity of macrophages as well as neutrophils, and an increase in the permeability of the intestinal barrier (Ramachandran and Balasubramanian, 2001).
The concept of probiotics probably dates back to 1908, when Nobel Prize winner Eli Metchnikoff suggested that the long life of Bulgarian peasants resulted from their consumption of fermented milk products (Dobrogosz et al., 2010). The term ‘probiotic’ was coined in 1965 by Lilly and Stillwell when they described ‘substances secreted by one organism which stimulate the growth of another’ (Lilly and Stillwell, 1965). Subsequently in 1989, Fuller proposed that probiotics were ‘a live microbial supplement which beneficially affects the host animal by improving its microbial balance’ (Fuller, 1989). Other descriptions of probiotics include ‘foods containing live bacteria which are beneficial to health’ (Salminen et al., 1998), and ‘microbial preparations or components of microbial cells that have a beneficial effect to health and well being’ (Marteau et al., 2002). Such definitions underpin the current popular commercial usage of various ‘friendly bacteria’ to secure non-specific benefits to health.

Essentially, probiotics are live microorganisms that have beneficial properties for the host. The list of such microorganisms continues to grow and includes strains of lactic acid bacilli (e.g. Lactobacillus and Bifidobacterium), a non-pathogenic strain of Escherichia coli (e.g. E.coli Nissle 1917), Clostridium butyricum, Streptococcus salivarius, and Saccharomyces boulardii.

COMMON PREPARATIONS OF PROBIOTICS

The common probiotic strains and their potential beneficial effects are listed in table 3.
Table 3: Common preparations of probiotics

<table>
<thead>
<tr>
<th>Strains</th>
<th>Potential benefits</th>
</tr>
</thead>
<tbody>
<tr>
<td>E coli Nissle</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>Lactobacillus (many strains)</td>
<td>Prevention and treatment of acute childhood diarrhoea, antibiotic-associated diarrhoea</td>
</tr>
<tr>
<td>Bifidobacterium spp</td>
<td>Childhood diarrhoea, ulcerative colitis</td>
</tr>
<tr>
<td>Sacchromyces boulardii</td>
<td>Prevention of antibiotic-associated diarrhoea</td>
</tr>
<tr>
<td>VSL#3 (combination of 8 species belonging to Lactobacillus, Bifidobacteria and Streptococcus)</td>
<td>Ulcerative colitis, prevention of pouchitis</td>
</tr>
</tbody>
</table>

Adapted from Sharma et al. (2013)
YAKULT™

Yakult was created by Dr Minoru Shirota in 1935. It is labelled as a dairy product and is readily available in many parts of the world. It contains fermented skimmed milk together with a minimum of $6.5 \times 10^9$ live cells of *Lactobacillus casei* Shirotia. Yakult™ was used in the probiotic study to be discussed later due to its availability as a food product, and the lack of any documented adverse event in the population, including cirrhotic patients (Stadlbauer et al., 2008).

PROBIOTICS: MECHANISMS OF BENEFIT

Mechanisms for the benefits of probiotics are not completely understood. However, four general benefits have been described (Sartor, 2004):

1. Suppression of growth or epithelial binding or invasion by pathogenic bacteria (Jones and Versalovic, 2009),

2. Improvement of intestinal barrier function (Yan et al., 2007),

3. Modulation of the immune system. These include the inducement of protective cytokines such as IL-10 (McCarthey et al., 2003) and TGF-beta, and suppression of proinflammatory cytokines such as TNF (Borruel et al., 2002). Suppression of T-Helper 1 cells migration has also been described (Dalmasso et al., 2006),

4. Modulation of intestinal pain perception by inducing expression of micro-opioid and cannabinoid receptors (Rousseaux et al., 2007).
PROBIOTICS IN LIVER DISEASES

This review on association of probiotics and liver diseases focuses on the following aspects:

1. Prevention of infection,
2. Hepatic encephalopathy,
3. Liver function in cirrhosis,
4. Non-alcoholic fatty liver disease.

PREVENTION OF INFECTION

Spontaneous bacterial infections such as SBP and bacteraemia are common in hospitalised cirrhotic patients. They also correlate with the severity of liver cirrhosis. These infections are associated with significant morbidity and mortality. For example, the prevalence of SBP in cirrhotic hospitalised patients with ascites is as high as 30% (Rimola et al., 2000), and despite aggressive therapy, 1 in 5 patients still die from SBP (Garcia-Tsao, 2001).

The causal link between bacterial translocation and SBP has been well demonstrated in an animal study (Llovet et al., 1998). Probiotic usage in this setting is attractive because of firstly its ability to modulate gut flora favouring protective anaerobic organisms, and secondly its effects in promoting gut barrier function (Versalovic, 2007). An animal study has shown that probiotic supplementation increases resistance to enteric infection in IL-10 deficient mice (Madsen et al., 2001). In clinical studies, probiotics have
demonstrated efficacy in reducing endotoxaemia in cirrhotics (Lata et al., 2007), which is an indicator of the bacterial translocation, by reducing the viable counts of potentially pathogenic Gram-positive and Gram-negative gut flora in patients with cirrhosis (Wiest and Garcia-Tsao, 2005). Probiotics have also been shown to reduce infections post-orthotopic liver transplantation (Rayes et al., 2005).

**HEPATIC ENCEPHALOPATHY**

Gut microflora-derived ammonia has been widely recognised as the major contributor in the pathogenesis of hepatic encephalopathy. Modulating the intestinal flora pharmacologically remains the mainstay of treatments currently. This includes lactulose, non-absorbable antibiotics such as neomycin and rifaximin.

As early as 1965, the idea of populating the colonic lumen with non–urease-producing bacteria as a treatment for hepatic encephalopathy was first put to test. An uncontrolled study suggested that high oral doses of Lactobacillus acidophilus might have a beneficial effect in patients with cirrhosis and hepatic encephalopathy (Macbeth et al., 1965). A year later, Lactobacillus acidophilus has also been used in another small study to show clinical improvement in encephalopathic patients refractory to neomycin alone (Read et al., 1966).

Enterococcus faecium was being investigated by Loguercio et al (Loguercio et al., 1995). Forty patients were randomised to probiotic or lactulose for three 4-week periods, each
separated by a 2-week washout. At the end of the study, patients receiving probiotic demonstrated reduced serum ammonia levels and demonstrated improvement in various neuro-cognitive tests, as well as improvements in mental status.

Liu et al (Liu et al., 2004) investigated the effect of synbiotic, which is a combination of a probiotic and probiotic. They demonstrated improvement in minimal hepatic encephalopathy in patients treated with synbiotic, as well as a reduction in gram-negative organisms via quantitative stool analysis. A surprising secondary end point for patients receiving synbiotic was an improvement in overall liver function, as measured by the Child-Pugh score.

Bifidobacterium longum was studied by Malaguarnera et al. in 60 cirrhotic patients with minimal hepatic encephalopathy (Malaguarnera et al., 2007). Significant improvement in neuropsychological testing and serum ammonia levels were found in those patients treated with probiotic.

The use of probiotics in common clinical practice, however, requires further studies. Recent Cochrane review concluded that current available trials suffered from a high risk of bias and play of chance (McGee et al., 2011). Although probiotics did appear to reduce plasma ammonia concentration, probiotics could not be concluded to be efficacious in altering clinically relevant outcomes.
LIVER FUNCTION IN CIRRHOSIS

Available studies suggest that probiotic therapy may improve liver function parameters in cirrhotic patients. In the study of synbiotic by Liu et al described earlier (Liu et al., 2004), improvement of Child-Pugh class was recorded in nearly half the patients randomised to taking synbiotic. The authors speculate that the liver synthetic function may be improved by the reduction in serum endotoxin levels.

In the study by Lata et al using Escherichia coli Nissle, similar, but statistically less robust, results were achieved (Lata et al., 2007). They found that patients treated with probiotic for 42 days did show a trend toward lower endotoxin levels and improvement in Child-Pugh score, although they were not statistically significant.

In another study, Loguercio et al demonstrated improvement in serum liver tests and also reduction in proinflammatory cytokines in non-alcoholic steatohepatitis related cirrhotics using VSL#3 which is a cocktail of probiotics (Loguercio et al., 2005).

NON ALCOHOLIC FATTY LIVER DISEASE

Evidence in animal studies shows that intestinal bacterial overgrowth plays a significant role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (Wigg et al., 2001). Furthermore, obese patients with bacterial overgrowth after jejuno-ileal bypass surgery experienced rapid worsening of their NAFLD (Drenick et al., 1982), suggesting a causal
Endotoxaemia worsens NAFLD by stimulating hepatic Kupffer cells to produce TNF-α which stimulates liver fibrosis (Yang et al., 1997).

To date there are only 2 human studies on probiotics in NAFLD. As outlined previously, Loguercio et al demonstrated improvement in serum liver tests and also reduction in proinflammatory cytokines in non-alcoholic steatohepatitis related cirrhotics using VSL#3, a cocktail of probiotics (Loguercio et al., 2005). A more recent study has shown that Lactobacillus bulgaricus and Streptococcus thermophilus improve liver aminotransferases levels in patients with NAFLD, although anthropometric parameters and cardiovascular risk factors remained unchanged after treatment (Aller et al., 2011). Cochrane systemic review has concluded that due to the lack of randomised controlled trials, the use of probiotics in NAFLD can neither be supported nor refuted (Lirussi et al., 2007).

PROBIOTICS: POTENTIAL RISK AND THE LACK OF BENEFICIAL EFFECT

Although probiotics are generally well tolerated, it is not without risk especially in patients with severe illness or in those who are immuno-compromised. A previous study showed that in patients with predicted severe acute pancreatitis, probiotics prophylaxis with a cocktail of six different strains (Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum, and Bifidobacterium Lactis) was actually associated with an increased risk of mortality (Besselink et al., 2008).
Published data in probiotics studies are undoubtedly positively biased. Nevertheless, there are some data to suggest that probiotics perhaps might not be as effective. Vleggaar et al found that in primary sclerosing cholangitis, treatment with a probiotics cocktail containing four Lactobacillus and two Bifidobacillus strains did not improve clinical symptoms or liver function tests (Vleggaar et al., 2008).

Saji et al. assessed the efficacy of probiotics in compensated cirrhotic patients with minimal hepatic encephalopathy. Treatment with a probiotics preparation containing Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium longum and Sacharomyces boulardi did not produce any significant improvement in the various parameters assessed in patients with minimal hepatic encephalopathy, when compared with placebo (Saji et al., 2011).

In decompensated liver cirrhosis, Jayakumar et al. evaluated the effect of VSL#3 treatment on portal pressure by assessing hepatic venous pressure gradient before and after therapy. No significant difference was detected (Jayakumar et al., 2013). Furthermore, the same study observed that there were no significant changes in the stool microbiota evaluated by terminal restriction fragment length polymorphism, before and after therapy.
CHAPTER 2

AIMS
AIMS

The aim of this thesis is to study the frequencies of MBL SNPs in patients with CLD, the relationship between MBL SNPs and the development of CLD complications (SBP, variceal bleed), and the usefulness of MBL SNPs as a prognostic marker. This thesis also aims to study, as a proof on concept, whether bacterial translocation and systemic inflammation in CLD is influenced by probiotics.

HYPOTHESES

1. MBL SNP increases bacterial translocation, systemic inflammation and eventually complications (variceal bleed, spontaneous bacterial peritonitis) in patients with CLD,

2. These MBL SNPs are a prognostic marker for patients with CLD,

3. Bacterial translocation and systemic inflammation are reduced by probiotics.
CHAPTER 3

METHODS
STUDY DESIGN AND METHOD

1. CASE CONTROLLED STUDY

Patients with CLD, diagnosed histologically or with biochemical (low platelet count, prolonged prothrombin time, decreased serum albumin) or radiological evidence of cirrhosis (nodular liver surface, coarse liver parenchyma, splenomegaly) were recruited from the liver ward and liver clinic at Royal Derby Hospital, from January 2009 to December 2009. Inclusion criterion was liver cirrhosis without acute decompensation. Exclusion criteria were primary immune deficiency, human immunodeficiency virus, ongoing chemotherapy, and post liver transplantation. Demographic and clinical data including age, aetiology and severity of CLD, previous occurrence of variceal bleed and SBP were collected. A venous blood sample of 10mls was taken, with aseptic technique, to measure MBL SNPs (real time PCR), LBP (ELISA), and TNF-α (ELISA). Patients with a known occurrence of complications of CLD (variceal bleed/SBP) were grouped together, and were compared to patients without, in a case controlled study.

2. LONGITUDINAL STUDY

From study (1), patients were stratified according to their MBL SNPs status, and were followed up until October 2010, or when liver related death (the measured end point) occurred.

3. OPEN LABEL PROOF OF CONCEPT STUDY

Patients with treated variceal bleed, 1 week after cessation of the standard antibiotic therapy, were recruited and given probiotics Lactobacillus casei Shirota (Yakult™) for 28
days (1 pot 3 times a day). Inclusion criteria were age >18, cirrhosis, presented with and treated for variceal bleeding. Exclusion criteria were antibiotics use within one week prior to recruitment, immunosuppressant medication including steroid, recent chemotherapy, primary immune deficiency, human immunodeficiency virus, recent chemotherapy and post liver transplantation.

LBP and TNF-α were determined at day 0 of probiotic therapy (baseline), and day 28 of probiotic therapy (end of treatment) to determine the effect of probiotics on these markers. Yakult™ were obtained from retail supermarket, and stored at 4–7 °C in a domestic refrigerator before consumption.

STATISTICAL ANALYSIS

Normally distributed data were presented as mean ±standard deviation (SD), and non parametric data are presented as median (inter quartile range [IQR]). Categorical variables were compared using Chi-Squared ($X^2$) test. Continuous variables were compared using Student T test and Mann Whitney U test, for normally distributed data and non parametric data, respectively. In study (1), correlations between 3 stages of CLD and LBP and TNF-α were analysed using Kruskal-Wallis test. Univariate analysis was used to compare risk factors between patients with previous CLD complications and those without. Multivariate analysis was performed using Binary Logistic Regression to determine independent risk factor. In study (2), survival was calculated using Kaplan Meier survival curves and the log rank test. Multivariate Cox regression analysis was used to test covariates as independent risk factors for liver related death. In study (3), no power calculation was performed as it was a ‘proof of concept’ study. Wilcoxon Signed
Rank test was used to compare changes of LBP and TNF-α at the end of therapy from baseline. All p-values calculated were 2 tailed, and p<0.05 was regarded as significant. All statistical analyses were performed using the SPSS version 20 software (Statistical Package for the Social Sciences, IBM, USA).

ETHICAL CONSIDERATION

Study (1) and (2) were reviewed and approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee. Study (3) was reviewed and approved by the Derbyshire Research Ethics Committee. All studies were approved by the Research and Development department at Royal Derby Hospital. All patients gave written informed consent.

LABORATORY ASSAYS

All the laboratory works were carried out in a laboratory within the Nottingham Derby Medical School.

MBL SNPs by Real time PCR

DNA was isolated from plasma samples with the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using 100 μl of elution buffer.

Three single nucleotide polymorphisms (SNPs) located within the MBL2 gene (MBL2 – D (codon 52, rs5030737), MBL2 –C (codon 57, rs1800451), MBL2 –B
(codon 54, rs1800450), were analyzed using real-time polymerase chain reaction (rt-PCR) with TaqMan SNP Genotyping Assays, as per the manufacturer’s instruction (Applied Biosystems, Foster City, CA) (Van Hoeyveld et al., 2004) (Mellbin et al., 2010). For all TaqMan assays, DNA amplifications were carried out in 25μl polymerase chain reactions containing 20 ng DNA, 0.9μM primers and 0.2μM TaqMan probes (final concentrations) amplified in 96-well plates. Reactions were performed with the following protocol on a GeneAmp PCR 9700 or a 7900 HT Sequence Detection System: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The genotyping was determined by measuring the end-point fluorescence on a 7900 HT Sequence Detection System using the SDS version 2.3 software.

**TNF-α and LBP by ELISA**

Serum TNF-α and LBP concentrations were measured with a commercially available ready to use ELISA kit (Hycult Biotech, The Netherlands), according to the manufacturer’s instruction.

In short, the human TNF-α ELISA and LBP ELISA are ready-to-use solid-phase enzyme-linked immunosorbent assays based on the sandwich principle. Samples and standards are incubated in microtiter wells coated with antibodies recognizing human TNF-α (or LBP). Biotinylated tracer antibody will bind to captured human TNF-α (or LBP). Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody. Streptavidin-peroxidase conjugate will react with the substrate, tetramethylbenzidine. The enzyme reaction is stopped by the addition of oxalic acid. The absorbance at 450 nm is measured
with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human TNF-α (or LBP) standards (log). The human TNF-α (or LBP) concentration of samples, which are run concurrently with the standards, are determined from the standard curve.
CHAPTER 4

RESULTS
CHAPTER 4.1

THE RELATIONSHIPS BETWEEN SINGLE NUCLEOTIDE POLYMORPHISMS OF MANNAN BINDING LECTIN AND COMPLICATIONS OF CHRONIC LIVER DISEASE
145 cirrhotic patients were recruited. 77 (53.1%) were Child-Pugh class A, 57 (39.3%) were class B, and 11 (7.6%) were class C.

MBL2 analysis found that 24 patients were heterozygous, and 3 were homozygous for B mutation. The corresponding numbers for C mutation and D mutation were 9, 1 and 6, 0 respectively (Table 4).

Frequency of A/A was 102 (70.3%), A/B was 24 (16.6%), A/C was 9 (6.2), A/D was 6 (4%) and O/O was 4 (2.8%).

The majority of patients in the study were Caucasians, some originating from mainland Europe and Republic of Ireland. There were only three non-Caucasian, and all three of them were British South Indians. If three of them (1 was of A/C MBL2 genotype) were excluded, the percentages were 70.4, 16.9, 5.6, 4.2 and 2.8 respectively.
Table 4: Frequency of MBL SNPs

<table>
<thead>
<tr>
<th>MBL</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>102</td>
<td>70.34</td>
</tr>
<tr>
<td>SNP MBL 52</td>
<td>6</td>
<td>4.14</td>
</tr>
<tr>
<td>SNP MBL 54</td>
<td>27</td>
<td>18.62</td>
</tr>
<tr>
<td>SNP MBL 57</td>
<td>10</td>
<td>6.90</td>
</tr>
<tr>
<td>TOTAL</td>
<td>145</td>
<td></td>
</tr>
</tbody>
</table>
Based on the MBL2 analysis, patients were categorised into 2 groups: AA patients (n=102) were homozygous for the wild-type MBL2 and AO/OO patients (n=43) were heterozygous with an A gene and one of the three structural mutations (B-D) in the other allele (n=39) or homozygous (O/O) for variant genotypes (n=4).

Patient characteristics are tabulated in Table 5. There is no statistical difference between these 2 groups except Child Pugh score (median, 7 vs. 6, p=0.02)

**Correlations between MBL SNPs and LBP & TNF-α**

There was no significant difference in LBP and TNF-α between AA group and AO/OO groups (Table 6).

**Correlations between stages of CLD and LBP & TNF-α**

There was no significant difference in LBP and TNF-α when 3 stages of CLD were compared using Kruskal-Wallis test (Table 7).
Table 5: Comparison of clinical characteristics between MBL SNPs and wild types

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AO/OO</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male/female)</td>
<td>103 (61/42)</td>
<td>42(30/12)</td>
<td>0.234</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.10±11.95</td>
<td>56.29±10.73</td>
<td>0.396</td>
</tr>
<tr>
<td>Child Pugh score</td>
<td>7 (5-8)</td>
<td>6 (5-6)</td>
<td><strong>0.020</strong></td>
</tr>
</tbody>
</table>

Aetiology

<table>
<thead>
<tr>
<th></th>
<th>AA</th>
<th>AO/OO</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALD</td>
<td>77 (74.8%)</td>
<td>30 (71.4%)</td>
<td>0.840</td>
</tr>
<tr>
<td>Viral</td>
<td>7</td>
<td>4</td>
<td>0.830</td>
</tr>
<tr>
<td>NASH</td>
<td>8</td>
<td>4</td>
<td>0.980</td>
</tr>
<tr>
<td>AIH</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Normally distributed data are given as mean and standard deviation. Non-parametric data are given as median (Inter Quartile Range). Analysis by chi-square for categorical data, t-test for parametric continuous data, and Mann Whitney U test for non-parametric continuous data.

ALD: alcoholic liver disease. NASH: non alcoholic steatohepatitis. AIH: autoimmune hepatitis
Table 6: Association of MBL SNPs and LBP & TNF-α

<table>
<thead>
<tr>
<th>Wild type AA</th>
<th>SNP AO/OO</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=103</td>
<td>N=42</td>
<td></td>
</tr>
<tr>
<td>LBP (ng/ml)</td>
<td>20.214 (13.619-37.053)</td>
<td>21.703 (12.044-32.175)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>58.622 (55.869-62.099)</td>
<td>56.797 (55.161-64.462)</td>
</tr>
</tbody>
</table>

Data are non-parametric and given as median (Inter Quartile Range). Analysis by Mann Whitney U test.

LBP: Liposaccharide binding protein, TNF-α: Tumour necrosis factor-alpha
Table 7: Association of different stages of CLD and LBP & TNF-α

<table>
<thead>
<tr>
<th></th>
<th>CPA</th>
<th>CPB</th>
<th>CPC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>77</td>
<td>57</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>58.622 (55.524-63.549)</td>
<td>57.349 (55.302-60.006)</td>
<td>59.831 (57.993-60.647)</td>
<td>0.568</td>
</tr>
</tbody>
</table>

Data are non-parametric and given as median (Inter Quartile Range). Analysis by Kruskal-Wallis test.

CPA: Child Pugh A, CPB: Child Pugh B, CPC: Child Pugh C

LBP: Liposaccharide binding protein, TNF: Tumour necrosis factor-alpha
ASSOCIATION BETWEEN MBL SNPs AND COMPLICATIONS OF CLD

There was a significant proportion of patients with prior history of variceal bleed or SBP (n=57, 39.3%) in the study population (Table 8).

Patient characteristics and clinical data were compared between the group with prior history of complications and the group without. The only significant difference found was that the group with prior history of complications had more severe underlying liver disease in the form of Child Pugh score (median, 8 vs. 6, p<0.001) (Table 9).

21.1% of the group with prior history had MBL2 genetic polymorphism AO/OO, whereas a higher percentage (34.1%) in the group without had MBL2 genetic polymorphism. The difference was not statistically significant. There was also no difference in the level of TNF-α or LBP between the two groups.

Multivariate logistic regression analysis revealed that Child-Pugh score (Hazard ratio=1.487, 95% CI [1.190-1.858], p=0.011) was an independent risk factor for the occurrence of prior CLD complications.
Table 8: The prevalence of previous variceal bleed and/or spontaneous bacterial peritonitis

<table>
<thead>
<tr>
<th>Presence/absence of previous complication</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous variceal bleed/SBP</td>
<td>57</td>
<td>39.31</td>
</tr>
<tr>
<td>No previous history</td>
<td>88</td>
<td>60.69</td>
</tr>
</tbody>
</table>
Table 9: Univariate analysis of risk factor between patients with previous CLD complications and patients without

<table>
<thead>
<tr>
<th>Prior complications</th>
<th>No prior complications</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=57</td>
<td>n=88</td>
<td></td>
</tr>
<tr>
<td>SNP MBL (AO/OO)</td>
<td>12 (21.1%)</td>
<td>30 (34.1%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.12±11.11</td>
<td>57.22±11.96</td>
</tr>
<tr>
<td>Male/female</td>
<td>36/21</td>
<td>55/33</td>
</tr>
<tr>
<td>Child Pugh score</td>
<td>8 (7-8)</td>
<td>6 (5-7.5)</td>
</tr>
<tr>
<td>ALD aetiology</td>
<td>46 (80.7%)</td>
<td>61 (69.3%)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>59.536 (55.584-62.736)</td>
<td>57.648 (55.464-62.692)</td>
</tr>
<tr>
<td>LBP (ng/ml)</td>
<td>19.834 (14.483-37.915)</td>
<td>20.698 (12.716-30.145)</td>
</tr>
</tbody>
</table>

Normally distributed data are given as mean and standard deviation. Non-parametric data are given as median (Inter Quartile Range). Analysis by chi-square for categorical data, t-test for parametric continuous data, and Mann Whitney U test for non-parametric continuous data.

ALD: alcoholic liver disease

LBP: Liposaccharide binding protein, TNF: Tumour necrosis factor-alpha
Chapter 4.2

PROGNOSTIC VALUE OF SINGLE NUCLEOTIDE POLYMORPHISMS
OF MANNAN BINDING LECTIN IN THE DEVELOPMENT OF LIVER
RELATED DEATH
All 145 patients were successfully followed up in this study. Median follow-up was 550 days (IQR 452-613). 26 patients (17.93%) died of liver related death. Causes of death are listed in table 10. 2 deaths were not liver related (myocardial infarction and cerebrovascular disease) and were therefore censored in the Kaplan-Meier survival analysis.

Kaplan-Meier survival curve showed that increasing severity of liver disease, as measured by Child Pugh classes, were found to be significantly associated with liver mortality (Figure 3). Patients with Child B and C stage cirrhosis were at higher risk for developing liver related death compared to patients with Child A disease (CPC vs. CPA: hazard ratio 12.284, p<0.001; CPB vs. CPA: hazard ratio 9.046, p=0.003, respectively). MBL SNPs, did not increase the overall liver related death (p=0.221) (Figure 4), nor did it increase the liver related death in each stage of the liver cirrhosis, as measured by Child Pugh class (p=0.147 for class A, p=0.617 for class B, p=0.564 for class C) (Figure 5, 6, 7). ALD aetiology (p=0.993) and previous CLD complications (p=0.207) were also not associated with liver related death.
Table 10: Causes of liver related death on follow up

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>End stage liver failure</td>
<td>6</td>
</tr>
<tr>
<td>Spontaneous bacterial peritonitis</td>
<td>5</td>
</tr>
<tr>
<td>Variceal bleed</td>
<td>4</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Pneumonia and liver failure</td>
<td>3</td>
</tr>
<tr>
<td>Urinary tract infection and liver failure</td>
<td>2</td>
</tr>
<tr>
<td>Alcoholic hepatitis</td>
<td>2</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>26</strong></td>
</tr>
</tbody>
</table>
Other univariate comparisons were made between patients who had liver related deaths and those who did not to identify additional risk factors. Age (median 63.5 [54-72] vs. 57 [49-64], p=0.023) was associated with increased liver related death. Factors that were not associated with liver related death were LBP (ng/ml) level (median, 38.716[17.095-54.577] vs. 25.022 [16.683-44.585], p=0.308), and TNF (pg/ml) level (median, 58.083 [56.543-61.463] vs. 57.648 [55.161-62.869], p=0.268).

Multivariate Cox regression analysis found that only Child Pugh C (p<0.001), Child Pugh B (p=0.008) and age (p=0.011) were independent predictors for liver related death (Table 11).
Figure 3: Association between Child Pugh class and liver related death

By Kaplan Meier survival curve and log rank analysis

CPA vs. CPB, \( p=0.003 \);
CPA vs. CPC, \( p=0.001 \);
CPB vs. CPC, \( p=0.194 \)
Figure 4: Association between MBL SNPs and liver related death

By Kaplan Meier survival curve and log rank analysis

P=0.221
Figure 5: Association between MBL SNPs and liver related death in Child Pugh class A

By Kaplan Meier survival curve and log rank analysis

P=0.147
Figure 6: Association between MBL SNPs and liver related death in Child Pugh class B

By Kaplan Meier survival curve and log rank analysis

P=0.617
Figure 7: Association between MBL SNPs and liver related death in Child Pugh class C

By Kaplan Meier survival curve and log rank analysis

P=0.564
Table 11: Risk factors independently associated with liver related death

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.048</td>
<td>1.011-1.087</td>
<td>0.011</td>
</tr>
<tr>
<td>Child Pugh B</td>
<td>4.053</td>
<td>1.445-11.370</td>
<td>0.008</td>
</tr>
<tr>
<td>Child Pugh C</td>
<td>11.121</td>
<td>3.137-39.424</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

By Cox regression analysis.
CHAPTER 4.3

THE EFFECT OF PROBIOTICS TREATMENT ON SYSTEMIC INFLAMMATION AND BACTERIAL TRANSLOCATION IN ACUTE DECOMPENSATED CHRONIC LIVER DISEASE
7 patients were recruited. Baseline characteristics are given in Table 12. Compliance with probiotic therapy was documented by patient home visit and bottle counting. By these methods, all patients were judged to be completely compliant with therapy. No patient discontinued treatment and no patient required antibiotic therapy during the trial period.

There was no significant difference in Child Pugh score, LBP level and TNF-α level before and after the probiotic therapy (Table 13). Individual patient data for LBP and TNF-α levels are presented in Figure 8.
Table 12: Baseline clinical characteristics

Age (years, mean age ±SE) 62.14±9.388

Male/female 5/2

Aetiology

<table>
<thead>
<tr>
<th>Aetiology</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic liver disease</td>
<td>5</td>
<td>(71.43%)</td>
</tr>
<tr>
<td>Cryptogenic</td>
<td>2</td>
<td>(28.57%)</td>
</tr>
</tbody>
</table>

Child Pugh score (mean ±SE) 7.00±1.00
Table 13: Effect of probiotic therapy on TNF-α, LBP and Child Pugh score

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>End of Rx</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>60.986 (59.674-64.551)</td>
<td>60.045 (57.494-61.995)</td>
<td>0.310</td>
</tr>
<tr>
<td>LBP (ng/ml)</td>
<td>39.219 (23.597-40.195)</td>
<td>32.000 (21.340-36.050)</td>
<td>0.874</td>
</tr>
<tr>
<td>Child Pugh score</td>
<td>13 (9-14)</td>
<td>14 (10-14)</td>
<td>0.461</td>
</tr>
</tbody>
</table>

Data are parametric and expressed in median (inter quartile range [IQR]). Analysis by Wilcoxon Signed Rank test.

LBP: Liposaccharide binding protein, TNF-α: Tumour necrosis factor-alpha
Individual patient data for LBP and TNF-α results from baseline to end of study.
CHAPTER 5

DISCUSSIONS
STUDY DESIGN

STUDY (1) & (2)

Study (1) was designed to compare CLD patients with MBL SNPs, and CLD patients without MBL SNPs, in the following areas: bacterial translocation marker, systemic inflammation marker, previous occurrence of CLD complications in the form of variceal bleed and SBP. Study (2) was designed to determine the risk of developing liver related death subsequently in a prospective manner. To answer these questions, a healthy control group was not required. Furthermore, the frequencies of MBL SNPs in a similar population in the UK has already been established (Garred et al., 2006). This could serve as historical controls for comparing frequencies.

Recruitment of participants were from both the in- and out-patient. Question might be raised regarding whether this difference in location, and hence potentially difference in liver disease stages and susceptibility to infection, could have skewed the results. To counteract this, only stable patients were included in the studies. Patients who had ongoing acute decompensation were excluded. Most of the recruited in-patients were stable, without any active infection and were about to be discharged from the hospital.

The other question about the study design was whether serum MBL level should have been included. It is recognised that serum MBL concentration is a dynamic measurement and it increases during acute phase response (Thiel et al, 1992). It is also affected by other factors including growth hormone (Hansen et al, 2001). On the other hand, MBL SNPs remain unchanged despite different clinical status, and the presence of MBL SNPs
has already been firmly established to correlate with a low serum MBL concentration (Garred et al., 2006). Therefore MBL serum concentration was not measured in this study to prevent unnecessary duplication and for cost saving.

With regards to the study end point in study (2), occurrence of infection was not used as an end point as it has already been established in the literature that among cirrhotic patients, MBL deficiency is an independent risk factor for infection (Altorjay et al, 2010). Therefore the focus in study (2) was on the unknown liver related death.

**STUDY (3)**

The major limitation of the study was the lack of a control group. The aim of this study was mainly to prove the concept that probiotics could influence LBP and TNF-α in CLD patients with acute decompensation. The comparison therefore was made within the same treatment group before and after treatment, and not with a control group.

**RESULTS**

**RESULTS: STUDY (1)**

Study (1) demonstrated that among patients with liver cirrhosis, frequency of A/A was 102 (70.3%), A/B was 24 (16.6%), A/C was 9 (6.2), A/D was 6 (4%) and O/O was 4 (2.8%). These numbers are not dissimilar to the published incidence of MBL2 genotype frequencies among normal British Caucasians. The corresponding percentages are 60, 21, 5, 10, and 4 respectively (Garred et al., 2006). It is already well established that MBL
levels in patients with liver cirrhosis of different aetiologies are similar to the general healthy population (Stadlbauer et al, 2008). Therefore it can be concluded that both frequencies of MBL SNPs and the associated MBL level among cirrhotics are rather similar compared to the general population.

In study (1), MBL SNPs did not alter TNF-α or LBP significantly. It is logical that as all recruited patients were clinically stable and did not suffer from any acute decompensation, there was no increased in bacterial translocation or endotoxin marker LBP. Subsequently, systemic inflammation (TNF-α) should not differ, regardless of the underlying MBL SNP status. TNF-α between MBL AA and MBL AO might only be different when they are being challenged with endotoxin, as a previous study found that in MBL SNPs patients, ex-vivo TNF-α production capacity was reduced only when challenged with gram-positive peptidoglycan, and gram-negative lipopolysaccharide (Babula et al., 2008).

There was no significant association between Child-Pugh classes and LBP. Previous study showed that in patients with liver cirrhosis, plasma endotoxin levels increased progressively as liver disease worsened (Chan et al., 1997). Study (1) did show an increasing LBP trend from Child Pugh class A to C, although the difference did not reach statistical significance.

MBL SNPs did not have any significant association between patients with a known occurrence of CLD complications (SBP/variceal bleed), and patients without. A previous
study found that MBL codon 54 mutation was significantly associated with risk of developing SBP in patients with chronic hepatitis B (Yuen et al., 1999). This relationship was not replicated in other types of CLD, neither did it achieve the same association in non-Chinese hepatitis B patients (Höhler et al., 1998) (Bellamy et al., 1998). Perhaps both arguments are relevant to study (1) as majority of them had ALD and none had hepatitis B. In addition, the lack of association can also be explained by the fact that more than 90% of MBL-deficient individuals do not actually suffer from recurrent infections (Petersen et al., 2001). It is thought that the phenotypic manifestation of MBL deficiency is observed only in association with another acquired (e.g. post chemotherapy) or genetically determined immunodeficiency (De Seta et al., 2007).

TNF-α and LBP did not predict the occurrence of CLD complications. This is not surprising as both are dynamic parameters, and therefore one off measurement is unlikely to predict long term outcome (Chan et al, 1997)

In both univariate and multivariate analysis, only Child-Pugh score was significantly associated with the occurrence of CLD complications. It is well established in the literature that severity of the liver disease is directly associated with the incidence of complications (Căruntu and Benea, 2006) (Sharara and Rockey, 2001).

**RESULTS: STUDY (2)**

Almost a fifth of the patients died during a median follow-up of 550 days (IQR 452-613). As the overall median Child Pugh Score for all recruited patients was 6 (IQR 5-8, class
A), the mortality rate was consistent with expected 2-year mortality rate of 20% among patients with this stage of liver disease (Infante-Rivard et al., 1987).

Kaplan-Meier survival analysis showed that MBL SNPs did not increase liver related death in the whole study population, or in each stage of the liver cirrhosis. It is possible that although a low serum MBL level is already a recognised risk factor for the development of all infections (Altorjay et al., 2010), not all infections lead to clinical significance such as death, especially in those patients with milder form of compensated CLD.

Cox regression analysis revealed that advanced liver cirrhosis (Child-Pugh class B and C) and older age were both independent predictors for liver related death. Advanced liver cirrhosis has long been recognised to associate with a lower survival probability (Pugh et al., 1973), and 2-year survival for Child-Pugh class B and C are 60% and 35%, respectively (Infante-Rivard et al., 1987). On the other hand, older age has been established to be an independent risk factor for liver related death in patients with both alcoholic (Masson et al., 2014) and non-alcoholic liver cirrhosis (Stepanova et al., 2013).

**RESULTS: STUDY (3)**

Study (3) showed that there was a trend that probiotic therapy reduced both TNF-α and LBP, although not statistically significant. Due to the very small sample size, a type-2 error cannot be entirely excluded here.
Previous probiotics studies showed reduction in endotoxaemia and cytokines mainly in alcoholic liver cirrhosis (Zhao et al., 2004) (Loguercio et al., 2005). In comparison, study (3) consisted of a high proportion of patients with non-alcohol aetiology (28%), which might have also accountable for the negative results.

**LIMITATIONS**

Several study limitations must be acknowledged. Firstly, the recruiting locations for study (1) and (2) involved both the ward and the clinic. This might have already introduced bias into the study in terms of patients’ characteristics and their susceptibility to the occurrence of CLD complications, as well as to the follow-up end point of liver related death. Stratification according to the recruiting location was not possible as the study was not powered to do so. However, for the ward patients, every effort was made to recruit only the stable ones who were nearly well enough to be discharged, to minimise such a bias.

Secondly, MBL serum levels were not measured in study (1) and (2). Although MBL SNPs correlate well with serum MBL levels (Altorjay et al., 2010), conflicting data has shown that there are a number of serologically MBL-deficient individuals who actually belong to the genetically non-MBL-deficient groups (Swierzko, et al., 2009). This supports the measurements of serum MBL levels.
For study (3), the major limitations were the lack of a controlled arm and the very small sample size. Therefore it was not possible to draw any conclusion from this proof of concept study.

A potential confounding factor in study (3) is the use of antibiotics prior to study enrolment. Prior antibiotics therapy (as part of the standard treatment for variceal bleed) might still have an effect on the bacterial flora even after 1 week of washout period. It might have therefore masked any effect of probiotics on endotoxin and the subsequent inflammation. 1 week of antibiotics washout period in study (3) was entirely arbitrary. Perhaps at least 30-day interval should be employed as per previous similar study (Stadlbauer et al, 2008).

**SUGGESTIONS FOR FUTURE STUDIES**

Current studies only examined the three major MBL SNPs as risk factor and as prognostic marker in patients with CLD. As serum MBL levels are also affected by several other SNPs in promoter 1 and 5’ untranslated regions, it would be of interest to examine three other common regulatory polymorphisms (H/L variant situated at position -550, X/Y variant at position -221, and P/Q variant at position +4), in a similar study. The study should include both healthy controls, and ‘disease (CLD)’ controls. It will be of great interest to include measurement of serum MBL levels in this future study.
As MBL only forms part of the innate immune system, it will be of great interest to conduct a study of similar nature to assess other SNPs such as the TLR2 and TLR4 SNPs to ascertain their roles as biomarkers for the development of complications in CLD.

Although not statistically significant, the probiotics study in acute decompensated CLD did show a small trend of reduction of inflammation and endotoxaemia. Clearly, a larger randomised controlled study powered to detect these changes will be needed before any conclusion can be drawn. An antibiotics washout interval of at least 4 weeks is advisable.

**CONCLUSION**

In conclusion, in patients with CLD, MBL SNPs does not increase the occurrence of complications (variceal bleed/SBP). In addition, MBL SNPs is not a risk factor or prognostic marker for liver related death.
CHAPTER 6

APPENDIX AND REFERENCES
APPENDIX

PAPERS & PRESENTATION ARISING FROM THIS THESIS

Published papers


Lo R, Austin AS, Freeman JG. Vasopressin in liver disease - should we turn on or off? Current Clinical Pharmacology 2008;3:156-65


Published abstract

Poster presentation European Association for the Study of the Liver (EASL) annual meeting Copenhagen, Denmark 2009
REFERENCES


to cytomegalovirus (re)infection after orthotopic liver transplantation. J. Hepatol. 55, 800–807.


